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Genome-Wide Association for Smoking Cessation Success: Participants in a Trial with Adjunctive Denicotinized Cigarettes

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The ability to quit smoking successfully displays substantial heritability in classical and molecular genetic studies. Twin studies suggest that some of the genetics for the ability to quit overlap with genetic components of nicotine dependence, but many do not. Genome-wide association (GWA) studies have demonstrated haplotypes that distinguish successful quitters from individuals who were not able to quit smoking in: i) clinical trials that employed nicotine replacement; ii) clinical trials that employed bupropion; and iii) community quitter samples. We now report novel GWA results from participants in a clinical trial that document the efficacy of adjunctive use of denicotinized cigarettes. These results buttress data from our prior GWA studies of smoking cessation. They suggest that ability to change smoking behavior using denicotinized cigarettes shares substantial underlying genetics with the ability to change this behavior in community settings or in response to treatments with nicotine replacement or bupropion.

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INTRODUCTION

Twin studies document that the ability to successfully abstain from smoking displays substantial heritability (1,2). Vulnerability to becoming dependent on addictive substances also displays substantial heritability that is largely shared between dependences on different addictive substances (3–7). Classical genetics suggest that some, but not most, of the influences on ability to quit smoking are shared with the genetics of developing dependence on an addictive substance (1,2).

We have reported recently genome-wide association (GWA) data for successfully quitting smoking in three independent samples of carefully monitored individuals who tried to quit smoking in the context of clinical trials that used placebo, nicotine replacement, or bupropion treatments (8), and in a fourth sample of research volunteers who reported achieving sustained abstinence in community settings (9). The molecular genetic results from these independent samples display substantial convergence with each other; the nominally positive results cluster in small chromosomal regions to extents much more than we would expect by chance. These results also display more modest convergence with molecular genetic data for vulnerability to dependence on nicotine and other addictive substances (8,10–14).

Reduced nicotine cigarettes are designed to progressively wean smokers from the smoking habit. Research demonstrating that smoking cessation requires withdrawal from nicotine, weaning from the habitual sensory, and behavioral reinforcement of smoking supports the idea that denicotinized cigarettes may help extinguish the reinforcing value of smoking-related behavioral and sensory cues. Recently, Becker *et al*. reported significantly increased success in smoking cessation when they added denicotinized cigarettes (Quest 1, Quest 2, and Quest 3; Vector Tobacco Inc., New York, NY, USA) to standard nicotine patch therapy (15). We now report GWA data that compares individuals from this trial who were successful in achieving biochemically monitored 4-week abstinence with those who were unsuccessful. Single nucleotide polymorphism (SNP) data from Affymetrix 6.0 (Affymetrix, Santa Clara, CA, USA) array studies of these samples identifies many of the same chromosomal regions previously identified by data for smoking ces-

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sation and for vulnerability to dependence on addictive substances. Copy number variant (CNV) data from this work provides an initial basis for future comparisons. We discuss the limitations of these modest-sized data sets, as well as the ways in which they buttress previous GWA results for the molecular genetics of smoking cessation from clinical trial and community-based samples of participants who attempted to quit using a variety of other behavioral and pharmacological strategies.

MATERIALS AND METHODS

Samples

DNA was available from most of the 262 participants in a study of denicotinized cigarettes who were followed for at least 4 wks after the end of treatment. Participants smoked ≥ 15 but < 60 cigarettes/d for at least 1 year prior to the study, displayed carbon monoxide measurements > 0.15 ppm, expressed motivation to quit, were 21 to 65 years of age, were recruited at 1 of 5 United States sites, and were generally healthy, as described (15). Participants were randomized to treatment in 1 of 3 conditions that utilized Quest (Quest 1, Quest 2, and Quest 3) denicotinized versus active control cigarettes as well as 24–h patches that contained 21-, 14-, or 7-mg nicotine doses, and standard behavioral support. Subjects were examined and evaluated every 2 wks after randomization until the end of wk 18 (3 months following the targeted quit date), and then 3 months later at the end of week 31 (6 months after the quit date). DNA extracted from the blood of self-reported European-American subjects (the largest self-reported ethnicity in this sample) was pooled into: i) three pools (n = 16 in each) containing DNA from all individuals who self reported abstinence, with biochemical confirmation, at the 4-wk follow-up time point ("successful quitters"); and ii) six pools of individuals who never displayed self-reported abstinence at any follow-up time ("unsuccessful quitters") who were selected based on matches to features noted for the successful quitters. Successful and unsuccessful quitters were similar for gender (% female 39 versus 42), baseline scores on the Fagerstrom Test for Nicotine Dependence (FTND) (5.8 versus 6.1), and the fraction who reported prior unsuccessful attempts to quit (92 versus 94%). Of these individuals who were abstinent for 4 wks, 73% and 65% both attended subsequent 3- and 6-month follow-up visits and reported continuous biochemically verified abstinence at these time points, respectively.

DNA Preparation and Assessment of Allelic Frequencies

DNA was prepared from blood (16–18) and carefully quantitated. DNA from groups of 16 individuals of the same phenotype was combined. Pooled genotyping reduced costs and allowed us to assess high densities of genotypes in these subjects while providing no threat of loss of genetic confidentiality to these individual research volunteers. Hybridization probes were prepared with precautions to avoid contamination, as described (Affymetrix assay 6.0, [19]). For each pool, 250 ng of pooled DNA was processed, labeled, and hybridized to Affymetrix 6.0 arrays according to the instructions of the manufacturer (Affymetrix) and previous studies (16,19,20). Quality controls for assays were performed as recommended (Affymetrix, Supplementary Information).

Identification of Nominally Positive SNPs

Allele frequencies for each SNP in each DNA pool were assessed based on hybridization to the 3–4 "perfect match" cells on each of three arrays, as described (19,20). We have validated this approach and identified approximately 0.98 correlations between SNP genotypes identified in individual versus pooled samples using this approach (19,20). We deleted data from SNPs on sex chromosomes, allowing us to combine data from male and female subjects and increase overall power. We also deleted data for SNPs whose chromosomal positions could not be determined adequately.

Identification of Genomic Regions and Genes that Contained Clustered Positive SNP Data from These Samples

We performed preplanned primary analyses as described previously. We identified SNPs that display t values with $P < 0.01$ "nominally positive" significance in comparison of data from quit success versus failure pools, and cluster in small chromosomal regions, so that at least four of these nominally positive SNPs lie within 25 kb of other reproducibly positive SNPs. A number of these clustered, nominally positive SNPs identify genes; many of them also lie between currently annotated genes. We identify different subsets of these clusters when we impose criteria for < 100 kb, < 50 kb, and < 10 kb distances between reproducibly positive SNPs.

To seek additional support for the chromosomal regions identified by these clusters of nominally positive SNPs, we sought additional association signals in these same regions from clustered, nominally positive SNPs identified in relevant independent GWA studies:

i) 500,000 to 600,000 SNP GWA studies of smokers who were successful versus unsuccessful in quitting in clinical trial settings (three independent samples from Uhl *et al*. [8]);
ii) 500,000 SNP GWA study of smokers who quit versus those who continued to smoke in community settings (Drgon *et al*. [9]);
iii) 38,000 SNP genome-wide association studies of nondependent (FTND) versus dependent (FTND) smokers (Bierut *et al*. [12]); and
iv) data from European-American subjects in studies of dependence on illegal addictive substances (21) (T Drgon, Q-R Liu, P-W Zhang, C Johnson, D Walther, C-Y Li, J Hess, and GR Uhl. Genome wide association for addiction: replicated results and comparisons of two analytic approaches [submitted]) (Table 1).

To provide insights into some of the genes likely to harbor variants that con-

Table 1. Genomic regions with variants likely to contribute to individual differences in smoking cessation success based on data from the current study. The data highlight the same small chromosomal regions identified by clustered nominally positive SNPs from at least one other GWA study of ability to quit smoking or of vulnerability to dependence on addictive substances.

chr[a]	bp:start	bp:end[b]	# SNPs[c]	Confirm sample[d]	# Confirm SNPs[e]	Genes[f]
1	29,920,187	29,948,179	4	C	2[g]	
1	***167,937,772***	***167,975,898***	***5***	***E***	***4***	***SELL, SELE***[h]
2	**9,192,143**	**9,207,808**	**7**	**E**	**6**	
2	*100,649,980*	*100,682,660*	*9*	*C*	*3*	
2	139,403,085	139,450,494	4	B	2	
2	**221,891,738**	**221,913,606**	**9**	**E**	**4**	
2	***224,903,889***	***224,953,132***	***7***	***F***	***2***	***FLJ22746***
2	***231,987,255***	***232,009,914***	***5***	***F***	***1***	
3	10,972,362	10,995,033	9	C	2	
3	***35,749,993***	***35,762,465***	***5***	***E***	***4***	***ARPP-21***[j], ***MIRN128B***
3	38,734,469	38,778,513	4	B	3	SCN10A
3	141,216,152	141,287,770	7	B	3	CLSTN2[j]
3	141,800,311	141,835,923	4	A	9	
3	**141,800,311**	**141,835,923**	**4**	**F**	**1**	
3	**144,085,356**	**144,113,673**	**4**	**E**	**16**	**PCOLCE2**
3	146,729,297	146,774,561	7	A	3	
4	***41,672,864***	***41,833,889***	***4***	***E***	***16***	***ATP1BL1***, ***CCD4***, ***SLC30A9***[k]
4	126,093,238	126,113,552	6	D, ***E***	4,***4***	
4	126,193,075	126,232,652	4	D	4	
4	168,191,419	168,193,048	4	C	3	SPOCK3[k]
5	168,177,383	168,207,535	5	C	3	SLIT3
6	**86,706,424**	**86,761,974**	**6**	**E**	**5**	
6	**98,227,102**	**98,268,675**	**5**	**E**	**4**	
6	105,161,737	105,193,915	4	B	2	
6	144,960,512	144,982,008	4	C	2	UTRN
7	**42,471,602**	**42,487,544**	**4**	**E**	**8**	
8	3,909,332	3,944,372	4	C,D,***E***	3,6,***9***	CSMD1
9	89,373,187	89,380,485	4	B	3	DAPK1
9	***102,617,616***	***102,659,836***	***6***	***E***	***12***	
9	***102,809,194***	***102,826,144***	***6***	***F***	***1***	***PRG-3***
10	2,734,009	2,811,707	8	B	2	[k]
10	49,758,250	49,810,114	6	A,B	4,8	LRRC18[k]
10	***49,758,250***	***49,810,114***	***6***	***F***	***1***	
10	***66,303,948***	***66,409,111***	***13***	***E***	***7***	
10	69,720,623	69,731,358	4	D	4	MAWBP
10	**88,086,066**	**88,108,932**	**5**	**F**	**3**	**GRID1**
10	108,879,748	108,964,249	9	B,C	3,2	SORCS1
10	118,957,450	118,976,703	4	B	4	KCNK18[k]
11	81,985,443	82,024,265	4	B,E	3,***4***	
11	***91,649,044***	***91,675,441***	***4***	***E***	***4***	
11	***108,260,231***	***108,310,082***	***7***	***E***	***4***	***DDX10***
11	117,090,924	117,119,023	4	C	2	DSCAML1[k]
12	221,728	248,990	5	B	2	SLC6A13[k]
12	**3,432,200**	**3,452,279**	**4**	**F**	**1**	
12	32,976,465	33,009,217	7	C,***F***	2,***1***	
12	33,039,131	33,072,493	6	C	2	
12	104,124,882	104,149,349	4	D	3	APPL2

Continued

tribute to individual differences in ability to quit, we identified candidate genes that are identified by overlapping clusters of positive SNPs from the current and at least one other quit success or addiction vulnerability sample in Table 1.

We compared observed results to those expected by chance using 10,000 Monte Carlo simulation trials, as described (21). For each trial, a randomly selected set of SNPs from the current data set was assessed to see if it provided results ≥ than the results that we actually observed. The number of trials for which the randomly selected SNPs displayed (at least) the same features as the observed results was then tallied to generate an empirical *P* value. These simulations thus correct for the number of repeated comparisons made in these analyses, and are an important consideration in evaluating these GWA data sets.

To assess the power of our current approach, we used current sample sizes and standard deviations, the program PS v2.1.31 (22,23) (see http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize) and $\alpha = 0.05$. To provide controls for the possibilities that abuser-control differences observed herein were due to either occult ethnic/racial allele frequency differences, or noisy assays, we assessed the overlap between the results obtained here and the SNPs that displayed the largest allele frequency differences between African-American versus European-American control individuals and the largest assay "noise."

Identification of Nominally Positive CNV Probes

Allele frequencies for each CNV probe in each DNA pool were assessed based on hybridization to the single "perfect match" cell on each of three arrays, as described (T Drgon, Q-R Liu, C Johnson, P-W Zhang, D Walther, and GR Uhl, Copy number variant probe data and vulnerability to substance dependence [in preparation]). In studies that have sought to validate this

Table 1. *Continued.*

chr[a]	bp:start	bp:end[b]	# SNPs[c]	Confirm sample[d]	# Confirm SNPs[e]	Genes[f]
12	**115,255,355**	**115,276,560**	**4**	**F**	**1**	
13	33,740,610	33,765,548	4	C	2	
13	44,202,415	44,209,971	4	C	2	
13	***60,112,584***	***60,160,245***	***15***	***E***	***6***	[k]
13	***61,658,287***	***61,687,708***	***4***	***F***	***1***	
14	***102,690,084***	***102,714,789***	***5***	***E***	***5***	***RPL21P12***
15	24,857,276	24,876,109	4	C	2	
16	4,508,770	4,534,672	4	B	2	C16 or f5, HMOX2[k]
16	80,978,225	81,035,927	6	A	3	
17	47,051,861	47,087,703	4	B	2	CA10[l]
18	4,636,261	4,670,052	4	A	6	
18	10,027,213	10,030,445	5	C	4	[k]
18	***54,104,584***	***54,136,429***	***4***	***E***	***5***	***NEDD4L***
20	17,715,035	17,728,921	6	B	4	
20	41,966,838	41,982,230	4	A	4	C20 or f100
20	53,283,389	53,307,708	5	B,D	2,5	[k]
21	32,721,019	32,799,427	9	A	3	C21 or f63
22	**48,374,819**	**48,392,082**	**4**	**B**	**2**	**C22 or f34**

[a]Chromosome for the genomic region identified by clustered nominally positive SNPs from the current study and supporting data from at least one other study.
[b]Basepair coordinates for the beginning and end of the genomic region.
[c]Number of nominally positive SNPs that lay in clusters within the region in the current sample
[d]Code for the confirmatory sample(s): A, Lerman; B, Brown; C, Rose from (8); D, Hamer (9); E, data from European-American samples from comparisons of substance dependent to control samples (Drgon submitted); and F, data from comparison of 38,000 SNPs from (12).
[e]Number of SNPs in the confirmatory sample that fall into the chromosomal region defined by the current sample.
[f]Annotated gene(s) that fall within the chromosomal region, if any.
[g]Boldfaced segments are those identified by data from at least two smoking cessation studies.
[h]Italicized segments are those identified by data from the current smoking cessation study and at least one addiction vulnerability GWA sample.
[i]Genes for which "NRT-selective" effects have been previously suggested in eTable 4 in (8).
[k]Genes (or chromosomal regions) for which some (but not all) of the nominally positive SNPs fall into the group of SNPs with the 2.5% largest differences between control European American versus African American research volunteers in prior studies (C Johnson, T Drgon, and GR Uhl, unpublished observations, 2008).

method, we have identified many CNV probes that display good (> 0.9) correlations between CNV genotypes in individual versus pooled samples using this approach, but also identified subsets of CNV probes with poor correlations (< 0.02) (Drgon *et al*. [same as above in this paragraph], in preparation). For primary analyses, we sought CNV probes that display t values with $P <$ 0.05 significance in quit success versus failure comparisons and cluster, so that 4 to 10 reproducibly positive CNV probes lie within 25 kb of other reproducibly positive CNV probes.

All supplementary materials are available online at www.molmed.org.

RESULTS

We assessed allele frequencies in multiple pools of DNA from smokers who remained abstinent for at least 4 wks after the end of treatment versus those who were not abstinent at any time point. For SNPs, there was modest variability among replicate arrays (n = 3) that assessed the same pool, the standard error of the mean (SEM) value was 0.015. There also was modest variability among the different pools that assessed the same phenotype; SEM was 0.024. For the quitter versus non-quitter comparison, these samples and these estimates of variance thus provided 0.17, 0.56, 0.89, and 0.98 nominal power to detect allele frequency differences of 2.5%, 5%, 7.5%, and 10%, respectively. The allele frequency differences used in these power calculations are comparable to the actual mean allele frequency differences for the nominally significant SNPs for unsuccessful versus successful quitters in these studies.

Unsuccessful Versus Successful Quitters

When we compared data from unsuccessful versus successful quitters, there was significant clustering of nominally positive SNPs in small chromosomal regions. There were 7,931 "nominally positive" SNPs that displayed allele frequency differences with nominal $P <$ 0.01. Of these nominally positive SNPs, 1,361 fell into 249 clusters of at least four nominally positive SNPs separated from each other by ≤ 25 kb. Monte Carlo P values for this degree of clustering were $P <$ 0.0001. By chance, we would have expected only 29 SNPs to cluster in this fashion. Applying differing sets of criteria based on distances of 100, 50, and 10 kb between nominally positive SNPs led to identification of 2,756, 2,041, and 661 clustered, nominally positive SNPs, in 464, 361, and 138 clusters, respectively (all $P <$ 0.0001, data not shown).

Overlap with Data from Four Previously Reported Quit Success Samples

These data for clustered, nominally positive SNPs from the current data set provide significant overlap with genes identified by other relevant data sets. They thus identify the same small chromosomal regions within genes that are identified by nominally positive results in other studies to extents much greater than

we would expect by chance. The clustered nominally positive SNPs from the current data set provide highly significant overlap with data from each of the three quit success samples reported by Uhl *et al*. (8) (for example, of ≥ four nominally positive SNPs within 25 kb of each other identified by comparisons between successful versus unsuccessful quitters). Overlaps with the samples from Lerman *et al*., Niaura *et al*., and Rose *et al*. identified 34, 80, and 98 clusters of nominally positive SNPs, respectively ($P < 0.0001$ for each comparison). These four SNP/25 kb clusters also displayed significant overlap with clustered, nominally positive SNPs reported by Drgon *et al*. (9). Overlaps with these data identified 27 clusters of nominally positive SNPs ($P < 0.0001$, 1 would have been expected by chance). These overlaps between the clustered, nominally positive SNPs from the current sample and the clustered, nominally positive SNPs from at least one other sample of successful versus unsuccessful quitters identify 40 chromosomal regions that contain 20 annotated genes (see Table 1).

Overlap with Data from Substance Dependence Phenotypes

The clusters of four nominally positive SNPs from the data that compare individuals who are successful versus those who are unsuccessful in quitting smoking in the current trial also overlap at greater than chance levels with data from studies that compare nondependent to dependent smokers and studies that compare substance dependent to nondependent individuals who have little lifetime use of any addictive substance. The overlaps between the clustered, nominally positive SNPs from the current sample and the clustered, nominally positive SNPs from at least one other sample comparing nondependent to dependent individuals identify 29 chromosomal regions that contain 13 annotated genes ($P < 0.001$, see Table 1).

Possible Alternative Explanations for Observed Results

We would anticipate the observed, highly significant clustering of SNPs that display nominally positive results if many of these reproducibly positive SNPs lay near, and were in linkage disequilibrium with functional allelic variants that distinguished subjects who were more able to quit smoking from those who were less able. We would not anticipate this degree of clustering if the results were due to chance. The Monte Carlo *P* values noted here are thus likely to receive contributions from both the extent of linkage disequilibrium among the clustered, nominally positive SNPs and the extent of linkage disequilibrium between these SNPs and the functional haplotype(s) that lead to the association with quit success.

Control for occult stratification was based on examining the overlap between the 1,361 clustered positive SNPs from the present quit success analyses with the 2.5% of the SNPs for which the racial/ethnic differences in control individuals from prior data sets were largest. There was a mild excess of such SNPs; we identified 64 when 35 would have been expected by chance. Principal components analyses did not provide evidence for overall stratification, however (data not shown). In Table 1, we thus indicate the SNP clusters that contain SNPs which do display sizable racial/ethnic differences, to allow additional cautions in interpreting these data. It is important to note that none of these genes were identified only by SNPs whose allele frequencies fell into those with the 2.5% largest differences between African-American versus European-American control individuals.

Controls for noisy SNPs identified many fewer than expected by chance. None of the clustered nominally positive SNPs overlapped with the set of SNPs that provided the largest variance, while 34 would be expected by chance.

Identification of Nominally Positive CNV Probes

Nominally significant differences between successful and unsuccessful quitters were displayed by 75,915 CNV probes. There was no significant difference from values expected by chance when we examined clusters of ≥ 4 to ≥ 8 nominally positive CNV probes. However, when we examined clusters of ≥ 9 nominally positive CNV probes, we identified 3,771 CNV probes that fell into 344 such clusters in ways that were found in only 79 of 10,000 Monte Carlo trials ($P = 0.0079$). The genes identified by these clusters of ≥ 9 nominally significant CNV probes are listed in Supplemental Table S1.

DISCUSSION

The current results provide independent support for GWA results from prior studies of smoking cessation success in clinical trial and community settings. The substantial overlaps between these data and those obtained previously support the idea that ability to quit in a circumstance in which the most participants used denicotinized cigarettes shares strong overall genetic influences with ability to quit when other pharmacological strategies were used. None of the current data exclude potential influences of allelic variants that are selective to success in using Quest denicotinized cigarettes. Confirmation of such allelic variants will require additional studies using independent samples, however. The current results also support a significant, though more modest, overlap between allelic variants that alter vulnerability to develop dependence on an addictive substance versus those that alter ability to quit smoking, as we have noted in prior analyses (24).

These observations can be discussed in light of the strengths and limitations of the current data sets. The data display several strengths: i) the similar clinical characteristics and the small pool-to-pool variation provide reassurance that these smoking cessation subjects may not differ markedly from the European-American smoking cessation subjects who were studied previously; ii) the subjects were followed carefully with biochemical monitoring of self-reported abstinence; iii) we used pooled genotyping methods that have been validated exten-

sively in prior work and that demonstrate small variance in the current studies; and iv) many more of the positive results from this work than we would expect by chance identify the same chromosomal regions that were identified by other studies of smoking cessation and/or vulnerability to develop substance dependence in European-American samples. In prior work, we have compared results from trials of nicotine replacement to results from trials of bupropion to tentatively nominate genes with "NRT-selective" and "bupropion-selective" effects. It is interesting that current results, from a study of reduced-nicotine cigarettes, identified three of the "NRT-selective" but none of the "bupropion-selective" genes (eTable 4 in [8]).

There are major and minor limitations of the present work. The major limitation is based on the relatively modest number of successful quitters in this trial for whom we have DNA available. Though we have worked to match these individuals to twice their number of nonquitters, limitations from this modest sample size reduce our confidence in the genes that are identified in this work but not in prior studies, and negative data concerning any gene that has been reproducibly identified in prior studies but not in the current work. In addition, the power of this sample is insufficient and does not allow us to split successful quitters based on the treatment to which they were assigned. While we can thus make general statements about the likelihood that the allelic variants that we identify here might impact individual differences in response to Quest or other denicotinized cigarettes based on the use of these cigarettes by most successful quitters, our results do include data from a small group who were able to quit without this aid. Finally, though we have provided extensive validation of the pooled genotyping methods for the SNP probes used here, the modest variance introduced by the pooling approach combined with the modest sample size does provide a modest additional limit to interpretation of data from SNP probes. The large number of CNV probes for which pooling data provides only a modest reflection of data that would be obtained from individual genotyping provides an even greater caution in interpreting the negative data from the CNV probe data sets. Conceivably, these differences could contribute to observations that significant results for the chromosomal clustering of these probes only arise when we require nominally significant differences in at least nine nearby CNV probes.

Despite these limitations, the current data add appreciably to the increasingly robust sets of studies that document clear molecular genetic contributions to the ability to quit smoking in research and community settings. It is conceivable that such findings also may identify some of the genetic determinants of generalized abilities to change health-related behaviors. Thus, for both dependent individuals and individuals with other health problems that can be modified through behavior change, this data adds to an increasingly rich basis for improved understanding and for development of personalized treatment strategies.

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DISCLOSURE

T Drgon, C Johnson, and D Walther report no conflict of interest for this study. JE Rose and GR Uhl are listed as inventors on a patent application filed by Duke University based on genomic markers that distinguish successful quitters from unsuccessful quitters in data from other clinical trials. AP Albino is an employee of Vector Tobacco Inc., which has a financial interest in the product upon which the work was based.

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cluster	chr	cluster start	cluster stop	cluster size	#cnv in cluster	gene	clustered cnv in gene	description
1	1	22,398,760	22,512,713	113,953	10			
2	1	25,289,336	25,343,403	54,067	9			
3	1	39,709,177	39,800,367	91,190	9	BMP8A	4	bone morphogenetic protein 8a
3	1	39,709,177	39,800,367	91,190	9	MACF1	4	microtubule-actin crosslinking factor 1
4	1	62,141,345	62,219,511	78,166	14	INADL	14	InaD-like (Drosophila)
5	1	65,037,729	65,162,358	124,629	22	JAK1	8	Janus kinase 1 (a protein tyrosine kinase)
5	1	65,037,729	65,162,358	124,629	22	RAVER2	4	ribonucleoprotein, PTB-binding 2
6	1	89,190,622	89,284,890	94,268	10	GBP3	4	guanylate binding protein 3
6	1	89,190,622	89,284,890	94,268	10	KAT3	4	kynurenine aminotransferase III
7	1	90,527,937	90,621,665	93,728	9			
8	1	113,258,086	113,345,893	87,807	10	SLC16A1	6	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)
9	1	113,854,842	113,949,669	94,827	10	MAGI3	10	membrane associated guanylate kinase, WW and PDZ domain containing 3
10	1	118,179,163	118,272,332	93,169	11	GDAP2	6	ganglioside induced differentiation associated protein 2
11	1	150,808,827	150,864,608	55,781	13	LCE3C	5	late cornified envelope 3C
11	1	150,808,827	150,864,608	55,781	13	LCE3D	5	late cornified envelope 3D
11	1	150,808,827	150,864,608	55,781	13	LCE3A	3	late cornified envelope 3A
11	1	150,808,827	150,864,608	55,781	13	LCE3B	3	late cornified envelope 3B
12	1	154,781,734	154,885,466	103,732	9	IQGAP3	4	IQ motif containing GTPase activating protein 3
12	1	154,781,734	154,885,466	103,732	9	HAPLN2	3	hyaluronan and proteoglycan link protein 2
13	1	156,694,163	156,793,196	99,033	11	OR6Y1	6	olfactory receptor, family 6, subfamily Y, member 1
14	1	161,133,127	161,186,023	52,896	9			
15	1	177,395,299	177,479,395	84,096	9	ABL2	7	v-abl Abelson murine leukemia viral oncogene homolog 2 (arg, Abelson-related gene)
16	1	191,405,979	191,480,085	74,106	14	CDC73	14	cell division cycle 73, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)
16	1	191,405,979	191,480,085	74,106	14	B3GALT2	7	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2
17	1	200,523,777	200,575,327	51,550	10	LGR6	8	leucine-rich repeat-containing G protein-coupled receptor 6
17	1	200,523,777	200,575,327	51,550	10	UBE2T	3	ubiquitin-conjugating enzyme E2T (putative)
18	1	211,769,716	211,840,529	70,813	9			
19	1	217,503,879	217,590,162	86,283	11			
20	1	225,034,057	225,159,365	125,308	10	PSEN2	5	presenilin 2 (Alzheimer disease 4)
21	1	244,038,573	244,182,584	144,011	13	SMYD3	13	SET and MYND domain containing 3
22	2	10,098,170	10,233,041	134,871	9	FLJ25102	4	hypothetical protein FLJ25102
23	2	17,977,502	18,087,291	109,789	13			
24	2	25,415,683	25,522,450	106,767	9	DTNB	7	dystrobrevin, beta
25	2	34,564,317	34,608,463	44,146	12			
26	2	35,426,486	35,538,200	111,714	12			
27	2	37,836,767	37,973,006	136,239	11			
28	2	54,906,806	54,995,856	89,050	10			
29	2	67,181,284	67,264,115	82,831	10			
30	2	71,192,304	71,226,817	34,513	10	MPHOSPH10	9	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
30	2	71,192,304	71,226,817	34,513	10	MCEE	4	methylmalonyl CoA epimerase
31	2	75,201,890	75,240,237	38,347	9	TACR1	9	tachykinin receptor 1
32	2	83,342,903	83,407,138	64,235	10			
33	2	83,779,296	83,866,270	86,974	9			
34	2	97,634,808	97,720,251	85,443	9	ZAP70	4	zeta-chain (TCR) associated protein kinase 70kDa
34	2	97,634,808	97,720,251	85,443	9	ACTR1B	3	ARP1 actin-related protein 1 homolog B, centractin beta (yeast)
35	2	113,314,394	113,437,799	123,405	9			
36	2	126,087,887	126,255,510	167,623	17			
37	2	132,983,679	133,039,177	55,498	9	GPR39	9	G protein-coupled receptor 39
38	2	146,594,853	146,686,713	91,860	12			
39	2	151,272,537	151,347,436	74,899	9			
40	2	152,865,952	152,921,371	55,419	9	FMNL2	3	formin-like 2
41	2	189,256,604	189,292,583	35,979	9			
42	2	191,890,367	192,046,482	156,115	9	MYO1B	7	myosin IB
43	2	194,499,883	194,662,308	162,425	14			
44	2	197,194,742	197,342,763	148,021	12	FLJ39660	9	hypothetical protein FLJ39660
45	2	203,004,008	203,051,032	47,024	11	BMPR2	11	bone morphogenetic protein receptor, type II (serine/threonine kinase)
46	2	205,011,363	205,054,797	43,434	10			
47	2	205,563,661	205,768,225	204,564	14	PARD3B	14	par-3 partitioning defective 3 homolog B
48	2	206,844,783	206,922,419	77,636	9	KIAA1571	5	KIAA1571 protein
49	2	208,048,050	208,088,022	39,972	9			
50	2	226,596,290	226,711,950	115,660	11			
51	2	237,428,008	237,478,236	50,228	9			
52	2	242,564,127	242,629,390	65,263	10			
53	3	5,928,426	6,012,091	83,665	9			
54	3	10,211,634	10,293,101	81,467	10	IRAK2	8	interleukin-1 receptor-associated kinase 2
54	3	10,211,634	10,293,101	81,467	10	TATDN2	5	TatD DNase domain containing 2
55	3	12,584,551	12,667,041	82,490	15	RAF1	14	v-raf-1 murine leukemia viral oncogene homolog 1
55	3	12,584,551	12,667,041	82,490	15	MKRN2	5	makorin, ring finger protein, 2
56	3	25,536,801	25,610,168	73,367	10	RARB	10	retinoic acid receptor, beta
56	3	25,536,801	25,610,168	73,367	10	TOP2B	3	topoisomerase (DNA) II beta 180kDa
57	3	41,214,098	41,254,695	40,597	19	CTNNB1	19	catenin (cadherin-associated protein), beta 1, 88kDa
58	3	45,769,696	45,887,234	117,538	9	SLC6A20	3	solute carrier family 6 (proline IMINO transporter), member 20
59	3	53,006,861	53,069,329	62,468	14	SFMBT1	13	Scm-like with four mbt domains 1
60	3	67,588,118	67,660,416	72,298	10	SUCLG2	10	succinate-CoA ligase, GDP-forming, beta subunit
61	3	89,420,895	89,509,195	88,300	12	EPHA3	12	EPH receptor A3
62	3	105,493,652	105,633,078	139,426	14			
63	3	109,186,288	109,265,686	79,398	11	CD47	4	CD47 molecule
64	3	132,803,354	132,877,309	73,955	9	CPNE4	9	copine IV
65	3	176,511,295	176,574,679	63,384	11	NAALADL2	11	N-acetylated alpha-linked acidic dipeptidase-like 2
66	3	178,177,748	178,229,845	52,097	11			
67	3	187,244,390	187,304,660	60,270	21	ETV5	21	ets variant gene 5 (ets-related molecule)
68	4	17,108,592	17,207,643	99,051	11	QDPR	3	quinoid dihydropteridine reductase
69	4	17,784,038	17,880,244	96,206	9			
70	4	23,063,614	23,158,504	94,890	10			
71	4	28,525,236	28,624,907	99,671	9			
72	4	43,481,804	43,610,871	129,067	12			

73	4	54,797,869	54,857,515	59,646	11	PDGFRA	11	platelet-derived growth factor receptor, alpha polypeptide
74	4	55,223,109	55,298,254	75,145	21	KIT	21	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
75	4	63,298,520	63,377,587	79,067	12			
76	4	64,380,052	64,390,841	10,789	14			
77	4	74,509,821	74,610,294	100,473	12	AFM	5	afamin
77	4	74,509,821	74,610,294	100,473	12	AFP	4	alpha-fetoprotein
78	4	75,239,929	75,360,322	120,393	9	MTHFD2L	9	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2-like
79	4	88,878,430	88,983,707	105,277	18	IBSP	4	integrin-binding sialoprotein (bone sialoprotein, bone sialoprotein II)
79	4	88,878,430	88,983,707	105,277	18	MEPE	3	matrix, extracellular phosphoglycoprotein with ASARM motif (bone)
80	4	108,287,735	108,351,836	64,101	9			
81	4	108,409,908	108,475,419	65,511	10			
82	4	115,331,847	115,433,402	101,555	13			
83	4	116,380,547	116,441,355	60,808	10			
84	4	140,199,359	140,273,278	73,919	16	ELF2	16	E74-like factor 2 (ets domain transcription factor)
85	4	152,027,500	152,159,796	132,296	11	LRBA	11	LPS-responsive vesicle trafficking, beach and anchor containing
86	4	154,842,482	154,940,775	98,293	9	RNF175	7	ring finger protein 175
87	4	157,027,780	157,104,373	76,593	10	CTSO	7	cathepsin O
87	4	157,027,780	157,104,373	76,593	10	TDO2	3	tryptophan 2,3-dioxygenase
88	4	161,161,181	161,289,626	128,445	11			
89	4	179,097,623	179,127,412	29,789	9			
90	4	188,098,031	188,142,047	44,016	9			
91	4	189,409,493	189,471,407	61,914	10			
92	4	189,951,258	190,037,170	85,912	12			
93	5	8,794,776	8,880,625	85,849	10			
94	5	19,015,612	19,121,529	105,917	10			
95	5	24,517,374	24,589,190	71,816	9	CDH10	9	cadherin 10, type 2 (T2-cadherin)
96	5	33,557,837	33,587,354	29,517	9	ADAMTS12	9	ADAM metallopeptidase with thrombospondin type 1 motif, 12
97	5	45,711,110	45,820,789	109,679	10	HCN1	3	hyperpolarization activated cyclic nucleotide-gated potassium channel 1
98	5	101,093,410	101,150,058	56,648	9			
99	5	101,208,001	101,234,776	26,775	9			
100	5	105,814,087	105,913,768	99,681	10			
101	5	112,169,610	112,205,555	35,945	9	APC	9	adenomatosis polyposis coli
102	5	114,701,746	114,759,557	57,811	9	CTNNAP1	4	catenin (cadherin-associated protein), alpha pseudogene 1
103	5	155,362,342	155,449,494	87,152	9			
104	5	179,593,618	179,664,674	71,056	9	MAPK9	7	mitogen-activated protein kinase 9
105	6	272,519	343,177	70,658	12	DUSP22	8	dual specificity phosphatase 22
106	6	11,403,401	11,465,302	61,901	9			
107	6	14,739,288	14,847,594	108,306	12			
108	6	22,085,386	22,146,846	61,460	10			
109	6	27,776,995	27,874,093	97,098	11			
110	6	37,377,553	37,462,660	85,107	9	RNF8	5	ring finger protein 8
110	6	37,377,553	37,462,660	85,107	9	TBC1D22B	4	TBC1 domain family, member 22B
111	6	51,280,194	51,380,710	100,516	11			
112	6	52,732,545	52,830,487	97,942	11	GSTA2	4	glutathione S-transferase A2
112	6	52,732,545	52,830,487	97,942	11	GSTA5	3	glutathione S-transferase A5
113	6	72,837,167	72,865,665	28,498	9	RIMS1	9	regulating synaptic membrane exocytosis 1
114	6	74,681,738	74,793,445	111,707	9			
115	6	79,057,989	79,082,851	24,862	11			
116	6	84,792,441	84,867,900	75,459	10	C6orf117	9	chromosome 6 open reading frame 117
117	6	124,233,783	124,312,262	78,479	10	NKAIN2	10	
118	6	135,401,714	135,501,429	99,715	10	HBS1L	4	HBS1-like (S. cerevisiae)
119	6	135,527,819	135,578,353	50,534	11	MYB	10	v-myb myeloblastosis viral oncogene homolog (avian)
120	6	148,292,165	148,370,451	78,286	12			
121	6	152,247,361	152,317,804	70,443	11	ESR1	11	estrogen receptor 1
122	7	3,326,142	3,418,230	92,088	9	SDK1	9	sidekick homolog 1 (chicken)
123	7	9,917,844	10,002,306	84,462	9			
124	7	13,085,532	13,130,874	45,342	10			
125	7	13,950,772	13,979,752	28,980	11	ETV1	11	ets variant gene 1
126	7	18,975,523	19,075,259	99,736	13	HDAC9	6	histone deacetylase 9
127	7	19,140,707	19,211,977	71,270	9			
128	7	28,969,528	29,146,202	176,674	16	CPVL	13	carboxypeptidase, vitellogenic-like
129	7	46,898,996	46,996,032	97,036	10			
130	7	55,094,409	55,186,676	92,267	17	EGFR	17	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)
131	7	57,460,390	57,521,568	61,178	11	ZNF716	4	zinc finger protein 716
132	7	63,271,509	63,323,784	52,275	9			
133	7	79,074,393	79,141,511	67,118	9			
134	7	87,022,520	87,103,244	80,724	11	ABCB1	11	ATP-binding cassette, sub-family B (MDR/TAP), member 1
134	7	87,022,520	87,103,244	80,724	11	RPIB9	3	Rap2-binding protein 9
135	7	92,117,539	92,299,353	181,814	18	CDK6	18	cyclin-dependent kinase 6
136	7	92,328,116	92,348,286	20,170	12			
137	7	101,440,780	101,552,899	112,119	10	CUTL1	10	cut-like 1, CCAAT displacement protein (Drosophila)
138	7	114,474,582	114,548,901	74,319	9			
139	7	115,659,732	115,784,213	124,481	13	TES	5	testis derived transcript (3 LIM domains)
140	7	116,136,240	116,221,845	85,605	13	MET	13	met proto-oncogene (hepatocyte growth factor receptor)
141	7	127,521,427	127,632,648	111,221	13	SND1	3	staphylococcal nuclease domain containing 1
142	7	127,666,668	127,715,932	49,264	9	LEP	3	leptin (obesity homolog, mouse)
143	7	140,147,483	140,268,920	121,437	16	BRAF	16	v-raf murine sarcoma viral oncogene homolog B1
144	7	140,469,629	140,558,092	88,463	9			
145	7	141,903,092	142,007,637	104,545	15	TRB@	15	T cell receptor beta locus
145	7	141,903,092	142,007,637	104,545	15	TRBV10-3	4	T cell receptor beta variable 10-3
145	7	141,903,092	142,007,637	104,545	15	TRBV11-3	4	T cell receptor beta variable 11-3
145	7	141,903,092	142,007,637	104,545	15	TRBV15	4	T cell receptor beta variable 15
145	7	141,903,092	142,007,637	104,545	15	TRBV12-5	3	T cell receptor beta variable 12-5
145	7	141,903,092	142,007,637	104,545	15	TRBV13	3	T cell receptor beta variable 13
145	7	141,903,092	142,007,637	104,545	15	TRBV14	3	T cell receptor beta variable 14
145	7	141,903,092	142,007,637	104,545	15	TRBV16	3	T cell receptor beta variable 16
145	7	141,903,092	142,007,637	104,545	15	TRBV5-8	3	T cell receptor beta variable 5-8
146	7	142,883,633	142,963,469	79,836	11			

147	7	143,216,390	143,361,591	145,201	17	OR6B1	4	olfactory receptor, family 6, subfamily B, member 1
148	7	144,901,737	144,981,307	79,570	9			
149	7	145,494,792	145,608,040	113,248	10	CNTNAP2	10	contactin associated protein-like 2
150	7	152,772,617	152,858,146	85,529	9			
151	7	153,736,584	153,789,952	53,368	9			
152	8	2,667,236	2,762,504	95,268	9			
153	8	4,125,364	4,181,926	56,562	11	CSMD1	11	CUB and Sushi multiple domains 1
154	8	6,023,064	6,158,627	135,563	24			
155	8	8,363,718	8,446,979	83,261	9			
156	8	24,520,028	24,566,066	46,038	10			
157	8	25,025,458	25,068,170	42,712	13			
158	8	32,710,696	32,838,994	128,298	12	NRG1	3	neuregulin 1
159	8	38,354,638	38,440,583	85,945	12	FGFR1	10	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)
160	8	42,226,250	42,325,129	98,879	15	IKBKB	12	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
160	8	42,226,250	42,325,129	98,879	15	POLB	4	polymerase (DNA directed), beta
161	8	60,867,420	60,981,931	114,511	10			
162	8	63,363,511	63,486,037	122,526	10	NKAIN3	10	
163	8	64,949,241	65,035,972	86,731	9			
164	8	85,391,046	85,466,753	75,707	14			
165	8	93,043,746	93,095,977	52,231	10	RUNX1T1	10	runt-related transcription factor 1; translocated to, 1 (cyclin D-related)
166	8	120,090,206	120,144,789	54,583	9			
167	8	126,640,170	126,713,113	72,943	9			
168	8	126,893,544	126,983,027	89,483	10			
169	8	131,985,423	132,071,897	86,474	9	ADCY8	9	adenylate cyclase 8 (brain)
170	8	134,190,336	134,248,176	57,840	12	TG	5	thyroglobulin
171	8	137,557,506	137,675,164	117,658	10			
172	8	141,551,417	141,647,658	96,241	9	EIF2C2	5	eukaryotic translation initiation factor 2C, 2
173	9	587,042	719,027	131,985	16	ANKRD15	16	ankyrin repeat domain 15
174	9	1,486,424	1,546,286	59,862	13			
175	9	2,953,302	3,002,218	48,916	9			
176	9	3,999,795	4,035,209	35,414	9	GLIS3	9	GLIS family zinc finger 3
177	9	4,971,257	5,124,615	153,358	20	JAK2	20	Janus kinase 2 (a protein tyrosine kinase)
177	9	4,971,257	5,124,615	153,358	20	IGHEP2	6	immunoglobulin heavy constant epsilon P2
178	9	8,533,416	8,625,992	92,576	10	PTPRD	10	protein tyrosine phosphatase, receptor type, D
179	9	10,508,294	10,561,886	53,592	9			
180	9	15,991,191	16,088,432	97,241	11			
181	9	16,662,901	16,735,580	72,679	11	BNC2	11	basonuclin 2
182	9	17,755,897	17,834,090	78,193	11			
183	9	71,219,408	71,230,364	10,956	9	APBA1	6	amyloid beta (A4) precursor protein-binding, family A, member 1 (X11)
184	9	77,398,330	77,512,144	113,814	9			
185	9	78,569,639	78,658,885	89,246	9	C9orf65	4	chromosome 9 open reading frame 65
186	9	82,952,058	83,039,528	87,470	9			
187	9	86,889,687	87,047,257	157,570	15			
188	9	91,743,956	91,828,958	85,002	10			
189	9	95,388,806	95,478,448	89,642	9	PHF2	9	PHD finger protein 2
190	9	96,798,603	96,907,384	108,781	9	C9orf3	7	chromosome 9 open reading frame 3
191	9	97,246,997	97,329,302	82,305	21	PTCH	19	patched homolog (Drosophila)
192	9	104,256,371	104,355,587	99,216	10			
193	9	105,855,524	105,889,881	34,357	9			
194	9	108,927,532	109,057,860	130,328	10			
195	9	113,336,124	113,457,280	121,156	9	bA16L21.2.1	3	DnaJ-like protein
195	9	113,336,124	113,457,280	121,156	9	ZNF483	3	zinc finger protein 483
196	9	117,570,409	117,673,598	103,189	9			
197	9	125,757,210	125,841,036	83,826	12	LHX2	7	LIM homeobox 2
198	9	131,587,721	131,688,908	101,187	9	USP20	6	ubiquitin specific peptidase 20
199	9	132,605,543	132,758,499	152,956	22	ABL1	22	v-abl Abelson murine leukemia viral oncogene homolog 1
200	9	134,710,807	134,823,571	112,764	16	TSC1	9	tuberous sclerosis 1
200	9	134,710,807	134,823,571	112,764	16	C9orf98	5	chromosome 9 open reading frame 98
200	9	134,710,807	134,823,571	112,764	16	C9orf9	3	chromosome 9 open reading frame 9
201	10	4,287,241	4,322,032	34,791	9			
202	10	4,411,971	4,502,216	90,245	11			
203	10	11,148,305	11,285,116	136,811	12	CUGBP2	12	CUG triplet repeat, RNA binding protein 2
204	10	12,961,507	13,046,588	85,081	9	CCDC3	8	coiled-coil domain containing 3
205	10	13,287,895	13,371,146	83,251	10	C10orf49	5	chromosome 10 open reading frame 49
205	10	13,287,895	13,371,146	83,251	10	RNU6B	3	RNA, U6B small nuclear
206	10	14,532,193	14,644,726	112,533	11	FAM107B	7	family with sequence similarity 107, member B
207	10	42,905,952	42,957,467	51,515	12	RET	11	ret proto-oncogene (multiple endocrine neoplasia and medullary thyroid carcinoma 1, Hirschspru
208	10	60,984,289	61,054,535	70,246	9			
209	10	82,435,703	82,509,035	73,332	12			
210	10	85,319,879	85,452,396	132,517	10			
211	10	86,117,837	86,267,641	149,804	13	KIAA1128	13	KIAA1128
212	10	89,669,390	89,781,762	112,372	16	PTEN	10	phosphatase and tensin homolog (mutated in multiple advanced cancers 1)
213	10	107,866,050	107,940,878	74,828	10			
214	10	123,247,250	123,345,467	98,217	14	FGFR2	14	fibroblast growth factor receptor 2 (bacteria-expressed kinase, keratinocyte growth factor receptor,
215	11	4,465,262	4,518,181	52,919	9	OR52M2P	3	olfactory receptor, family 52, subfamily M, member 2 pseudogene
216	11	5,438,657	5,519,955	81,298	9	OR51A10P	4	olfactory receptor, family 51, subfamily A, member 10 pseudogene
217	11	8,344,714	8,427,981	83,267	9	STK33	6	serine/threonine kinase 33
218	11	17,063,755	17,128,652	64,897	9	PIK3C2A	9	phosphoinositide-3-kinase, class 2, alpha polypeptide
219	11	18,908,495	18,954,001	45,506	10	MRGPRX1	5	MAS-related GPR, member X1
220	11	29,408,632	29,474,353	65,721	10			
221	11	35,153,927	35,214,241	60,314	9	CD44	9	CD44 molecule (Indian blood group)
222	11	36,046,586	36,147,047	100,461	10	LDLRAD3	10	low density lipoprotein receptor class A domain containing 3
223	11	68,231,032	68,364,574	133,542	9	CPT1A	6	carnitine palmitoyltransferase 1A (liver)
223	11	68,231,032	68,364,574	133,542	9	MTL5	4	metallothionein-like 5, testis-specific (tesmin)
224	11	74,767,766	74,834,323	66,557	9	FLJ33790	3	hypothetical protein FLJ33790
224	11	74,767,766	74,834,323	66,557	9	GDPD5	3	glycerophosphodiester phosphodiesterase domain containing 5
224	11	74,767,766	74,834,323	66,557	9	RPS3	3	ribosomal protein S3
224	11	74,767,766	74,834,323	66,557	9	SNORD15A	3	small nucleolar RNA, C/D box 15A

225	11	75,124,134	75,222,859	98,725	10	DGAT2	5	diacylglycerol O-acyltransferase homolog 2 (mouse)
225	11	75,124,134	75,222,859	98,725	10	UVRAG	3	UV radiation resistance associated gene
226	11	81,970,044	82,027,319	57,275	9			
227	11	85,497,874	85,585,125	87,251	10			
228	11	87,233,068	87,302,214	69,146	9			
229	11	90,636,839	90,757,499	120,660	9			
230	11	100,414,310	100,510,884	96,574	13	PGR	13	progesterone receptor
231	11	114,316,116	114,382,576	66,460	9			
232	11	117,859,556	117,898,066	38,510	9	MLL	9	myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
233	11	119,236,742	119,383,437	146,695	10			
234	11	124,160,645	124,245,269	84,624	9	C11orf61	3	chromosome 11 open reading frame 61
235	11	127,834,819	127,919,017	84,198	17	ETS1	16	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)
236	12	2,363,938	2,442,056	78,118	9	CACNA1C	9	calcium channel, voltage-dependent, L type, alpha 1C subunit
237	12	11,670,476	11,834,613	164,137	26	ETV6	24	ets variant gene 6 (TEL oncogene)
238	12	12,385,309	12,519,249	133,940	13	LOH12CR1	12	loss of heterozygosity, 12, chromosomal region 1
239	12	12,554,607	12,653,049	98,442	11	DUSP16	7	dual specificity phosphatase 16
240	12	22,095,602	22,145,860	50,258	11	CMAS	4	cytidine monophosphate N-acetylneuraminic acid synthetase
241	12	25,110,642	25,141,730	31,088	9	LRMP	9	lymphoid-restricted membrane protein
242	12	27,518,374	27,542,077	23,703	9			
243	12	33,185,915	33,229,238	43,323	14			
244	12	64,897,766	65,001,497	103,731	9			
245	12	65,184,570	65,281,353	96,783	9	GRIP1	9	glutamate receptor interacting protein 1
246	12	67,501,000	67,529,968	28,968	10	MDM2	10	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)
247	12	72,433,505	72,524,546	91,041	9			
248	12	73,008,797	73,116,391	107,594	10			
249	12	83,463,665	83,594,153	130,488	11			
250	12	104,584,553	104,679,268	94,715	9			
251	12	107,303,950	107,355,808	51,858	9			
252	12	114,704,316	114,802,782	98,466	11			
253	12	116,257,826	116,317,106	59,280	9	NOS1	4	nitric oxide synthase 1 (neuronal)
254	12	125,358,436	125,430,302	71,866	10			
255	13	27,518,109	27,598,174	80,065	15	FLT3	14	fms-related tyrosine kinase 3
256	13	31,785,226	31,839,661	54,435	13	BRCA2	13	breast cancer 2, early onset
257	13	42,088,164	42,130,775	42,611	9			
258	13	47,755,041	47,854,190	99,149	13	RB1	11	retinoblastoma 1 (including osteosarcoma)
259	13	66,114,145	66,232,454	118,309	11	PCDH9	11	protocadherin 9
260	13	69,619,642	69,680,559	60,917	11			
261	13	79,453,594	79,514,996	61,402	9			
262	13	82,685,952	82,750,389	64,437	9			
263	13	101,381,936	101,490,849	108,913	10	FGF14	10	fibroblast growth factor 14
264	13	109,343,725	109,460,996	117,271	9			
265	14	29,035,872	29,156,729	120,857	10	PRKD1	5	protein kinase D1
266	14	32,657,458	32,788,358	130,900	10	NPAS3	10	neuronal PAS domain protein 3
267	14	50,484,071	50,560,634	76,563	10	TRIM9	9	tripartite motif-containing 9
268	14	52,796,130	52,895,069	98,939	13			
269	14	55,229,785	55,313,673	83,888	10			
270	14	60,524,781	60,608,660	83,879	10	SLC38A6	8	solute carrier family 38, member 6
271	14	62,941,082	63,040,274	99,192	9	PPP2R5E	9	protein phosphatase 2, regulatory subunit B (B56), epsilon isoform
272	14	63,766,162	63,830,070	63,908	13	ESR2	13	estrogen receptor 2 (ER beta)
273	14	77,421,158	77,496,277	75,119	9	ADCK1	5	aarF domain containing kinase 1
274	15	18,846,092	18,887,010	40,918	11			
275	15	19,911,384	20,018,451	107,067	11	OR4N3P	3	olfactory receptor, family 4, subfamily N, member 3 pseudogene
276	15	31,495,128	31,572,436	77,308	10	RYR3	10	ryanodine receptor 3
277	15	54,518,953	54,579,181	60,228	10	MNS1	5	meiosis-specific nuclear structural 1
278	15	64,207,043	64,315,055	108,012	10	MEGF11	10	multiple EGF-like-domains 11
279	15	66,457,383	66,519,155	61,772	10	ITGA11	10	integrin, alpha 11
280	15	72,067,051	72,114,524	47,473	10	PML	10	promyelocytic leukemia
281	15	74,678,920	74,729,056	50,136	9	ZNF291	9	zinc finger protein 291
282	15	90,567,835	90,621,172	53,337	11			
283	15	92,761,399	92,828,961	67,562	10	MCTP2	10	multiple C2 domains, transmembrane 2
284	16	23,460,282	23,541,590	81,308	9	EARS2	4	glutamyl-tRNA synthetase 2 (mitochondrial)(putative)
284	16	23,460,282	23,541,590	81,308	9	NDUFAB1	4	NADH dehydrogenase (ubiquinone) 1, alpha/beta subcomplex, 1, 8kDa
284	16	23,460,282	23,541,590	81,308	9	FLJ21816	3	hypothetical protein FLJ21816
284	16	23,460,282	23,541,590	81,308	9	UBPH	3	ubiquitin-binding protein homolog
285	16	26,829,376	26,912,543	83,167	9			
286	16	27,594,759	27,651,007	56,248	10	KIAA0556	10	KIAA0556 protein
287	16	45,921,236	45,969,018	47,782	9	ITFG1	9	integrin alpha FG-GAP repeat containing 1
288	16	52,031,300	52,080,916	49,616	11	RBL2	11	retinoblastoma-like 2 (p130)
289	16	63,189,755	63,322,472	132,717	12			
290	16	63,474,329	63,523,723	49,394	9			
291	16	64,310,429	64,388,652	78,223	9			
292	16	66,951,731	67,055,002	103,271	10	SMPD3	8	sphingomyelin phosphodiesterase 3, neutral membrane (neutral sphingomyelinase II)
293	16	72,737,649	72,833,460	95,811	9			
294	16	73,968,251	74,092,861	124,610	12	CFDP1	8	craniofacial development protein 1
294	16	73,968,251	74,092,861	124,610	12	CHST6	3	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6
295	16	76,418,571	76,488,521	69,950	10	KIAA1576	10	KIAA1576 protein
296	16	77,561,023	77,620,362	59,339	11			
297	16	80,949,422	81,055,188	105,766	10			
298	16	83,221,941	83,301,074	79,133	10	C16orf44	4	chromosome 16 open reading frame 44
299	16	83,915,731	83,994,389	78,658	12			
300	16	85,069,964	85,165,188	95,224	9	MTHFSD	4	methenyltetrahydrofolate synthetase domain containing
301	17	9,292,495	9,385,394	92,899	10	STX8	10	syntaxin 8
302	17	19,443,132	19,506,432	63,300	9	ALDH3A2	4	aldehyde dehydrogenase 3 family, member A2
303	17	24,217,422	24,312,447	95,025	10	PHF12	5	PHD finger protein 12
303	17	24,217,422	24,312,447	95,025	10	FLOT2	3	flotillin 2
304	17	28,461,208	28,566,650	105,442	11	ACCN1	11	amiloride-sensitive cation channel 1, neuronal (degenerin)
305	17	35,092,923	35,136,457	43,534	12	ERBB2	12	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogen
305	17	35,092,923	35,136,457	43,534	12	PERLD1	3	per1-like domain containing 1

306	17	50,678,634	50,846,295	167,661	14	HLF	5	hepatic leukemia factor
306	17	50,678,634	50,846,295	167,661	14	MMD	4	monocyte to macrophage differentiation-associated
307	17	59,657,640	59,782,626	124,986	14	PECAM1	5	platelet/endothelial cell adhesion molecule (CD31 antigen)
307	17	59,657,640	59,782,626	124,986	14	TEX2	5	testis expressed sequence 2
308	17	71,285,826	71,358,969	73,143	10	UNC13D	7	unc-13 homolog D (C. elegans)
308	17	71,285,826	71,358,969	73,143	10	ZC3H5	6	zinc finger CCCH-type containing 5
308	17	71,285,826	71,358,969	73,143	10	WBP2	4	WW domain binding protein 2
309	17	73,907,139	73,971,572	64,433	10	DNAH17	7	dynein, axonemal, heavy polypeptide 17
309	17	73,907,139	73,971,572	64,433	10	PGS1	5	phosphatidylglycerophosphate synthase 1
310	18	6,704,940	6,821,084	116,144	9			
311	18	28,108,406	28,194,436	86,030	9	FAM59A	9	family with sequence similarity 59, member A
312	18	32,350,975	32,475,536	124,561	10	FHOD3	10	formin homology 2 domain containing 3
313	18	33,747,148	33,842,056	94,908	10			
314	18	43,676,378	43,724,211	47,833	12	SMAD2	10	SMAD, mothers against DPP homolog 2 (Drosophila)
315	18	46,789,968	46,858,273	68,305	16	SMAD4	14	SMAD, mothers against DPP homolog 4 (Drosophila)
316	18	55,785,417	55,863,079	77,662	9			
317	18	58,922,895	59,028,058	105,163	13	BCL2	12	B-cell CLL/lymphoma 2
318	18	60,537,521	60,598,022	60,501	9			
319	19	9,438,447	9,553,898	115,451	9	ZNF121	3	zinc finger protein 121 (clone ZHC32)
319	19	9,438,447	9,553,898	115,451	9	ZNF560	3	zinc finger protein 560
320	19	39,609,295	39,658,792	49,497	9	UBA2	9	SUMO-1 activating enzyme subunit 2
321	19	39,776,121	39,878,749	102,628	10			
322	19	45,447,531	45,522,149	74,618	13	AKT2	11	v-akt murine thymoma viral oncogene homolog 2
322	19	45,447,531	45,522,149	74,618	13	MIRN641	5	microRNA 641
323	20	1,399,414	1,595,999	196,585	19	SIRPB1	8	signal-regulatory protein beta 1
323	20	1,399,414	1,595,999	196,585	19	SIRPD	4	signal-regulatory protein delta
323	20	1,399,414	1,595,999	196,585	19	SIRPG	4	signal-regulatory protein gamma
323	20	1,399,414	1,595,999	196,585	19	SIRPB2	3	signal-regulatory protein beta 2
324	20	7,240,792	7,386,321	145,529	13			
325	20	13,514,384	13,624,652	110,268	10	TASP1	5	taspase, threonine aspartase, 1
326	20	18,174,249	18,225,382	51,133	9	ZNF133	5	zinc finger protein 133
327	20	23,461,514	23,597,312	135,798	10			
328	20	24,898,844	25,016,552	117,708	10	C20orf3	5	chromosome 20 open reading frame 3
328	20	24,898,844	25,016,552	117,708	10	ACSS1	4	acyl-CoA synthetase short-chain family member 1
329	20	35,136,102	35,155,757	19,655	9	RBL1	9	retinoblastoma-like 1 (p107)
330	20	36,421,307	36,488,906	67,599	9	LBP	3	lipopolysaccharide binding protein
331	20	43,481,436	43,571,313	89,877	9	WFDC2	3	WAP four-disulfide core domain 2
332	20	48,562,736	48,639,539	76,803	20	PTPN1	20	protein tyrosine phosphatase, non-receptor type 1
332	20	48,562,736	48,639,539	76,803	20	MIRN645	5	microRNA 645
333	20	51,746,113	51,798,208	52,095	9			
334	20	54,090,327	54,212,606	122,279	10			
335	21	45,948,558	45,999,568	51,010	9			
336	22	17,405,213	17,466,749	61,536	9	DGCR2	9	DiGeorge syndrome critical region gene 2
337	22	19,389,671	19,510,261	120,590	23	PIK4CA	23	phosphatidylinositol 4-kinase, catalytic, alpha polypeptide
337	22	19,389,671	19,510,261	120,590	23	SERPIND1	9	serpin peptidase inhibitor, clade D (heparin cofactor), member 1
338	22	20,804,538	20,834,033	29,495	12	IGL@	12	immunoglobulin lambda locus
339	22	21,469,389	21,551,264	81,875	10	IGL@	10	immunoglobulin lambda locus
339	22	21,469,389	21,551,264	81,875	10	IGLV2-8	4	immunoglobulin lambda variable 2-8
339	22	21,469,389	21,551,264	81,875	10	IGLV3-10	4	immunoglobulin lambda variable 3-10
339	22	21,469,389	21,551,264	81,875	10	IGLV3-9	4	immunoglobulin lambda variable 3-9
339	22	21,469,389	21,551,264	81,875	10	MIRN650	4	microRNA 650
340	22	24,041,845	24,133,213	91,368	10	LRP5L	3	low density lipoprotein receptor-related protein 5-like
341	22	25,257,185	25,367,383	110,198	9	TPST2	5	tyrosylprotein sulfotransferase 2
342	22	39,820,180	39,949,460	129,280	15	EP300	12	E1A binding protein p300
343	22	47,510,034	47,583,523	73,489	11	FAM19A5	8	family with sequence similarity 19 (chemokine (C-C motif)-like), member A5
344	22	49,451,008	49,530,707	79,699	13	SHANK3	12	SH3 and multiple ankyrin repeat domains 3
344	22	49,451,008	49,530,707	79,699	13	ACR	3	acrosin

Genetic Architecture of Smoking Behavior: A Study of Finnish Adult Twins

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Both genetic and environmental factors affect smoking initiation and maintenance, but less is known about the genetic architecture of various other smoking-related behaviors. The aim of this study is to examine the genetic architecture of smoking behavior in a large twin cohort. Questionnaires with an extensive smoking history section were mailed to same-sex adult twins of the Finnish twin cohort. The final study population included 2923 monozygotic and 6018 dizygotic twin pairs aged 24 to 88 years. Two-stage bivariate genetic modeling of age at initiation with amount smoked (less than 20 cigarettes per day vs. 20 or more) and age at initiation with smoking cessation was done by using the Mx statistical package. For men the heritability estimate for age at initiation was .59 (95% confidence interval [CI] .49–.69), for amount smoked .54 (95% CI .45–.62) and for smoking cessation .58 (95% CI .50–.65). For women the heritability estimates were .36 (95% CI .28–.43), .61 (95% CI .46–.70) and .50 (95% CI .39–.60), respectively. The genetic correlations between age at initiation and amount smoked or smoking cessation were at most .22 in magnitude, indicating that genetic influences in age at initiation accounted for at most about 4% of the genetic factors in amount smoked or in cessation. Genetic factors are important in amount smoked and smoking cessation and they are largely independent of genetic influences on age at initiation. This has implications for defining phenotypes in searches for genes underlying smoking behaviors.

The genetic architecture of many features of smoking behavior based on twin and family studies is fairly well characterized (Li et al., 2003). The results, however, vary. Some studies have suggested that there is a strong genetic contribution to variation in smoking behavior (Edwards et al., 1995; Heath et al., 1993; Kaprio et al., 1981; Kendler et al., 1999), but there are also studies which have found little evidence of a genetic effect (Heath et al., 1993; Heath et al., 1999). Most of the studies on the genetic architecture of smoking behavior have examined smoking initiation and smoking cessation, while quantitative genetic studies on other smoking behaviors and related traits are fewer.

Earlier studies have revealed a substantial genetic contribution to risk of smoking initiation, and the heritability estimates in these studies range from .32 to .78 (Edwards et al., 1995; Heath et al., 1993; Heath et al., 1999; Heath et al., 1998; Kendler et al., 1999; Madden et al., 2004; True et al., 1997; Vink, 2004). This variation is not surprising, taking into account that the role of genetic factors probably varies with time and place (Kendler et al., 1999). A meta-analysis by Li et al. (2003) showed that on average heritability of smoking initiation appears to be higher in adult women (55%) than in adult men (37%) as it did in studies of Madden et al. (1999) and Heath et al. (2002).

Another well studied aspect of smoking behavior is smoking cessation versus persistence among regular smokers. In the previous studies, heritability estimates of smoking cessation have varied from .11 to .74 (Heath et al., 1993; Heath et al., 1999; Heath et al., 1998; Heath et al., 2002; Kaprio et al., 1981; Kendler et al., 2000; Madden et al., 1999; Medlund et al., 1977; True et al., 1997). However, only a few of these studies include both men and women from the same population. Genetic effects on smoking cessation seem to be higher among men than among women (Li et al., 2003; Madden et al., 2004; Maes et al., 2004).

The amount smoked has been studied both as a trait in itself and as a proxy measure for nicotine dependence. Analyses of amount smoked have been done mostly among men (Carmelli et al., 1990; Swan et al., 1996; Swan et al., 1997; Swan et al., 1990). Smoked cigarettes per day has been examined in three ways: highest number of cigarettes ever smoked per day (Carmelli et al., 1990; Swan et al., 1996; Swan et al., 1990; among men, $h^2 = .52$ to $.56$), as a dichotomy of light or heavy smokers (Swan et al., 1997; among men, $h^2 = .49$) or the number of cigarettes smoked per day as a categorical variable (Vink et al., 2004; among both sexes, $h^2 = .51$). Nicotine dependence is determined either by the Fagerström test

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for nicotine dependence (Heatherton et al., 1991) or the criteria of *Diagnostic and Statistical Manual of Mental Disorders* (4th ed.; DSM-IV; American Psychiatric Association, 1994) and DSM-III-R (3rd ed., rev.; American Psychiatric Association, 1987) nicotine dependence. In these studies, heritability estimates in adults have varied from .60 among men (True et al., 1999) to .72 among women (Kendler et al., 1999) and .56 among both men and women (Lessov et al., 2004).

Many of the previous studies have examined only one smoking behavioral factor at a time. Bivariate analyses have been conducted for initiation and dependence (Kendler et al., 1999), initiation and smoking persistence (Madden et al., 1999), and a trivariate analysis has been conducted for initiation, regular tobacco use and nicotine dependence (Maes et al., 2004). Anyhow, we lack studies comparing genetic influences in age at initiation, amount smoked per day and smoking cessation in the same study. Thus, the previous quantitative genetic studies on smoking behavior give little information on the mutual associations between these different smoking behavioral factors. For example, it has been found that persons who start to smoke in young age smoke also, on average, more cigarettes per day than those who start to smoke later (Broms et al., 2004; Jarvis, 2004). However, it is not known whether this trait correlation is because of common genetic factors, common environment, or direct associations between age at initiation and amount smoked per day. Another problem in the previous studies on the genetic architecture of smoking behavior is that only a few studies have included both smokers and nonsmokers (Heath et al., 2002; Kendler et al., 1999; Maes et al., 2004), whereas others have been targeted to concordant smoking pairs only. Heath et al. (2002) have pointed out that if the same genetic and environmental factors that determine variation in risk of dependence among users also determine risk of initiation, then excluding never smokers, that is, discordant pairs and concordant never-smoker pairs, from analyses, discards genetic information. In analyses of twin data, it will also lead to biased estimates of genetic and environmental effects on risk of dependence.

The aim of this study is to examine the genetic architecture of smoking behavior by using a large Finnish data set of adult male and female twins. We address the following specific research questions: (1) What is the effect of genetic and environmental factors on age at initiation, smoked cigarettes per day and smoking cessation? and (2) Are there same genetic and environmental factors behind these phenotypes?

Materials and Methods

The Older Finnish Twin Cohort was established to examine the genetic, environmental, and psychosocial determinants of chronic diseases and health behaviors (Kaprio & Koskenvuo, 2002), and includes data on same-sex twin pairs born before 1958 with both members alive in 1967. The study was accepted by the Ethical Committee of the University of Helsinki and followed the rules and principles of the Helsinki Declaration. The first questionnaire survey was carried out in 1975. The second questionnaire was sent in the autumn of 1981 to all twins then alive in the cohort, including ones nonrespondent in 1975. Earlier heritability estimates of smoking behaviors from the 1975 questionnaire have been reported (Heath et al., 1998; Kaprio et al., 1981; Madden et al., 1999; Madden et al., 2004). This is the first time we report heritability estimates based on the 1981 questionnaire.

In the 1981 survey, the number of respondents was 24,053 and the response rate was 84%. Both twins responded from 9880 pairs, who formed the basic study population, and were aged 23 up to 88 years (mean age 40 years).

Zygosity was determined by asking two questions about the similarity of appearance at school age. The reliability of the questionnaire method for zygosity was assessed by using 11 blood markers in a subsample of 104 twin pairs classified as monozygous (MZ) or dizygous (DZ; Sarna et al., 1978). The agreement between the blood tests and the questionnaire was 100%. It was estimated that the probability of misclassification based on the questionnaire method was 1.7%.

We excluded from the analyses the pairs with unknown zygosity (866 pairs) and the pairs lacking smoking cessation information on either twin (73 pairs). The final study population included 2923 MZ and 6018 DZ twin pairs. The high DZ to MZ ratio reflects the high rate of DZ twinning in the first half of the 20th century in Finland.

In the questionnaire, current smokers were classified as persons who had smoked at least 5 to 10 packs of cigarettes over their lifetime and who were smoking daily or almost daily at the time of the study (Kaprio & Koskenvuo, 1988; Broms et al., 2004). Two questions were asked: 'Have you ever smoked more than 5 to 10 packs of cigarettes in your lifetime?' Those responding positively were then asked: 'Do you smoke or have you smoked cigarettes regularly, daily, or almost daily during your lifetime?' Regular smokers were classified for smoking cessation as follows: former smokers and current smokers. Never and occasional smokers were considered to have missing data for the smoking cessation item.

Age at initiation of smoking was asked by the question: 'What age were you when you started to smoke regularly?' (Broms et al., 2004). Smokers were classified as early starters if they reported to start to smoke regularly when they were 18 years old or younger and as late starters if they had reported to start to smoke regularly when they were older than 18 years. Never smokers and occasional smokers were placed in the category of no initiation. Thus, there were three categories of the age at initiation variable. The correlation between age at initiation as reported in health questionnaire surveys conducted in 1975 and 1981 was .84 among all smokers, with no variation by smoking status or sex.

Smokers were asked a question about the amount of smoking as follows: 'How many cigarettes do you smoke daily on average?' The response alternatives were: none, less than 5, 5–9, 10–14, 15–19, 20–24, 25–39, and more than 40. In the questionnaire, the question for former smokers was same as above but it was in the past tense. We classified amount of smoked cigarettes per day in two categories as follows: less than 20 cigarettes, that is, less than one pack, and 20 cigarettes or more, that is, one pack or more.

The data were analyzed using quantitative genetic methods based on linear structural modeling. Twin modeling is based on the assumption that MZ twins are genetically identical whereas DZ twins share on average 50% of their segregating genes. Thus, a greater similarity for MZ twins compared with DZ twins gives support to the hypothesis that genetic transmission is a component of importance, under the assumption that MZ and DZ share to the same extent their trait-relevant environmental experiences (Boomsma et al., 2002; Neale et al., 2003; Posthuma et al., 2003). Environmental factors are divided into factors shared by a twin pair (shared environment) and factors unique to each twin individual (unique environmental effects), which also includes measurement error. The correlations for the shared environmental factors are 1 and for unique environmental factors 0 within both MZ and DZ twin pairs. The Mx statistical package (Neale et al., 2003) was used to estimate the proportion of trait variance accounted for by additive genetic factors (A), by shared environmental factors (C) and by factors not shared (unique) with the co-twins (E). The ACE model was selected as a starting point of the modeling based on twin correlations. First, a full model was fitted including ACE effects for both age at initiation and amount smoked and the correlations between the genetic and environmental components affecting these two phenotypes. Second, we studied the statistical significance of each component of the base model by fixing them zero to find the most parsimonious model, which was used in the future modeling.

We first used univariate modeling and then the two-stage modeling approach developed by Heath et al. (2002). Two-stage modeling gives results of genetic and environmental variation and correlation of two variables in the situation of structural missing data in one variable. This model permits including the information from never smokers to the analysis. We based our analyses on the Mx scripts given by Heath et al. (2002). All models were fitted using Mx software, using the option for ordinal data-analysis (Neale et al., 2003).

Results

Descriptive Statistics

Table 1 summarizes the distribution of smoking behavior data in the Older Finnish Twin Cohort in 1981. Forty-six per cent of all participants were men and 54% women. Among men 28% were former smokers and 37% current smokers whereas in women the proportions were 15% and 21%, respectively. Approximately equal amounts of subjects had started smoking early or late, and there were no major differences in these proportions by smoking cessation or sex. The number of smoked cigarettes per day was higher among men than among women. The great majority (73%) of smokers smoked less than 20 cigarettes per day. The proportion of those who smoked 20 or more cigarettes was greater among men and current smokers compared with women and former smokers.

Table 1

Numbers and Percentages by Smoking Cessation Age at Initiation and Amount Smoked per Day in the 1981 Questionnaire of Finnish Adult Twins Born Before 1958

Smoking cessation	Men		Women	
	N	%	*N*	%
Never smoked regularly	3229	35.6	6889	64.4
Former smoker	2513	27.8	1615	15.1
Current smoker	3320	36.6	2194	20.5
Total number	9062		10,698	
Total percentage	45.9		54.1	
Smoking cessation and early or late age at initiation				
Never smoked regularly	3229	35.6	6889	64.4
Former smoker, started at age 18 or earlier	1453	16.0	921	8.6
Former smoker, started older than 18	1060	11.7	694	6.5
Current smoker, started at age 18 or earlier	2117	23.4	1184	11.1
Current smoker, started older than 18	1203	13.3	1010	9.4
Total	9062		10,698	
Smoking cessation and smoked less than 20 cigarettes or 20 and more cigarettes per day				
Never smoked regularly	3229	35.6	6889	64.4
Former smoker, smoked less than 20 cigarettes per day	1665	18.4	1424	13.3
Former smoker, smoked 20 cigarettes or more per day	848	9.4	191	1.8
Current smoker, smoked less than 20 cigarettes per day	1988	21.9	1822	17.0
Current smoker, smoked 20 cigarettes or more per day	1332	14.7	372	3.5
Total	9062		10,698	

Table 2 shows the data used in the modeling. It presents the twin and co-twin status in four zygosity groups, according to age at initiation and amount smoked (upper part of the table) or by age at initiation and smoking cessation (lower part of the table).

The correlations of the three variables of age at initiation (continuous), amount smoked (all categories) and smoking cessation are shown in Table 3 for pairs concordant for ever smoking. MZ correlations were higher compared with DZ correlations among both men and women for all traits.

Table 2

Twin Pair Smoking Cessation for Age at Initiation of Smoking (≤ 18 Yrs, 18+, Never), Amount Smoked per Day (< 20 vs. 20+ Cigarettes/Day) and Smoking Cessation (Former, Current, Never), in the 1981 Questionnaire Survey, for Complete Pairs Born Before 1958

Twin status		Co-twin status		Number of pairs			
Age at initiation	Amount smoked	Age at initiation	Amount smoked	MZM	DZM	MZF	DZF
—	N	—	N	350	578	958	1679
≤ 18	< 20	≤ 18	< 20	161	239	169	276
—	N	> 18	> 18	102	287	165	348
≤ 18	≥ 20	≤ 18	≥ 20	80	138	10	20
> 18	< 20	> 18	< 20	80	136	89	119
≤ 18	≥ 20	≤ 18	< 20	60	117	18	27
—	N	≤ 18	< 20	59	274	64	265
≤ 18	< 20	≤ 18	≥ 20	55	150	20	38
≤ 18	< 20	> 18	< 20	42	96	44	78
> 18	< 20	≤ 18	< 20	36	85	27	73
—	N	> 18	≥ 20	31	113	14	54
—	N	≤ 18	≥ 20	30	169	9	42
> 18	≥ 20	> 18	≥ 20	26	54	3	5
> 18	< 20	> 18	≥ 20	21	36	5	14
> 18	≥ 20	> 18	< 20	20	36	6	15
≤ 18	≥ 20	> 18	≥ 20	19	37	4	3
≤ 18	≥ 20	> 18	< 20	17	46	4	11
> 18	< 20	≤ 18	≥ 20	17	51	11	11
> 18	≥ 20	≤ 18	< 20	15	33	0	5
> 18	≥ 20	≤ 18	≥ 20	12	35	4	2
≤ 18	< 20	> 18	≥ 20	11	33	4	8
Age at initiation	Smoking cessation	Age at initiation	Smoking cessation	MZM	DZM	MZF	DZF
—	N	—	N	350	578	958	1679
≤ 18	C	≤ 18	C	175	278	90	150
≤ 18	F	≤ 18	C	107	251	78	144
≤ 18	F	≤ 18	F	87	133	60	80
—	N	> 18	F	82	194	86	215
> 18	F	> 18	F	56	87	34	31
—	N	> 18	C	53	215	101	289
> 18	C	> 18	C	51	79	48	63
—	N	≤ 18	F	46	198	45	156
> 18	F	> 18	C	43	107	23	67
—	N	≤ 18	C	43	250	31	153
≤ 18	F	> 18	C	37	96	29	51
≤ 18	C	> 18	C	34	75	21	33
> 18	C	≤ 18	C	33	96	30	32
> 18	F	≤ 18	C	24	72	9	34
> 18	F	≤ 18	F	23	42	8	25
≤ 18	F	> 18	F	20	46	8	19

Note: ≤ 18 = Age at initiation of smoking ≤ 18 yrs; > 18 = age at initiation of smoking older than 18 yrs; — = never smoked regularly; < 20 = smoked less than 20 cigarettes per day; ≥ 20 = smoked 20 cigarettes or more per day; N = never smoked regularly; F = former smoker; C = current smoker; MZM = male monozygotic pair; DZM = male dizygotic pair; MZF = female monozygotic pair; DZF = female dizygotic pair.

Univariate Modeling

We started the modeling by univariate modeling with continuous variables of age at initiation and amount smoked. Heritability estimate of amount smoked was .49 (95% confidence interval [CI] .44–.54) among men and .52 (95% CI .46–.57) among women. Heritability estimate of age at initiation among men was .36 (95% CI .21–.51) and among women .67 (95% CI .60–.72).

Modeling of Initiation and Amount Smoked

We continued modeling by two-stage analyses of categorized variables of age at initiation and amount

Table 3

The Intrapair Correlations and Confidence Intervals (and Prevalence) for Age at Initiation, Amount Smoked and Smoking Cessation Among MZ and DZ Twin Pairs and Sex Among Ever-Smoker Pairs

	Men		Women	
	MZ	DZ	MZ	DZ
Age at initiation (years)	.49	.34	.55	.36
	.43, .55	.29, .39	.48, .61	.30, .42
	(695)	(1385)	(441)	(747)
Amount smoked	.45	.27	.50	.29
	.39, .51	.22, .32	.43, .57	.22, .36
	(676)	(1343)	(426)	(714)
Cessation (former in 1981)	.56	.31	.50	.25
	.51, .61	.26, .36	.43, .57	.18, .32
	(695)	(1389)	(445)	(738)

smoked. When compared to the full ACE model, the most parsimonious model among men included ACE effects for age at initiation, AE effects for amount smoked and the correlation between the two additive genetic components ($\Delta\chi^2$ = 4.526, p = .21, Δdf = 3). Among women two models offered adequate fit: (1) ACE effects for age at initiation, AE effects for amount smoked and the correlations between the additive genetic and unique environmental factors ($\Delta\chi^2$ = 4.613, p = .100, Δdf = 2); and (2) ACE effects for age at initiation, CE effects for amount smoked and the correlations between the common and unique environmental factors ($\Delta\chi^2$ = 1.548, p = .461, Δdf = 2). Thus, for amount smoked both AE and CE submodels offered adequate fit. In the subsequent modeling, we fitted both of these models for women.

Table 4 shows the estimated genetic and environmental variances and correlations with their 95% confidence intervals in the full ACE two-stage bivariate model and in the most parsimonious models described above. In the best fitting model among men, additive genetic variance was .59 (95% CI .49–.69) and shared environmental variance .19 (95% CI .10–.27) for age at initiation. In the same model, amount smoked had an estimated additive genetic variance of .54 (95% CI .45–.62). There was only a weak genetic correlation between age at initiation and amount smoked (r_a = –.22, 95% CI –.28, –.15).

Among women the estimate for the effect of additive genetic factors was .36 (95% CI .28–.43) and for shared environmental factors .50 (95% CI .43–.57) of the variation of age at initiation. Under the AE model for amount smoked, additive genetic variance was .61 (95% CI .46–.70). Both the additive genetic correlation (r_a = .17, 95% CI –.23, –.04) and the nonshared environmental correlation (r_e = –.04, 95% CI –.09, –.01) between age at initiation and amount smoked were weak. In the CE model for amount smoked, shared environmental variance was .47 (95% CI .36–.58). The shared environmental correlation in the CE model among women between age at initiation and amount smoked was –.15 (95% CI –.24, –.05) and that between unique environmental factors –.07 (95% CI –.13, –.02).

Modeling of Age at Initiation and Smoking Cessation

We continued the analyses by conducting the corresponding modeling for age at initiation and smoking cessation. Compared to the full ACE model, the most parsimonious model included ACE effects for age at initiation, AE effects for smoking cessation and the correlations between additive genetic and unique environmental factors ($\Delta\chi^2$ = 2.772, p = .250, Δdf = 2). Among women the best model included ACE effects for age at initiation, AE effects for amount smoked and the correlation between the unique environmental factors ($\Delta\chi^2$ = .229, p = .973, Δdf = 3).

Table 5 shows the estimated genetic and environmental variances and correlations with their 95% confidence intervals under a two-stage bivariate genetic model for age at initiation and smoking cessation. In the best fitting model among men, additive genetic variance was .59 (95% CI .49–.68) and shared environmental variance was .19 (95% CI .11–.28) for age at initiation. In the same model, smoking cessation had an estimated additive genetic variance of .58 (95% CI .50–.65). There was only a weak additive genetic correlation (r_a = .22, 95% CI .16, .29) and only a weak unique environmental correlation (r_e = –.08, 95% CI –.12, –.04).

The best model for smoking cessation among women was an AE model. Age at initiation had an estimate of additive genetic variance of .34 (95% CI .28–.42) and of shared environmental variance of .51 (95% CI .45–.58). For smoking cessation, additive genetic variance was .50 (95% CI .39–.60). Phenotypic correlation in the AE model among women between age at initiation and smoking cessation was explained only a little by unique environmental factors (r_e = –.13, 95% CI –.15, –.08), and no significant genetic correlation was found.

We also tested the parameters estimates for men and women in the two stage models by equating the parameters and assessing the change in model fit. Models for both age at initiation and amount smoked ($\Delta\chi^2$ = 71.227, p < .001, Δdf = 9) and also age at initiation and smoking cessation ($\Delta\chi^2$ = 89.933, p < .001, Δdf = 9) showed a highly significant difference between men and women.

Discussion

In the present study, we found a strong genetic influence for age at initiation, amount smoked and smoking cessation. These findings are in accordance with results of previous quantitative genetic studies on smoking initiation and cessation (Heath et al., 2002; Li et al., 2003; Madden et al., 2004; Maes et al., 2004) and amount smoked (Vink et al., 2004).

We found in this study that for men the heritability estimate for age at initiation was .59 and for women

Table 4

Proportion of the Variation with 95% Confidence Intervals of Age at Initiation (≤18 Yrs, 18+ Yrs or Never) and Amount Smoked (< 20 vs. 20+ Cigarettes/Day) Accounted for Genetic and Environmental Factors and the Correlations Between These Effects Under a Two-Stage Bivariate Model

	Additive genetic variance	95% CI	Shared environmental variance	95% CI	Unique environmental variance	95% CI
Men						
ACE model for both age at initiation and amount smoked						
Age at initiation	.59	.49, .69	.19	.11, .28	.22	.19, .25
Amount smoked	.32	.06, .57	.17	.0, .36	.51	.41, .61
Genetic–environmental correlation	–.14	–.28, .01	–.06	–.17, .04	–.03	–.08, .03
ACE model forage at initiation and AE model for amount smoked (best model)						
Age at initiation	.59	.49, .69	.19	.10, .27	.22	.19, .25
Amount smoked	.54	.45, .62	—	—	.46	.38, .55
Genetic–environmental correlation	–.22	–.28, –.15	—	—	—	—
Women						
ACE model for both age at initiation and amount smoked						
Age at initiation	.35	.28, .43	.51	.44, .57	.14	.12, .17
Amount smoked	.26	.00, .66	.29	.00, .56	.45	.30, .62
Genetic–environmental correlation	–.06	–.24, .12	–.11	–.25, –.03	–.06	–.13, –.01
ACE model for age at initiation and AE model for amount smoked (best model)						
Age at initiation	.36	.28, .43	.50	.43, .57	.14	.12, .17
Amount smoked	.61	.46, .70	—	—	.39	.30, .54
Genetic–environmental correlation	–.17	–.23, –.04	—	—	–.04	–.09, –.01
ACE model for age at initiation and CE model for amount smoked (best model)						
Age at initiation	.35	.28, .43	.51	.44, .57	.14	.12, .17
Amount smoked	—	—	.47	.36, .58	.53	.42, .64
Genetic–environmental correlation	—	—	–.15	–.24, –.05	–.07	–.13, –.02

Note: The full ACE model and the best models presented.

.34–.36. These estimates are at the same level as the estimates found in the previous studies in which the heritability estimates ranged from .32 to .78 (Edwards et al., 1995; Heath et al., 1993; Heath et al., 1999; Heath et al., 1998; Kendler et al., 1999; Madden et al., 1999; Madden et al., 2004; True et al., 1997; Vink, 2004). However, in contrast to previous studies (Li et al., 2003; Madden et al., 1999), we found that the heritability of smoking initiation appears to be higher among men than among women. This sex difference may, however, be associated with different definitions of smoking initiation. In our study, age at initiation was classified as early initiation, late initiation and no initiation to smoke, whereas in many previous studies initiation was dichotomized as ever or never smoked.

When studying amount smoked, we found the heritability estimates of .54 for men and .61 for women. Previous quantitative genetic studies on amount smoked have been done mostly among men (Carmelli et al., 1990; Swan et al., 1996; Swan et al., 1997; Swan et al., 1990; Vink et al., 2004), and the heritability estimates in these studies were at the same level than in our study. In a previous study where both men and women were included (Vink et al., 2004) heritability estimates were also at the same level than in the present study. The study of adolescent and young adults of Koopmans and her colleagues (Koopmans et al., 1999) were reported higher heritability estimates (.86, 95% CI .70–.94) of amount smoked.

The heritability estimate for smoking cessation was .58 among men and .50 among women. In the previous studies, where only a few include both men and women from the same population, heritability estimates of smoking cessation have varied from .11 to .74 (Heath et al., 1993; Heath et al., 1999; Heath et al., 1998; Heath et al., 2002; Kaprio et al., 1981; Kendler et al., 2000; Madden et al., 1999; Medlund et al., 1977; True et al., 1997). Previous studies show that genetic effects on smoking cessation is higher among men than among women (Li et al., 2003; Madden et al., 2004; Maes et al., 2004). We found a

Table 5

Proportion of the Variation With 95% Confidence Intervals of Age at Initiation (≤ 18 Yrs, 18+ Yrs or Never) and Smoking Cessation (Former vs. Current Smoker) Accounted for Genetic and Environmental Factors and the Correlations Between These Effects Under a Two-Stage Bivariate Model

	Additive genetic variance	95% CI	Shared environmental variance	95% CI	Unique environmental variance	95% CI
Men						
ACE model for both age at initiation and smoking cessation						
Age at initiation	.59	.49, .68	.19	.11, .25	.22	.19, .26
Smoking cessation	.45	.21, .64	.10	.00, .26	.45	.36, .54
Genetic–environmental correlation	.23	.16, .29	—	—	–.09	–.13, –.04
ACE model for age at initiation and AE model for smoking cessation (best model)						
Age at initiation	.59	.49, .68	.19	.11, .28	.22	.19, .25
Smoking cessation	.58	.50, .65	—	—	.43	.35, .50
Genetic–environmental correlation	.22	.16, .29	—	—	–.08	–.12, –.04
Women						
ACE model for both age at initiation and smoking cessation						
Age at initiation	.34	.27, .42	.51	.45, .58	.15	.12, .17
Smoking cessation	.50	.19, .60	.00	.00, .23	.50	.40, .63
Genetic–environmental correlation	—	—	–.00	–.10, .06	–.13	–.17, –.08
ACE model for age at initiation and AE-model for smoking cessation (best model)						
Age at initiation	.34	.28, .42	.51	.45, .58	.15	.12, .17
Smoking cessation	.50	.39, .60	—	—	.50	.40, .61
Genetic–environmental correlation	—	—	—	—	–.13	–.15, –.08

Note: The full ACE model and the best models presented.

similar sex difference in this study as well, but it was not very large.

The trait correlation between age at initiation and amount smoked in men was explained solely by the genetic correlation (r_a = –.22). A similar genetic correlation was found also for age at initiation and smoking cessation (r_a = .22). Thus, there are partly the same genes to predispose these traits, but this proportion is not large. These correlations suggest that genetic influences to age at initiation accounted for at most about 4% of the genetic factors in amount smoked or in smoking cessation. In the study of Heath and his colleagues (2002) on age at initiation and smoking cessation, a weak and statistically nonsignificant genetic correlation was found in men (r_a = .11). In the present study, the genetic correlation between age at initiation and amount smoked among women was .17 if the AE model was used, indicating that genetic influences on initiation accounted for about 2% of the genetic factors in amount smoked. In the study of Heath and his colleagues (2002), the correlation was somewhat higher (r_a = .28) in women, but still account for less than 8%. In the present study, environmental correlation was weak or did not exist among men and women in age at initiation and amount smoked or smoking cessation. We also found that age at initiation and smoked cigarettes per day as well as initiation and smoking cessation might share some genetic factors. This would suggest that linkage analyses could identify such common genes. Vink and her colleagues (2004) showed in Dutch linkage analysis that on chromosome 10 there was a peak in the same region for both number of cigarettes and smoking initiation traits, but the specific genes are not yet known. However, it is likely that in the most part different genes will be responsible for the liability to smoking initiation and to the ability to quit smoking or amount smoked. Thus, careful phenotypic assessment of subjects in linkage and association studies is needed.

Our study has several important strengths. Our study population is large, which gives adequate power to study not only of smoking behavioral traits but also the associations between these traits. Earlier studies have used smaller number of twin pairs with a notable exception of the study of Madden and her colleagues (2004), which used pooled twin data from Australian, the United States and Finland. The data come from retrospective self-reports of age at initiation of substance abuse. Self-reported data on substance use, particularly age at initiation, can be unreliable (Johnson & Mott, 2001). However in our data, age at

initiation was found to be quite reliable: the correlation between age at initiation as reported in 1975 and 1981 health questionnaires was high and did not vary by smoking status or sex (r = .84 among all smokers). The strength of the present study is also that we had both men and women in our data whereas many previous studies were targeted to men only. We have only limited information on smoking behavior based on self-report, and we do not have, for example, any measure of nicotine dependence. Another limitation of our study is that our data do not include opposite-sex twin pairs, and thus we are not able to examine sex-limitation effects in the heritability of smoking behavior.

In conclusion, the present results confirmed that genetic factors are important in amount smoked and smoking cessation and these genetic factors are largely independent of genetic influences on age at initiation. This has implications for defining phenotypes in searches for genes underlying smoking behaviors.

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Defining nicotine dependence for genetic research: evidence from Australian twins

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ABSTRACT

Background. Whether current criteria used to define nicotine dependence are informative for genetic research is an important empirical question. The authors used items of the DSM-IV and of the Heaviness of Smoking Index to characterize the nicotine dependence phenotype and to identify salient symptoms in a genetically informative community sample of Australian young adult female and male twins.

Method. Phenotypic and genetic factor analyses were performed on nine dependence symptoms (the seven DSM-IV substance dependence criteria and the two Heaviness of Smoking Index (HSI) items derived from the Fagerström Tolerance Questionnaire, time to first cigarette in the morning and number of cigarettes smoked per day). Phenotypic and genetic analyses were restricted to ever smokers.

Results. Phenotypic nicotine dependence symptom covariation was best captured by two factors with a similar pattern of factor loadings for women and men. In genetic factor analysis item covariation was best captured by two genetic but one shared environmental factor for both women and men; however, item factor loadings differed by gender. All nicotine dependence symptoms were substantially heritable, except for the DSM-IV criterion of 'giving up or reducing important activities in order to smoke', which was weakly familial.

Conclusions. The salient behavioral indices of nicotine dependence are similar for women and men. DSM-IV criteria of tolerance, withdrawal, and experiencing difficulty quitting and HSI items time to first cigarette in the morning and number of cigarettes smoked per day may represent the most highly heritable symptoms of nicotine dependence for both women and men.

INTRODUCTION

Nicotine is a psychoactive substance and dependence on nicotine is recognized as a mental disorder (APA, 1994). Despite the enormous public health problem and cost associated with smoking (CDC, 2002) and the tremendous addictive potential of smoking (Stolerman & Jarvis, 1995; Henningfield & Jude, 1999; Dani *et al.* 2001), empirical evidence about the validity of current criteria used to define nicotine dependence is scant.

Although the harmful effects of smoking were first described in the 1964 Surgeon General report (USDHHS, 2000), tobacco-related research has surged only in the past decade (Swan, 1999). Clinically, nicotine dependence is most often defined using the seven dependence criteria of the DSM-IV, the eight-item Fagerström Tolerance Questionnaire (FTQ; Fagerström, 1978), and its modified six-item version, the Fagerström Test for Nicotine Dependence (FTND; Heatherton

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et al. 1991). The DSM-IV dependence criteria are applied to a wide range of substances (Cottler *et al*. 1995). With the exception of nicotine-specific withdrawal symptoms described in the DSM-IV, the degree to which each DSM-IV substance dependence criterion is appropriate for nicotine dependence is unclear.

Unlike the DSM-IV, the FTQ was designed specifically to assess physical dependence on nicotine (Fagerström, 1978). Two of the eight original FTQ items, that assess (1) the time to first cigarette of the day after waking up and (2) the number of cigarettes smoked per day, were found to account for most of the variance of the FTQ (Lichtenstein & Mermelstein, 1986). In addition, these items were found to be superior in predicting biochemical and behavioral indices of smoking (Heatherton *et al*. 1989, 1991; Kozlowski *et al*. 1994; Breslau & Johnson, 2000; Prokhorov *et al*. 2000) and Heatherton and colleagues (1989) created a scale of just these two items, the Heaviness of Smoking Index (HSI). It has been suggested that time to first cigarette in the morning and number of cigarettes smoked per day may be the most useful and powerful indicators of nicotine dependence (Heatherton *et al*. 1989, 1991; Haddock *et al*. 1999). Individual differences in lifetime DSM-defined nicotine dependence, in number of cigarettes smoked per day, and in other tobacco use measures have been found to be under considerable genetic control. Heritability of DSM-III-R nicotine dependence was substantial in both a sample of Vietnam veteran male twins (60%; True *et al*. 1999) and in a sample of Minnesota adolescents (44%; McGue *et al*. 2000). In Virginia young adult female twins, Kendler and colleagues (1999) tested a causal common pathway model for lifetime regular smoking and nicotine dependence (based mostly on FTQ items) and found substantial heritability for regular smoking (85%), substantial overlap in liability for regular smoking and nicotine dependence (60%), and moderate residual genetic effects for nicotine dependence (22%). Lifetime number of cigarettes smoked per day shows substantial heritability in adults (49–54%; Kaprio *et al*. 1984; Carmelli *et al*. 1990; Swan *et al*. 1990; Hettema *et al*. 1999) and adolescents (84%; Koopmans *et al*. 1999). Other tobacco use measures such as smoking initiation, lifetime regular smoking, and smoking persistence (current rather than ex-smoker at the time of assessment) are also strongly genetically influenced in adults (Heath *et al*. 1993, 1999; Heath & Martin, 1993; True *et al*. 1997; Madden *et al*. 1999; Kendler *et al*. 2000) and in adolescents (Han *et al*. 1999; Koopmans *et al*. 1999; Maes *et al*. 1999). Family and adoption data also show that smoking initiation, persistence, and lifetime heavy smoking are correlated among biological siblings (Eaves & Eysenck, 1980; Bierut *et al*. 1998; Osler *et al*. 2001).

The purpose of the present analysis was to examine the genetic and environmental influences on lifetime symptoms of nicotine dependence, assessed using DSM-IV criteria and HSI items, individually and jointly, to determine the genetic architecture of the clinical syndrome of nicotine dependence in a community sample of Australian adult female and male twins.

METHOD

Sample

Between 1980 and 1982, in response to media appeals and appeals through the Australian school system, twins born between 1964 and 1971 were registered with the Australian twin panel maintained by the Australian National Health and Medical Research Council. Twins either volunteered themselves or were volunteered by their parents. This twin cohort was first targeted for assessment in 1989–1992 ('1989 cohort') by mailed questionnaire survey (Gillespie *et al*. 2000; Heath *et al*. 2001). Between 1996 and 2000, the 1989 cohort was interviewed by telephone using a structured diagnostic assessment for DSM-IV alcohol dependence, nicotine dependence, major depression, panic disorder, and childhood conduct disorder (APA, 1994) as well as non-diagnostic sections for social anxiety, suicidality, a screening for bipolar disorder, and a history of the consumption of alcohol, cigarettes and other forms of tobacco, and illicit drugs. The interview was a modified version of the diagnostic Semi-Structured Assessment for the Genetics of Alcoholism (Bucholz *et al*. 1994); the smoking section was modified from the Composite International Diagnostic Interview (Robins *et al*. 1988; Cottler *et al*. 1989). Telephone interview data were available from a total of 6257 individual twins (3454 women and

2803 men). The overall individual response rate was 73·3%, with a higher response rate for women than men (77·7% *v.* 68·6%; Heath *et al.* 2001). At the time of interview, all respondents ranged in age from 24 to 36 years old (mean age ± S.D.: women 30·0 ± 2·48; men 29·9 ± 2·45).

This was a well educated sample, with 26·3% of women and 25·2% of men holding a university degree or higher, in addition to another 28·3% of women and 29·3% of men holding a high-school diploma plus additional technical training. Australian census data show that 17·5% of 25- to 34-year-olds had bachelor's degrees in 1997, with this percentage rising to 20·1% in 1999 and 24% in 2001; and that Australians with high-school diploma plus additional training comprised 31·4% in 1997, 32·8% in 1999, and 33·6% in 2001 (Australian Bureau of Statistics, 2001). Thus the 1989 twin sample was slightly better educated than their general population cohort. Forty-eight and a half per cent of the women and 87·4% of the men were employed full-time; for the women, an additional 22·7% were employed part-time (only 5·6% of the men) and 23·6% were homemakers (0·4% of the men).

Analyses of smoking and nicotine dependence variables were performed on data available from a total of 6249 individual twins (3449 women and 2800 men) who answered either of the first two items of the smoking history interview section, which assessed whether respondents had ever experimented with cigarettes. Phenotypic analyses were restricted to twins who reported they had ever tried smoking or had experimented with cigarettes at least once (ever smokers; $n = 3027$ women and 2553 men). The majority of the twins in this sample were ever smokers (87·8% of the women and 91·2% of the men; $p < 0{\cdot}01$). Data on cigarette smoking history were missing for five female and three male twins. Genetic analyses were conducted using twin pairs who were concordant ever smokers. Data on history of cigarette-smoking and nicotine dependence were available from both twins of 558 monozygotic (MZ) female, 419 MZ male, 424 dizygotic (DZ) female, 333 DZ male, and 559 DZ female-male twin pairs who were concordant ever smokers. We did not exclude from the analyses twins who had been exposed to nicotine, but never smoked regularly (i.e. experimenters). By including non-regular smokers in the genetic analysis, we sought to minimize the risk of obtaining biased estimates of the genetic contribution to the covariance structure of nicotine dependence symptoms, if some of the same genetic factors that contribute to risk of nicotine dependence symptoms also influence probability of becoming a regular smoker (Heath *et al.* 2002).

Zygosity was determined through standard questions about twin physical similarity and confusion by teachers and strangers. This method of zygosity determination shows 95% agreement with assignment based on blood tests (Eaves *et al.* 1989).

Measures

Lifetime DSM-IV nicotine dependence criteria

We used interview items that directly corresponded to the seven DSM-IV dependence criteria: tolerance to nicotine (assessed in response to an open-ended question about the largest number of cigarettes ever smoked in a single day: reporting 20 or more cigarettes was defined as tolerance); nicotine withdrawal symptoms; smoking more or over longer periods of time than intended; experiencing a persistent desire or unsuccessful attempts to cut down or quit smoking; spending a great deal of time smoking (operationalized as chain-smoking); reducing or giving up important activities because of an inability to smoke at such times; and continued smoking despite physical or psychological problems caused or exacerbated by smoking (APA, 1994).

Lifetime DSM-IV nicotine dependence diagnosis

DSM-IV nicotine dependence was defined as experiencing three or more of the seven criteria within the same 12-month period.

Lifetime Heaviness of Smoking Index items

Time to first cigarette in the morning (TTF) and number of cigarettes smoked per day (CPD) were assessed for the respondents' heaviest period of smoking. TTF was a four-category measure: smoking less than 5 min, 6–30 min, 31–60 min, or more than 60 min, after waking up; CPD was a five-category measure: 1–5, 6–10, 11–15, 16–19, and 20+.

Lifetime Heaviness of Smoking Index-defined nicotine dependence

TTF and CPD categories were coded as ordinal variables. Summing over TTF and CPD categories produced an eight-level scale ranging from light smokers at the lowest level who smoked maximum five CPD and had their first cigarette no earlier than one hour after waking up to smokers who smoked 20+ CPD and had their first cigarette within 5 min of waking up. We collapsed this scale into four mutually exclusive categories of what we called non-dependent, mildly dependent, moderately dependent, and dependent individuals (see note in Table 1 for details).

Statistical analyses

Phenotypic data analysis

Prevalence estimates of nicotine dependence symptoms and dependence definitions were computed for ever smokers; gender differences were evaluated using binomial and multinomial logistic regression in STATA (StataCorp, 2001), with the Huber–White robust estimator of variance (Huber, 1967; White, 1980, 1982) used to correct for non-independence of observations on twin pairs.

Exploratory factor analysis in ever smokers was performed using the structural equation-modeling program Mx (Neale *et al*. 2002). Polychoric correlations and asymptotic covariance matrices of the nine nicotine dependence items were estimated using PRELIS (Jöreskog & Sörbom, 1995), separately for women and men. The computation of polychoric correlations for categorical variables assumes an underlying normally distributed latent liability for each measure, with thresholds superimposed to yield the discrete response categories. Factor loadings were estimated by asymptotic weighted least squares. Factor loadings generated in Mx were rotated using the SAS PROMAX option (SAS, 1999) to allow for a correlated factor structure. Internal consistency of resulting factor scales was evaluated using Cronbach's alpha.

Genetic data analysis

Genetic analyses were conducted using data from the total sample of twins who had ever smoked cigarettes, including experimenters. To the extent that there are overlapping genetic influences on progression from experimentation to regular smoking and on risk of nicotine dependence symptoms in those who became regular smokers, exclusion of twin pairs where one or both twins have never smoked regularly could lead to biased estimates of genetic and environmental parameters (Heath *et al*. 2002). Analyses were also repeated using all twin pairs, including twins who had never smoked even a single cigarette, but since the latter group was small, results were similar.

Univariate genetic analysis. Using data from twin pairs reared together, phenotypic variance can be decomposed into at least three sources: (1) additive effects of genes (A); (2) shared environmental effects common to members of a twin pair (C); and (3) environmental effects unique to each twin, including error variance (E). In place of C, we can also test for non-additive genetic effects (D) such as dominance (interaction between alleles of the same gene) or epistasis (gene–gene interaction). The reader is referred to a recent review by Boomsma and colleagues (2002) for details on twin methodology. The variance component designations A, C (or D) and E reflect the square of the regression coefficients of the variance component latent parameter on the measured variable (a^2, c^2, d^2, and e^2 respectively). The ACE (or ADE) models were fit to the observed data (summary MZ and DZ twin pair covariance matrices for the quasi-continuous HSI measure; summary MZ and DZ twin pair contingency tables for all other measures) by the method of maximum-likelihood, with the goodness of fit of each model assessed by the χ^2 test. Significance of the A or C (or D) variance component parameters was tested by fixing A, C (or D), or both parameters simultaneously to zero and comparing the fit of the reduced models to the full model using likelihood-ratio χ^2 test. A significant likelihood-ratio χ^2 test with degrees of freedom equal to the degrees of freedom difference between the full and reduced models indicates a significant deterioration of fit of the reduced, relative to the full, model, indicating that the parameter cannot be equated to zero. In order to test for gender differences in A, C (or D), or E parameter estimates, we compared models in which estimates were free to vary across gender with

reduced models in which estimates were equated across gender using the likelihood-ratio χ^2 test. Genetic analyses were performed in Mx (Neale *et al*. 2002).

Multivariate genetic analysis. We tested two genetic mechanisms (cf. Kendler *et al*. 1987) via which covariance among nicotine dependence symptoms might arise. One mechanism, the independent (or general) pathway model, postulates that genetic and environmental factors act independently from one another in their influence on nicotine dependence symptom covariation. A second mechanism, the common pathway model, postulates that dependence symptom covariation can be explained by one or more intermediate latent phenotypes (e.g. some underlying mechanism, such as nicotine metabolism) that are themselves determined by genetic and environmental factors; thus, in the common pathway model, genetic and environmental factors contribute in a fixed ratio (correspondent to the heritability of the latent factor) to symptom covariation. Independent and common pathway models were fitted to summary MZ and DZ twin pair polychoric correlation matrices, using associated asymptotic covariance matrices as weight matrices, in Mx, by the method of asymptotic weighted least squares. Because the common pathway model represents a series of constraints on the independent pathway model, the two can be formally compared using the χ^2 difference test. Gender differences in parameter estimates were evaluated as in univariate analyses.

In the genetic factor model, failing to account for correlated measurement errors for different nicotine dependence items could result in biased estimates of genetic and environmental effects. In order to minimize the probability of misspecification of the genetic and shared environmental estimates, and to ensure that all error item variances and covariances were accounted for, we specified nine non-shared environmental factors (equal to the number of nicotine dependence items) loading in a triangular decomposition (Cholesky) pattern, where the first factor loaded on the first nicotine dependence item and on all subsequent items, the second factor loaded on the second dependence item and on all subsequent items, and so on. The general factor model applied to the additive genetic and shared environmental parameters is a submodel of a Cholesky decomposition model.

For both univariate and multivariate models, model parsimony (best fit with fewest parameters estimated) was assessed based on χ^2 difference tests and the Aikake Information Criterion statistic (AIC; Akaike, 1987), calculated as model χ^2 minus twice the degrees of freedom; lower AIC indicates better model fit.

Table 1. *Lifetime prevalence* (%) *of DSM-IV and Heaviness of Smoking Index* (*HSI*) *nicotine dependence measures in female and male ever smokers* (*i.e. experimented with cigarettes at least once*)

DSM-IV nicotine dependence criteria	Women ($n=3027$)	Men ($n=2553$)	OR (95% CI)
Tolerance	37·9	48·1	1·5 (1·4–1·7)
Withdrawal	24·1	24·6	N.S.
Smoking more than intended	42·2	40·8	N.S.
Difficulty quitting	49·4	53·0	1·2 (1·02–1·3)
Ever chain-smoked	20·9	22·1	N.S.
Gave up activities to smoke	6·0	5·9	N.S.
Smoked despite physical or emotional problems	19·4	18·3	N.S.
Nicotine dependence diagnosis	33·0	36·0	1·1 (1·01–1·3)
HSI items and HSI-defined nicotine dependence			
TTF: <5 min	6·7	9·6	1·6 (1·3–2·0)
6–30 min	11·9	15·9	1·5 (1·3–1·8)
31–60 min	9·8	10·8	1·2 (1·02–1·5)
60+ min	71·5	63·7	1·0
CPD: 20+	18·4	27·1	1·7 (1·4–1·9)
16–19	7·6	8·8	1·3 (1·1–1·6)
11–15	10·1	9·3	N.S.
6–10	10·5	7·7	N.S.
1–5	53·2	47·1	1·0
HSI dependence			
Dependent	12·2	19·1	1·8 (1·5–2·1)
Moderately dependent	13·8	16·5	1·4 (1·2–1·6)
Mildly dependent	13·2	12·3	N.S.
Non-dependent	60·7	52·1	1·0

Odds ratios (OR) and 95% confidence intervals (CI) indicate significant gender differences.

TTF and CPD categories were coded as ordinal variables by order of severity (0–3 for TTF, i.e. 60+ min to <5 min; and 0–4 for CPD, i.e. 1–5 to 20+ cigarettes per day), and summed across TTF and CPD categories to yield an HSI dependence score: non-dependent (0–1), mildly dependent (2–3), moderately dependent (4–5), and dependent (6–7).

TTF, time to first cigarette in the morning; CPD, cigarettes per day.

RESULTS

Prevalence

Prevalence estimates of lifetime DSM-IV and HSI nicotine dependence items and nicotine

Table 2. *Phenotypic factor loadings of DSM-IV and HSI nicotine dependence items for female (n = 3027) and male (n = 2553) ever smokers, and inter-factor correlations (r)*

Items	Women		Men	
	F1 ($r = 0{\cdot}75$)	F2	F1 ($r = 0{\cdot}74$)	F2
(1) Tolerance	0·81	0·19	0·79	0·24
(2) Withdrawal	0·29	0·62	0·16	0·75
(3) More than intended	0·44	0·56	0·40	0·56
(4) Difficulty quitting	0·35	0·72	0·31	0·78
(5) Ever chain-smoked	0·32	0·47	0·36	0·43
(6) Gave up activities	0·00	0·68	0·27	0·41
(7) Smoke despite problems	0·25	0·58	0·10	0·74
(8) TTF	0·77	0·11	0·75	0·16
(9) CPD	0·95	0·08	0·91	0·14

TTF, time to first cigarette in the morning; CPD, cigarettes per day.

dependence definitions for female and male ever smokers are shown in Table 1. Significantly more men than women smokers reported tolerance (48·1% *v.* 37·9%), difficulty quitting smoking (53·0% *v.* 49·4%), and met DSM-IV criteria for nicotine dependence (36% *v.* 33%). Additionally, more men than women smoked within the first hour after waking (36·3% *v.* 28·4%), more men than women were heavier smokers, averaging 16 or more cigarettes per day (35·9% *v.* 26·1%), and, accordingly, more men than women were in the more severe HSI nicotine dependence categories.

Factor analysis

Phenotypic factor analysis performed on the seven DSM-IV nicotine dependence symptoms and the two HSI items in ever smokers identified a three-factor model as the best-fitting model in both women and men. However, only a single item, 'ever chain-smoked', had a substantial loading on the third factor in both genders. Therefore the two-factor solution was chosen as the most interpretable (Table 2). Although the pattern of item loadings across the two factors was similar across gender, with only the magnitude of loadings of some items ('gave up activities' and 'smoke despite problems') differing somewhat in women and men, the model constraining item loadings to be equal across gender was rejected ($p < 0{\cdot}001$). For both women and men, DSM-IV nicotine tolerance together with TTF and CPD loaded highly on the first factor; four other DSM-IV dependence measures loaded more highly on the second factor – withdrawal, smoking more than intended, experiencing difficulty quitting, and smoking despite physical or psychological problems; DSM-IV ever chain-smoked loaded equally strongly on the two factors. Thus for both genders, the two-factor solution seemed to distinguish a tolerance/quantity smoked factor (on which time to first cigarette also had a strong loading), and a second factor associated with withdrawal and difficulty quitting smoking. However, the two factors were highly correlated ($r = 0{\cdot}75$ in women, $r = 0{\cdot}74$ in men). Restricting analysis to regular smokers (100+ cigarettes lifetime; results not shown) showed that item loadings could be constrained across gender and the pattern and magnitude of factor loadings across the two latent factors were nearly identical to those shown for the men in Table 2. Thus, factor analysis results suggest a similar pattern of endorsement of nicotine dependence symptoms between women and men, particularly among lifetime regular smokers.

Internal consistency

Internal consistency was acceptable for both factor scales for both female and male ever smokers. For the tolerance/quantity smoked factor, Cronbach's alpha was 0·78 for the women and 0·79 for the men. For the withdrawal/difficulty quitting factor, alpha estimates were 0·78 for the women and 0·78 for the men; 'gave up activities' had the lowest correlation with total scale score for both women (0·30) and men (0·27) compared to the remaining dependence items loading on this factor (range 0·50–0·75) and internal consistency improved to 0·81 and 0·80 for women and men, respectively, when this item was excluded.

Univariate genetic analysis

In the sample of twin pairs concordant for ever smoking, Table 3 shows that for all dependence symptoms, except for 'difficulty quitting', A, C, and E effects could be equated across gender; additionally, shared environmental effects did not contribute significantly to phenotypic variance, although their point estimates and associated 95% confidence intervals are shown in

Table 3. *Univariate estimates (%) of additive genetic (A), shared environmental (C), and non-shared environmental (E) influences and associated 95% CI for DSM-IV and HSI nicotine dependence items and definitions in twin pairs concordant for ever smoking*

	Variance component estimates			Model fit statistics			
Items	A (95% CI)	C (95% CI)	E (95% CI)	χ^2	df	*p*	AIC
(1) Tolerance	73 (54–79)	1 (0–17)	26 (21–32)	16·2	7	0·02	2·2
(2) Withdrawal	53 (37–61)	0 (0–12)	47 (39–55)	3·8	7	0·80	−10·1
(3) More than intended	62 (43–69)	0 (0–16)	37 (31–45)	11·9	7	0·11	−2·1
(4) Difficulty quitting							
Women	68 (60–75)	0 (0–60)	32 (25–40)	12·9	5	0·02	2·9
Men	54 (28–89)	26 (0–49)	20 (14–28)				
(5) Ever chain-smoked	45 (23–54)	0 (0–16)	55 (46–65)	9·4	7	0·22	−4·6
(6) Gave up activities	26 (0–45)	0 (0–27)	74 (55–93)	7·7	7	0·36	−6·3
(7) Smoke despite problems	39 (7–51)	2 (0–25)	59 (49–72)	10·0	7	0·19	−4·0
(8) TTF	68 (51–73)	0 (0–13)	32 (27–38)	83·7	58	0·02	−32·3
(9) CPD	70 (56–75)	0 (0–12)	30 (25–34)	147·7	94	<0·001	−40·3
Nicotine dependence							
DSM-IV	56 (40–63)	0 (0–13)	44 (36–51)	9·8	7	0·21	−4·3
HSI	71 (59–75)	0 (0–10)	29 (25–34)	109·4	58	<0·001	−6·6

Table 3. All individual symptoms were significantly heritable (45–73%; except for 'gave up activities') as were DSM-IV (56%) and HSI (71%) definitions of nicotine dependence. For 'gave up activities', it was not possible to distinguish between additive genetic *versus* shared environmental influences because there was no significant deterioration of model fit when either A or C were individually dropped from the model; however, dropping both simultaneously significantly reduced model fit, suggesting significant familial influences.

There was no evidence for a significant contribution of non-additive genetic effects to phenotypic variance for any item.

Multivariate genetic analysis

In a four-group analysis of data from pairs who were concordant ever smokers, excluding unlike-sex twin pairs, the best-fitting model estimated a single genetic and a single shared environmental common factor (AIC = 779·46; likelihood-ratio test *versus* full Cholesky model: $\chi^2 = 56{\cdot}20$, df = 108, $p = 0{\cdot}99$). Adding a second genetic common factor (AIC = 791·42) or a second shared environmental common factor (AIC = 787·55) worsened the fit of the model by AIC, and did not produce a significant improvement in fit by likelihood-ratio χ^2 test ($\chi^2 = 24{\cdot}04$, df = 18, $p = 0{\cdot}15$; $\chi^2 = 27{\cdot}91$, df = 18, $p = 0{\cdot}06$). A model which constrained genetic and environmental factor loadings to be the same in females and males (AIC = 1176) and a common pathway model (AIC = 1090) both gave substantially worse fits. Genetic common factor loadings under the best-fitting model were substantial (data not shown), and somewhat higher in males (0·67–0·89) than in females (0·61–0·81), with the lowest loadings observed for 'ever chain-smoked' and 'gave up activities' in both genders (0·53 and 0·74 for females, 0·67 and 0·71 for males, respectively); and, in females, for 'smoke despite problems' (0·61) and 'withdrawal' (0·68), with all other loadings being 0·75 or higher. Non-zero genetic specific variances were obtained for only three items, and in females only (TTF: 0·27; withdrawal: 0·26; 'ever chain-smoked': 0·21). Shared environmental common factor loadings were generally higher in females (zero for 'gave up activities', 0·18–0·42 for other items) than in males (zero for 'more than intended', 0·10–0·35 for other items), but were substantially lower than the corresponding genetic common factor loadings. A single non-zero shared environmental specific variance was obtained in each gender (female: 0·12 for 'more than intended'; males: 0·20 for 'withdrawal').

The five-group analysis of pairs who were concordant ever smokers uncovered important genotype × gender interaction effects. The fit of a model estimating a single genetic and a single shared environmental common factor for each gender ($\chi^2 = 2268{\cdot}59$, df = 621, AIC = 1026) was

improved on either by allowing the correlation between shared environmental effects, rC, in unlike-sex pairs, to deviate from unity (AIC = 1017) or by allowing the correlation between genetic effects in unlike-sex pairs, rG, to take values less than 0·5 (AIC = 1010). Compared to the one genetic and one shared environmental factor model with rC = 1 and rG = 0·5, estimating a second genetic common factor (AIC = 995) or a second shared environmental common factor (AIC = 1009) yielded an improvement in fit, with the two genetic factor model giving the better fit by AIC. A model that estimated two genetic and two shared environmental common factors (AIC = 1002) gave a substantial improvement in fit over the model with two shared environmental and a single genetic common factor ($\chi^2 = 43{\cdot}25$, df = 18, $p < 0{\cdot}001$) but only a marginal improvement in fit over the two genetic/one shared environment common factor model ($\chi^2 = 29{\cdot}30$, df = 18, $p = 0{\cdot}045$), and a worsening of fit compared to the latter by AIC. Adding a third genetic factor did not give any further improvement over the latter model (AIC = 1016; $\chi^2 = 15{\cdot}40$, df = 18, $p = 0{\cdot}63$). Thus the two genetic/one shared environmental common factor model was identified as the best-fitting model in the five-group analyses. Allowing correlations between the first or second genetic factors in unlike-sex pairs to take values less than 0·5 gave no change in fit. However, genetic factor loadings on the second genetic factor under the best-fitting model were opposite in sign in males *versus* females (equivalent to a negative correlation between genetic effects associated with the second factor in males *versus* females). Likewise, shared environmental common factor loadings were opposite in sign. Genetic and shared environmental specific factor loadings were similar to those observed in the four-group analysis.

Unrotated common factor loadings, under the best-fitting five-group model, are summarized in Table 4. The first genetic factor in each gender had high loadings on all items, with a pattern of loadings very similar to that seen for the single genetic factor estimated in the four-group solution. In females, the second genetic factor had high loadings only on items 'gave up activities' and 'difficulty quitting'; whereas in males the second genetic factor had moderate loadings on items broadly related to amount smoked (TTF, CPD, tolerance) as well as 'gave up activities' and 'more than intended'.

Table 4. *Factor loadings of DSM-IV and HSI nicotine dependence items on two orthogonal additive genetic factors (AF1 and AF2) and one shared environmental factor (C), for women and men ever smokers*

	Women			Men		
Items	AF1	AF2	C	AF1	AF2	C
(1) Tolerance	0·88	−0·09	0·03	0·77	0·22	0·26
(2) Withdrawal	0·67	−0·15	−0·15	0·78	0·12	0·22
(3) More than intended	0·83	−0·08	−0·16	0·80	0·18	0·28
(4) Difficulty quitting	0·76	−0·31	−0·08	0·78	0·10	0·27
(5) Ever chain-smoked	0·70	0·05	−0·06	0·57	0·09	0·12
(6) Gave up activities	0·57	−0·49	−0·05	0·64	0·23	0·27
(7) Smoke despite problems	0·71	0·00	−0·17	0·79	0·00	0·46
(8) TTF	0·78	0·03	−0·12	0·81	0·32	0·32
(9) CPD	0·86	−0·08	−0·04	0·80	0·22	0·26

TTF, time to first cigarette in the morning; CPD, cigarettes per day.

Item non-shared environmental variances and correlations under the best-fitting model are summarized in Table 5. Inter-item correlations are much higher in males than in females, with many estimated correlations in males 0·9 or higher in magnitude. This suggests that in males, non-shared environmental influences are largely associated with differences in the extent of progression of cigarette smoking, whereas in females non-shared environmental contributions to symptom profile are observed.

DISCUSSION

The DSM-IV criteria that are used to diagnose substance dependence have been applied to nicotine, as well as to alcohol and illicit drugs (APA, 1994). The Heavy Smoking Index items time to first cigarette in the morning and number of cigarettes smoked per day (Heatherton *et al.* 1989) comprise two of the original eight items of the Fagerström Tolerance Questionnaire (Fagerström, 1978), which was designed to assess physical dependence to nicotine. The goal of this paper was to conduct multivariate genetic analysis of DSM-IV and HSI items to define heritable aspects of nicotine dependence, thereby adding to the body of knowledge on the construct validity and properties of

Table 5. *Proportion of variance* (%) *attributable to non-shared environmental factors for each nicotine dependence item* (*shown across the diagonal in bold*), *and pair-wise non-shared environmental correlations among nicotine dependence items* (*shown in the off diagonal*) *for women* (*top*) *and men* (*bottom*) *ever smokers*

	Tolerance	Withdrawal	Intend	Quit	Chain	Activity	Problems	TTF	CPD
Women									
Tolerance	**21·6**								
Withdrawal	0·84	**42·3**							
Intend	0·79	0·89	**27·5**						
Quit	0·90	0·89	0·92	**32·3**					
Chain	0·70	0·50	0·67	0·66	**46·9**				
Activity	0·83	0·83	0·91	0·99	0·61	**26·8**			
Problems	0·60	0·57	0·52	0·62	0·28	0·60	**48·3**		
TTF	0·72	0·65	0·47	0·74	0·29	0·71	0·33	**31·1**	
CPD	0·91	0·72	0·65	0·79	0·44	0·82	0·45	0·74	**25·1**
Men									
Tolerance	**27·3**								
Withdrawal	0·85	**23·7**							
Intend	0·90	0·85	**29·3**						
Quit	0·96	0·93	0·96	**30·9**					
Chain	0·82	0·67	0·94	0·82	**44·8**				
Activity	0·98	0·91	0·94	0·99	0·82	**44·5**			
Problems	0·90	0·93	0·92	0·98	0·74	0·96	**31·3**		
TTF	0·84	0·79	0·61	0·81	0·42	0·83	0·79	**17·7**	
CPD	0·96	0·73	0·78	0·86	0·75	0·89	0·75	0·81	**26·4**

Intend, more than intended; Quit, difficulty quitting; Chain, ever chain-smoked; Activity, gave up activity; Problems, smoking despite problems; TTF, time to first cigarette; CPD, cigarettes per day.

the nosology currently used to define nicotine dependence.

Phenotypic factor analysis showed clustering of symptoms of tolerance to nicotine together with the two heavy smoking index items on one factor for women and men, which is not surprising considering that both tolerance and number of cigarettes smoked per day were operationalized in terms of quantity of cigarettes smoked (albeit different items), and that time to first cigarette upon waking and cigarettes smoked per day have been shown to be highly correlated in other general population studies (Etter *et al*. 1999; Haddock *et al*. 1999; Radzius *et al*. 2001). The weak loading of the time to first cigarette item on the second factor, on which nicotine withdrawal had a strong loading, suggests that contrary to what has previously been suggested (APA, 1994), time to first cigarette may not be a useful measure of nicotine withdrawal. Alternatively, our retrospective measure of withdrawal may not adequately capture symptoms of withdrawal following overnight abstinence.

In the second factor 'gave up activities' was the only item that loaded significantly in women but not in men. However, 'gave up activities' had the lowest correlation with total factor scale score, and when this measure was excluded, factor scale internal consistency improved in women and men. In another study, 'gave up activities' did not load on either of two factors identified in a factor analysis of DSM-III-R nicotine dependence symptoms in young adult female and male lifetime smokers (current and ex-smokers) similar in age and year of assessment, as reported in this study (21–30 years old; interviewed at home in 1989; Johnson *et al*. 1996), and the authors excluded this item from subsequent creation of factor scales. Additionally, in our analyses, 'gave up activities' was the least commonly endorsed nicotine dependence item by women and men, and was found in genetic analyses of these data to be mainly influenced by non-shared environmental factors. Taken together, these results suggest that the DSM-IV substance dependence criterion of 'important social, occupational, or recreational activities are given up because of substance use', as measured in our assessment, may not be a salient indicator of nicotine dependence in adults. Additionally, the importance of this criterion will likely vary in response to social and cultural changes in regulation of smoking practices.

'Ever chain-smoked' did not preferentially load on one phenotypic factor or the other, but showed significant and high loadings on a third factor for women (0·97) and men (0·83), indicating that 'ever chain-smoked' is not endorsed in a pattern that correlates with endorsement of the remaining nicotine dependence symptoms. It may be that 'ever chain-smoked' is too broad a measure or that it may occur only intermittently for many smokers, such as at times of stress, rather than represent a consistent, specific pattern of smoking behavior. Additionally, this item, which corresponds to the DSM-IV dependence criterion 'a great deal of time spent in using the substance', assessed at a general population level, may be more relevant to illicit substance use. The DSM-IV distinguishes between physiological dependence associated with tolerance or withdrawal and non-physiological (psychological) dependence associated with the lack of tolerance and withdrawal (APA, 1994). Since tolerance and withdrawal each loaded on separate factors, it appears that phenotypic factor analysis captured two aspects of physiological dependence to nicotine, and psychological symptoms of nicotine dependence loaded on the same factor as withdrawal. The clustering of these nicotine dependence items has been termed 'failed cessation' by others (Johnson *et al*. 1996).

There was evidence for substantial genetic influences accounting for individual differences in nicotine dependence measures (range 45–73%; except for 'gave up activities' where there was only evidence for weak familial effects). Our genetic estimate for DSM-IV nicotine dependence (56%) is similar to that for DSM-III-R nicotine dependences assessed in Vietnam veteran male twins (60%; True *et al*. 1999); the heritability estimate for daily cigarette consumption was high (70%) when the analysis included twin pairs concordant for ever smoking, relative to other estimates (50%; Hettema *et al*. 1999). Heritability was more modest, however, when analysis was restricted to regular smokers (51%; data not shown) and agreed with heritability estimates (about 50%) reported previously in adult current and ex-smoker lifetime regular smokers (Kaprio *et al*. 1984; Carmelli *et al*. 1990; Swan *et al*. 1990, 1996, 1997). Exclusion of twin pairs, where one or both twins has never smoked, or never smoked regularly, will lead to a biased estimate of the importance of genetic influences if in fact some of the same genetic factors that influence risk of nicotine dependence also influence risk of experimentation with cigarettes, or risk of becoming a regular smoker. In many cases, for traits where shared environmental influences are modest or negligible, it will lead to an underestimate of the importance of genetic influences. Exclusion of groups of types of smokers would be valid only in the case where liability for smoking experimentation or occasional smoking is uncorrelated with liability for regular smoking or nicotine dependence (Prescott & Kendler, 1995). In fact, two-stage modeling of dimensions of smoking initiation and smoking persistence (Heath, 1990; Heath & Martin, 1993; Heath *et al*. 1998; Madden *et al*. 1999) and of smoking initiation and number of cigarettes smoked per day in adolescents and young adults (Koopmans *et al*. 1999) have shown evidence of shared genetic risk. Heritability estimates derived under a two-stage model (Heath *et al*. 2002) that estimates jointly genetic effects on risk of becoming a regular smoker, and correlated genetic effects on individual symptoms of nicotine dependence in those who have become regular smokers, should yield a less biased estimate of heritability, but this is beyond the scope of the current paper.

The univariate genetic models did not fit the data for time to first cigarette in the morning and number of cigarettes smoked per day, or for their joint definition of nicotine dependence, suggesting that different genetic and environmental influences may determine risk of lower levels of smoking or smoking right after waking in the morning *versus* higher levels of smoking or smoking later in the day.

In the multivariate genetic models, although the results of four-group and five-group analyses appeared somewhat different, with the best-fitting model in the four-group analysis including a single genetic factor but that for the five-group analysis including two genetic factors in women and men, there was a trend for the addition of a second genetic factor to improve goodness-of-fit in the four-group analysis by likelihood-ratio χ^2 test criterion ($p = 0{\cdot}045$). In both four- and five-group analyses, results were consistent with the existence of a general genetic factor with high loadings of all items, albeit with

somewhat reduced loadings for items 'gave up activities' in both genders, and for 'withdrawal' in women and 'ever chain-smoked' in men. The second genetic factors had loadings that were opposite in sign in males and females, implying a negative genetic correlation, and were characterized by somewhat different items ('gave up activities' and 'difficulty quitting' in women; items related to amount smoked in men). Until this pattern has been replicated, these loadings should be interpreted with caution: once the first genetic common factor is controlled for, power for resolving the residual genetic structure would be expected to be somewhat low. However, the fact that a similar structure for the second genetic factor was observed in four-group (data not shown) as well as five-group analyses indicates that it is not simply an idiosyncratic pattern of cross-twin pair correlations in unlike-sex pairs that is leading to the observed pattern of genetic factor loadings.

The present analyses add to growing evidence for substantial genetic influence on nicotine dependence (Sullivan & Kendler, 1999; True *et al.* 1999). DSM-IV substance dependence criteria, which were developed to be applicable to a range of substances (Cottler *et al.* 1995), also appear to be useful indicators of nicotine dependence. Five nicotine dependence symptoms, in particular, had high phenotypic and genetic factor loadings as well as high heritability: tolerance, time to first cigarette in the morning, number of cigarettes smoked per day, withdrawal, and difficulty quitting. What makes some of these items particularly attractive is that they can be investigated in animal models. Animal studies show that development of tolerance to nicotine and quantity of self-administered nicotine are under significant genetic influences (Marks *et al.* 1991; Robinson *et al.* 1996) and are associated with changes in nicotine binding in the brain (Collins & Marks, 1989, 1991; Pietilä *et al.* 1998). Nicotine withdrawal can be considered an index of nicotine dependence in rodents and has been shown to be mediated by central nicotinic receptors and a number of neurotransmitter systems (Kenny & Markou, 2001).

Several limitations of this study should be considered. This is a large-scale community study, but conclusions drawn may be specific to the age range studied (24–36), to the time of assessment (1996–2000), and to the country of Australia. Genetic or environmental factors affecting aspects of cigarette use and dependence may change with advancing age or may be different during adolescence (or may be associated with length of time smoked), across birth cohorts, or across societies. For example, it has been shown that an overlap in genetic and environmental risk factors for starting to smoke and for continuing to smoke decreases with age when comparing age cohorts of 18–25-year-old and 36–46-year-old female and male twins (Madden *et al.* 1999). Evidence for greater importance of shared environmental effects on starting to smoke in Scandinavian relative to Australian men (Madden *et al.* 1999) further suggests cultural differences in cigarette-smoking.

Our data are based on retrospective self-report and are thereby liable to recall bias, particularly for respondents who are not current smokers. However, lifetime symptoms and diagnoses of nicotine and other substance use have been shown to have acceptable reliability (Cottler *et al.* 1989; Langenbucher *et al.* 1994) and self-reports of smoking have been found to be accurate when validated through biochemical measures (Patrick *et al.* 1994).

Inclusion of never smokers in the multivariate genetic modeling represents a confound of the genetic and environmental influences on smoking initiation and on risk of nicotine dependence in those who have become smokers and may introduce bias in parameter estimates. In the univariate approach, there are data-analytic strategies that can be applied under certain strong but testable assumptions to more rigorously separate genetic influences associated with initiation of regular smoking and genetic influences associated with risk of dependence in those who have become regular smokers (e.g. Heath *et al.* 2002), avoiding some of the somewhat arbitrary assumptions that have been used in the past to achieve model identification (e.g. Heath & Martin, 1993). While generalization of such an approach to the estimation of multivariate models is achievable in theory, this rapidly becomes numerically intractable in the multivariate case unless the number of variables being analyzed is small (four or fewer), and thus could not be applied in the present analysis. Since we consider it plausible that there are important genetic influences that are common to

both progression from experimentation to regular smoking and risk of dependence in those who become regular smokers (for example, conduct disorder: cf. Madden *et al.* 1997), we have preferred to include experimenters as well as smokers in our multivariate genetic analyses, to avoid the potential underestimation of the importance of genetic influence that would arise by excluding such individuals. Results when never smokers as well as experimenters were included in our analyses were similar (not shown), but numbers of never smokers were relatively small.

The twin method relies on the assumption that trait-relevant environmental influences contribute equally to the resemblance of MZ and DZ twin pairs. Environmental influences that are more similar for MZ compared to DZ twin pairs may be associated with a trait of interest and could account for greater similarity between MZ than DZ twins (i.e. inappropriately inflating estimates of trait heritability). Such characteristics, for example, physical twin similarity (Hettema *et al.* 1995), self-perceived zygosity (Xian *et al.* 2000), perceived zygosity and associated parental approach to rearing their twins (Kendler *et al.* 1993, 1994; Kendler & Gardner, 1998; Cronk *et al.* 2002), and physical and emotional closeness between the twins (LaBuda *et al.* 1997; Kendler & Gardner, 1998; Cronk *et al.* 2002) have been shown to be uncorrelated with zygosity differences in a number of psychiatric disorders such as alcohol and illicit drug dependence, major depression, anxiety, and externalizing disorders, supporting the validity of the equal environmental assumption in twin studies assessing these disorders. The equal environment assumption has also been found to be valid for DSM-III-R nicotine dependence in female (Kendler & Gardner, 1998) and male twins (Xian *et al.* 2000). However, greater sharing of peer group in adolescence in MZ compared to DZ twins is associated with greater MZ compared to DZ twin similarity for smoking initiation (Kendler & Gardner, 1998), which may not be surprising considering that peer smoking is a strong and important predictor of adolescent smoking initiation (Chassin *et al.* 2000; Mayhew *et al.* 2000; Kobus, 2003), and that MZ twins are more likely to share peers compared to DZ twins. Our sample of twins included young adults who were past adolescence, when the majority of adult smokers initiate smoking (Upadhyaya *et al.* 2002), and considering that nicotine dependence can be observed shortly after the start of regular smoking (DiFranza *et al.* 2002), the possible violation of the equal environment assumption with respect to smoking initiation would not be relevant to our analyses. We tested, therefore, the equal environment assumption with respect to DSM-IV and HSI definitions of nicotine dependence. We separated the sample into two groups, those who reported always sharing the same friends in childhood and early adolescence and those who reported sharing the same friends less often. We found no group differences in estimates of the relative contribution of genetic and shared environmental influences on DSM-IV and HSI definitions of dependence, suggesting that the equal environment assumption is valid for these measures of nicotine dependence in our data.

In conclusion, we found that nearly all symptoms of nicotine dependence were moderately to highly heritable and that nicotine dependence symptom covariance was influenced by two genetic and one shared environmental factors in both women and men, with symptoms on the second genetic factors loading opposite in sign in women *versus* men, suggesting a negative genetic correlation and implying that different genetic factors may, in part, operate in women compared to men. Our analyses suggest that the two DSM-IV criteria of giving up or reducing important activities in order to smoke and spending a lot of time using nicotine (i.e. chain-smoking) may contribute little to our measurement of nicotine dependence for the purpose of genetic research; while the DSM-IV criteria tolerance, withdrawal, and experiencing difficulty quitting, along with the two HSI index items may best signify nicotine dependence, and, considering their substantial heritability, they may also be fruitful phenotypes for gene-mapping efforts.

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Treating smokers before the quit date: Can nicotine patches and denicotinized cigarettes reduce cravings?

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The present study investigated whether treatment with the combination of denicotinized cigarettes and 21-mg nicotine patch for 2 weeks before a designated quit date could lessen cravings for smoking, thereby helping smokers abstain from smoking. The study was a randomized controlled clinical trial conducted at Roswell Park Cancer Institute, Buffalo, New York, in 2004 and 2005. Patients included 98 adult heavy smokers (using 20 or more cigarettes/day). Half of the subjects received 2 weeks of combination of denicotinized cigarettes (Quest 3) and 21-mg nicotine patch for 2 weeks before the quit date. The remaining smokers were switched to light cigarettes (Quest 1) during the 2 weeks before the quit date. After the quit date, all subjects received counseling for smoking cessation and were provided nicotine patches for up to 8 weeks after the quit date. Self-reported cravings for smoking, withdrawal symptoms, and smoking abstinence were measured at predetermined intervals using phone-based surveys and in clinical visits. The group that used denicotinized cigarettes and nicotine patch before quitting reported less frequent and less intense cravings for cigarettes in the 2 weeks before and after the designated quit date. Self-reported withdrawal symptoms and quit rates did not differ significantly between the groups. The use of a denicotinized cigarette combined with the nicotine patch appears to lessen cravings to smoke in the immediate postcessation period. A larger, better-powered study is needed to test if this treatment combination has merit for increasing quit rates.

Introduction

The rapid delivery of nicotine to the brain is one of the reasons many smokers find smoking pleasurable and find giving up smoking so difficult (Henningfield & Keenan, 1993; U.S. Department of Health and Human Services, 1988). However, if only the nicotine reinforced smoking behavior, the use of nicotine medications would make it possible for smokers to quit easily. Although nicotine medications have been shown to reduce withdrawal symptoms and increase the odds of quitting, most individuals who use them relapse back to smoking (Fiore et al., 2000).

Previous studies have demonstrated that smoking cigarettes that have very low levels of nicotine are still reinforcing for smokers (Shahan, Bickel, Badger, & Giordano, 2001; Shahan, Bickel, Madden, & Badger, 1999). The reinforcing effects of cigarette smoking in the absence of nicotine may be the result of past learning, whereby the repeated linked exposure of nicotine and smoke inhalation becomes associated with the act of smoking itself (Rose, Behm, Westman, & Kukovich, 2006). Thus the mere presence of smoke in the airway becomes a secondary reinforcer to the smoker because the sensory feel of the smoke becomes associated with the delivery of nicotine. This theory may help explain why very low nicotine delivery cigarettes reduce nicotine withdrawal symptoms (Buchhalter, Acosta, Evans, Breland, & Eissenberg, 2005; Buchhalter, Schrinel, & Eissenberg, 2001; Butschky, Bailey, Henningfield, & Pickworth, 1995; Gross, Lee, & Stitzer, 1997; Pickworth, Fant, Nelson, Rohrer, & Henningfield, 1999).

Smoking also becomes associated with many cues and triggers in the smokers' environment that serve as reminders to smoke (David et al., 2005; Due, Huettel, Hall, & Rubin, 2002); this helps explain why

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relapse to smoking is often associated with common smoking triggers such as drinking, stress, and socializing (Cummings, Jaen, & Giovino, 1985). Drug-seeking behavior may be extinguished better by nonrewarding drug administration than by discontinuation of drug use (Conkin & Tiffany, 2002). For example, in a rat model, removing the lever associated with cocaine injection does not extinguish the lever-pressing behavior; when the lever is returned to the chamber, the animal resumes lever pressing. However, if the lever is pressed and saline is injected instead of cocaine, lever pressing is extinguished (Koeltzow & Vezina, 2005). Another example is the administration of varenicline, a partial nicotine agonist, which seems to turn smoking into a nonrewarding experience and improve quit rates compared with placebo (Gonzales et al., 2006; Jorenby et al., 2006).

Based on these observations, we tested the hypothesis that combining steady-delivery nicotine replacement in the form of a nicotine patch with the sensory-motor reinforcing aspect of smoking without nicotine (i.e., Quest 3 cigarettes), compared with switching to light cigarettes (Quest 1), during the immediate precessation period would result in less cravings for a cigarette after quitting. We also tested a secondary hypothesis that less frequent cravings following cessation would increase quit rates, thereby favoring the combined patch plus Quest 3 over switching to light cigarettes during the precessation period.

Method

A randomized trial with 98 adult heavy smokers (using 20 or more cigarettes/day) was conducted in 2004 and 2005. Figure 1 provides a schematic of the study design. In the precessation part of the treatment, half the smokers in the trial received a very low nicotine yield cigarette (Quest 3, containing <0.05 mg of nicotine) along with a 21-mg nicotine patch for 2 weeks before quitting smoking. The other half were allowed to smoke a light cigarette (Quest 1 containing 0.6 mg of nicotine) for 2 weeks before the quit date. We deliberately chose to have all subjects switch from their usual cigarette brand to one of the Quest cigarette brands so that there was equivalence in the process of switching subjects away from their usual cigarette brand. Also, Quest 1 and Quest 3 cigarettes are identical in tar yield (10 mg, based on FTC method) and filter characteristics (no filter vents). During the 2 weeks before the quit date, subjects were asked to restrict themselves to smoking no more than one pack of the Quest cigarettes per day and not to smoke any other cigarettes except the Quest cigarettes we provided them.

After 2 weeks, all subjects were asked to stop smoking and received standard postcessation stop smoking treatment involving 8 weeks of 24-hour nicotine patch therapy (4 weeks of 21-mg patches, 2 weeks of 14-mg patches, and 2 weeks of 7-mg patches). Subjects were given their nicotine patches in 2-week increments, and all subjects participated in three 1-hr group cessation classes, plus individual counseling on

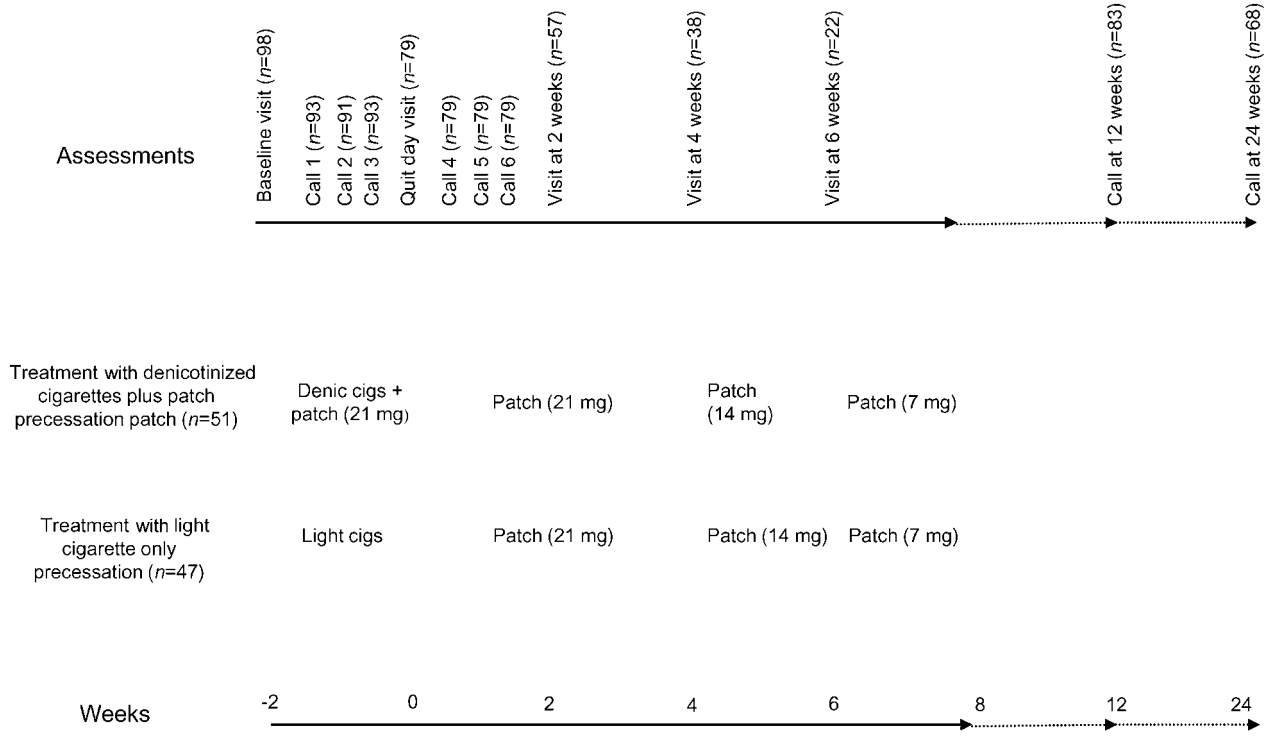


Figure 1. Study design of the randomized trial on denicotinized cigarettes (denic cigs) plus nicotine patch versus light cigarettes before the quit date (2004–2005).

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subsequent clinic visits (each individual counseling session lasting about 20 min). The study protocol was approved by the Roswell Park Cancer Institute human subjects' research committee.

Recruitment of study subjects

Several methods were used to recruit subjects for this study. Flyers were posted around Roswell Park Cancer Institute to recruit employees and visitors who smoked. We also sent out a press release to local media and sent invitation letters to people who had previously attended the Roswell Park stop smoking clinic and were known to be smoking still. All promotional materials directed interested participants to contact a phone number to learn about the study. We received 150 calls from smokers interested in the study. Figure 2 shows the flow of subjects through different stages of the study.

Those who called the phone number were screened for eligibility for inclusion in the study. Inclusion criteria included currently smoking 20 or more cigarettes/day, age 18–65 years, commitment to complete the scheduled assessments, and for female smokers, willingness to avoid pregnancy during the study period. Exclusion criteria included active treatment for mental health conditions, current use of other stop smoking treatments, and contraindications for using the nicotine patch (i.e., recent heart attack, pregnancy). During the baseline visit, the subject's general health condition was assessed and eligibility for participation in the study confirmed.

Randomization to treatment conditions

When subjects called to enroll in the study, they were allowed to choose from one of six clinic group dates. Three of the groups were assigned randomly to the denicotinized cigarette plus patch precessation treatment, and three were assigned to the light cigarette precessation treatment. All groups received standard postcessation patch treatment and behavioral counseling. A total of 98 subjects were enrolled into the study; 51 were assigned to the denicotinized cigarettes plus patch precessation treatment group and 47 were assigned to the light cigarette only precessation treatment group. At the time of the first visit, subjects were told about the study and their assigned treatment group because there was no feasible way for blinding subjects or investigators to treatment group assignment.

Outcome measures

Outcome measures included self-reports of cigarette cravings, nicotine withdrawal symptoms, and smoking abstinence. These items were measured repeatedly

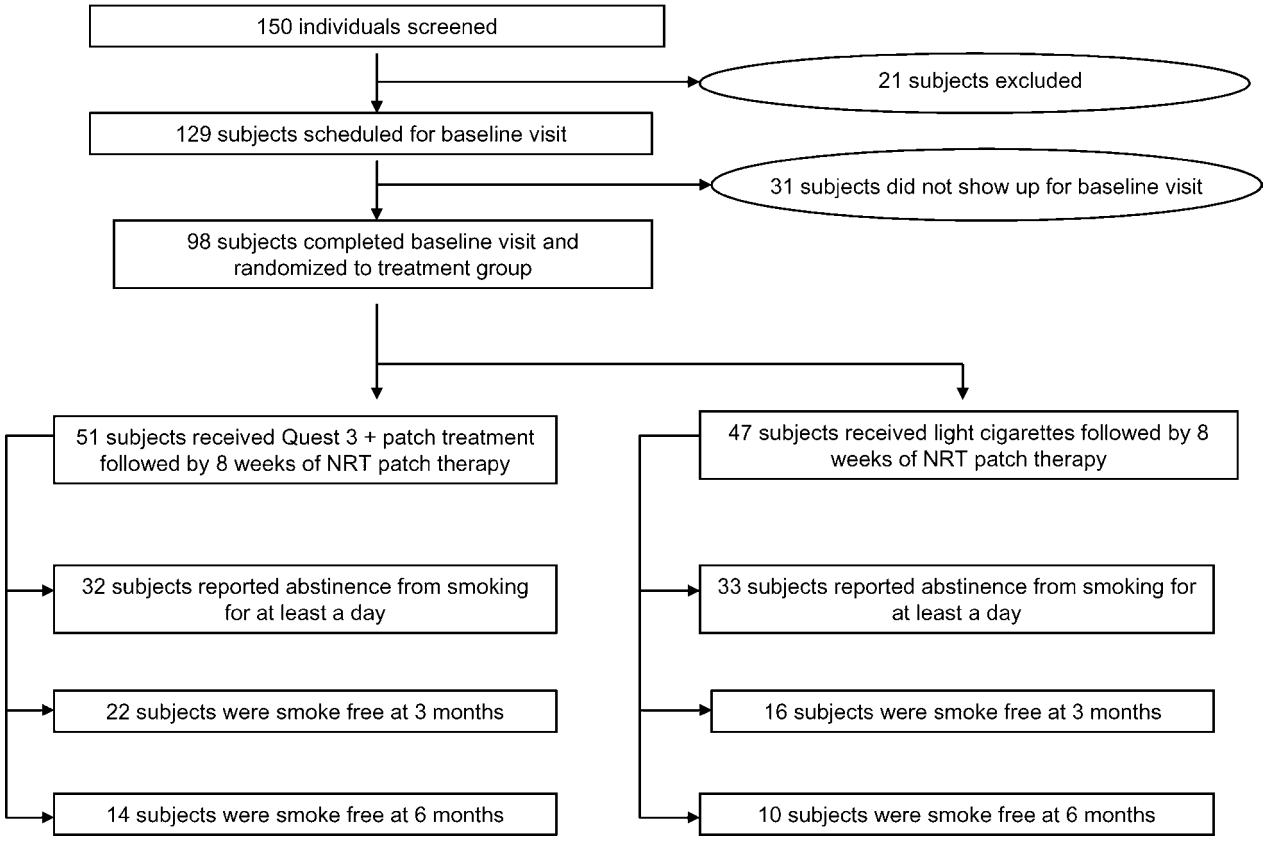


Figure 2. Flow of subjects through different stages of the study.

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before and after the quit date. Efforts were made to reach participants within 48 hr of their originally scheduled appointment or phone call. These measures were obtained by asking subjects to report their experience during the 24 hr prior to the assessment and are described below:

Cravings index. The questions used for assessment of craving were adopted from the Questionnaire of Smoking Urges (Tiffany & Drobes, 1991) and included two items. These two items asked about the frequency and severity of cigarette cravings based on a 10-point scale on which 1 was the lowest score and 10 was the highest score. An index of cigarette craving was computed by multiplying the frequency and severity of craving scores at each assessment.

Self-reported craving for smoking in general was obtained at each of the four assessments (phone calls 1, 2, 3, and quit date visit) during the 2 weeks before the quit date, and at the four assessments (phone calls 4, 5, 6, and follow-up visit) during the 2 weeks after the quit date.

Withdrawal symptoms index. Self-reported withdrawal symptoms were obtained using the Minnesota Withdrawal Scale (Hughes & Hatsukami, 1986) during the four assessments during the 2 weeks before the quit date and at the four assessments during the 2 weeks after the quit date. Each of the assessments included questions about the following symptoms: anger, nervousness, distractibility, impatience, hunger, sleep problems, depression, and desire to smoke. Each of these items was given a score of 1 (lowest severity) to 5 (highest severity). The sum of the score to these eight items was used as a measure for severity of withdrawal from smoking and was considered as the secondary outcome measure (Hughes & Hatsukami, 1986). The Cronbach's alpha for the eight items in the withdrawal scale was .82.

Self-reported quit rate. Point prevalence of smoking abstinence after the quit date was assessed based on the self-report during the phone surveys scheduled 3 and 6 months after the quit date. Subjects who did not smoke during the 7 days prior to assessment were classified as quitters. Those who did not complete the survey were considered as smokers.

Evaluable subjects

The analysis was done in two subsets of participants, depending on how far along each participant made it in the study. The first set of analyses used data collected on all 98 study participants who consented to join the study and who were randomized into one of the two treatment groups. The second set of analyses was restricted to data collected on the 65 evaluable participants who (a) completed the baseline visit, (b) completed the quit date visit, (c) made a quit attempt of at least 24 hr (self-report) during the initial 2-week period following the quit date, and (d) completed at least four of the first six phone surveys scheduled during the 2-week periods before and after the quit date. These 65 subjects were eligible to continue the nicotine replacement therapy; the others were dropped from the study after 2 weeks following the quit date.

Comparison of characteristics of the 65 evaluable subjects with the other 33 subjects revealed that the former smoked fewer cigarettes per day at baseline (24.6 vs. 30.0, $p=.019$) and were less likely to live with a smoker (34% vs. 70%, $p=.001$) but were otherwise comparable on baseline characteristics. The proportion of evaluable subjects was similar for the denicotinized cigarette plus nicotine patch group compared with the subjects who switched to the Quest 1 light cigarettes before quitting (i.e., 63% vs. 70%, $p=.435$). After restriction of analysis to the 65 evaluable subjects, the results and conclusions remained the same. Therefore, we present the data for the larger sample of 98 subjects.

Data analyses

The study was designed to obtain four measures for craving and withdrawal during the 2 weeks before the quit date and four measures of those variables during the 2 weeks after the quit date for each participant. To take into account the dependent nature of the measurements, the two arms of the study were compared using linear mixed model analysis (Wright & Wolfinger, 1996). The outcome variable was placed as the dependent variable, the group assignment variable was placed as the independent variable, two other variables indicating the subject and the order of assessment of the response variable were considered in the constructed mixed linear model. The beta coefficient obtained for the group assignment variable and the p value for this coefficient represent the comparison of the two treatment arms. A negative value for the beta coefficient indicates a lower score of the response variable in the group treated with denicotinized cigarettes plus patch precessation.

Point prevalence of smoking abstinence was measured at 3 and 6 months after the quit date. These proportions were compared using a chi-square test between the two study arms.

Characteristics of subjects were measured at the baseline visit. As shown in Table 1, random assignment of the treatment led to two groups that were comparable on most characteristics, with the exception that those in the denicotinized cigarette plus nicotine patch precessation treatment group were slightly younger than those in the light cigarette only

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Table 1. Comparison of selected demographic and tobacco use characteristics by treatment group.

Group	Denicotinized cigarettes plus patch precessation	Light cigarettes only precessation
Mean cigarettes/day (*SD*)	27.5 (1.5)	25.3 (1.1)
Mean FTND score (*SD*)	7.7 (0.3)	7.8 (0.3)
Mean combined craving score (*SD*)	60.06 (4.01)	65.09 (4.04)
Mean withdrawal score (*SD*)	18.42 (0.99)	20.70 (0.99)
Mean age, years (*SD*)	42.4 (1.5)	48.5 (1.2)
Gender		
Male	45%	43%
Female	55%	57%
Education, years		
<12	8%	6%
12	33%	43%
13–15	26%	38%
16+	33%	13%
Desire to quit		
Not at all	—	2%
A little	—	—
Somewhat	8%	15%
A lot	92%	83%
Smoker in house		
Yes	45%	47%
No	55%	53%
Tar content		
Regular	53%	53%
Light	37%	36%
Ultra-light	10%	11%
Attempts to quit in past year		
0	35%	28%
1	39%	36%
2+	25%	35%
Race		
White	86%	79%
Black	12%	17%
Other	2%	4%
Ethnicity		
Hispanic	4%	2%
Non-Hispanic	96%	98%
Income		
<$10,000	10%	13%
$10,000–$24,999	28%	23%
$25,000–$39,999	20%	36%
$40,000–$59,999	22%	11%
$60,000+	22%	17%
Marital status		
Never married	18%	19%
With partner	8%	13%
Married	53%	40%
Separated	18%	21%
Widowed	4%	6%

Note. Dashes indicate no responses in this category. FTND, Fagerström Test for Nicotine Dependence; *SD*, standard deviation. The only statistically significant group differences was for age (p=.002).

precessation treatment group. No other factors were significantly different between the two groups at the p<.05 level.

Results

Craving index

Figure 3 shows the difference between the two study groups in regard to the combined craving score. The group using the denicotinized cigarettes plus nicotine patch precessation treatment had lower combined craving score during the 2 weeks before (beta=−17, p<.001) and after (beta=−8, p=.013) the quit date.

Withdrawal symptoms index

Figure 4 shows the comparison of the two study arms in regard to the withdrawal score. The two study arms were similar in regard to the severity of withdrawal symptoms during the 2 weeks before (beta=0.74, p=.286) and after (beta=–1.05, p=.188) the quit date.

Self-reported quit rate

A shown in Figure 5, both study arms had comparable rates of self-reported point prevalence of smoking abstinence at the 3- and 6-month follow-ups. Differences in self-reported quit rates did not reach statistical significance.

As expected, quitters at 3 and 6 months, regardless of treatment group membership, had statistically significant lower average craving scores during the 2-week postcessation period (one-sided Mann–Whitney *U*-test p value=.05 for both cessation measures).

Discussion

Findings from this study indicate that combining use of denicotinized cigarettes plus a 21-mg nicotine patch for a brief period before quitting smoking can reduce cigarette cravings in the immediate postcessation period, compared with using the nicotine patch only after quitting. This result is compatible with the findings of Rose et al. (2006) that cravings were reduced in the denicotinized cigarette plus patch arm relative to usual brand plus patch.

The present study had a small sample size to start with and lost nearly one-third of the subjects because they were unable to remain smoke free for at least 24 hr after the designated quit date; thus it was not sufficiently powered to test for long-term differences in quit rates between treatment groups. The group treated with denicotinized cigarettes plus nicotine patch precessation had a slightly higher self-reported quit rate at 3 months (43% for denicotinized cigarettes plus patch vs. 34% for light cigarettes) and 6 months (28% vs. 21%, respectively), although the difference was not statistically significant at the p>.05 level. This self-reported quit rate differential is comparable with those of two other similar studies (Rose et al., 2006; Schuurmans, Diacon, van Biljon, & Bolliger, 2004). Rose et al. (2006) studied 98 subjects and found that the group treated with nicotine patch before the quit date had a significantly

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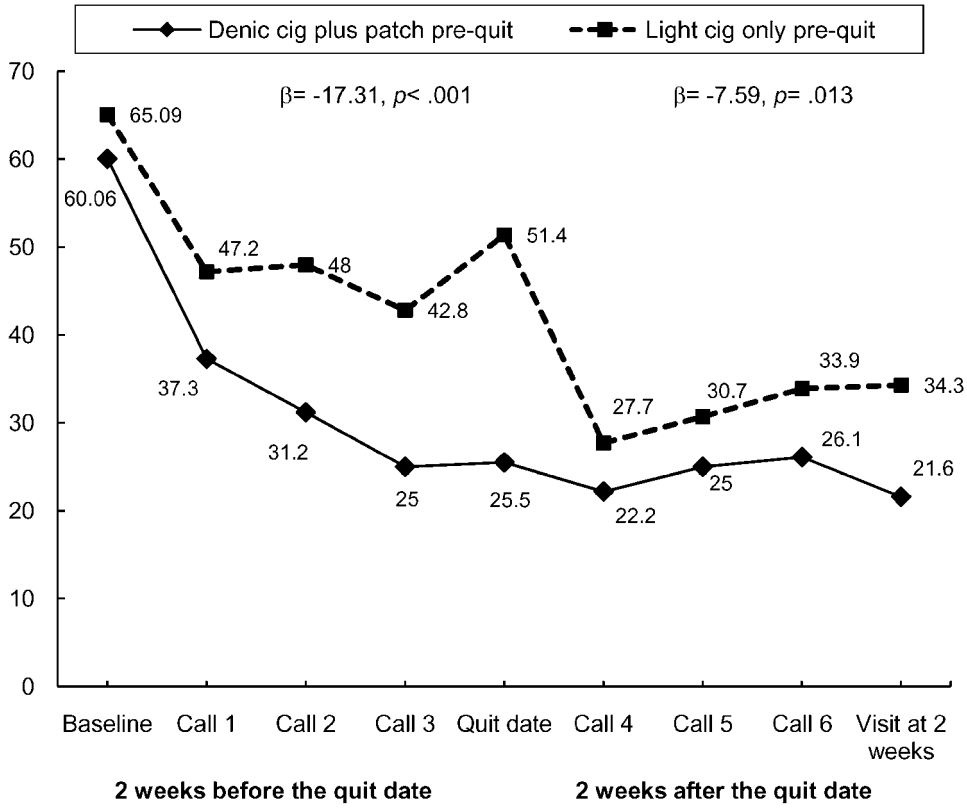


Figure 3. Combined craving score by treatment (N=98). Score=severity of craving × frequency of craving. Beta and p values obtained from mixed-model analysis.

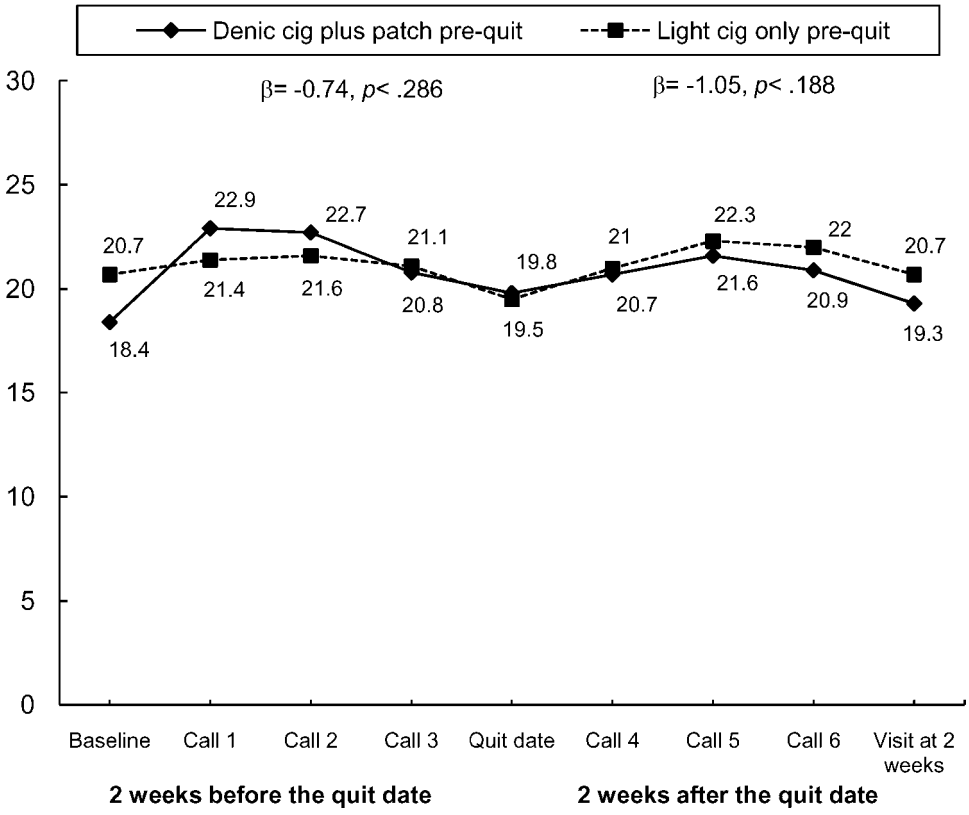


Figure 4. Withdrawal score by treatment (n=98). Score=sum of scores for anger+nervousness+distractibility+impatience+appetite+insomnia+depression+desire to smoke (Koeltzow & Vezina, 2005). Beta and p values obtained from mixed-model analysis (adjusted for baseline values).

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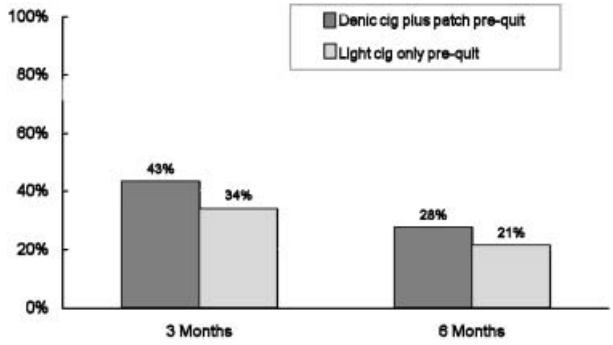


Figure 5. Self-reported quit rates by treatment arm at 3 and 6 months (N=98). Quitting defined as abstinence from smoking during the 7 days preceding the assessment. Nonsignificant p values obtained from chi-square test.

higher quit rate (50% vs. 20%) at 4 weeks. Schuurmans et al. (2004) studied a sample of 200 subjects and found that use of nicotine patch for 2 weeks before the quit date was associated with a significant increase (22% vs. 12%) in sustained abstinence at 24 weeks. Based on the effect size observed in the present study, we estimate that an adequately powered trial for smoking cessation would require a sample of about 430 subjects per treatment group.

Consistent with other published studies, we did not find evidence that combining the nicotine patch and denicotinized cigarette altered reports of nicotine withdrawal symptoms (Gonzales et al., 2006; Rose et al., 2006). Withdrawal symptoms may be related to decreased dopaminergic activity in the nucleus accumbens as a result of discontinuation of nicotine administration (Watkins, Koob, & Markou, 2000). Chronic exposure to nicotine may lead to neuron adaptations that can explain development of withdrawal symptoms when nicotine administration is discontinued. Nicotine replacement therapy has been shown to decrease the withdrawal symptoms and aid smoking cessation (Silagy, Lancaster, Stead, Mant, & Fowler, 2002). In the present study, subjects were receiving similar doses of nicotine either by the patch or by smoking the assigned light cigarettes, which could explain the similar Minnesota withdrawal scores (Hughes & Hatsukami, 1986) in both arms.

The Minnesota scale comprises different items that can be examined separately. One of the items measures the craving for smoking. The group treated with denicotinized cigarettes plus nicotine patch precessation had a significantly lower score for this single item of the scale during the 2 weeks before and after the quit date (data not shown). This finding confirmed our results from analysis of the craving scale presented in Figure 3.

Four of the items included in the withdrawal scale that were measuring anger, nervousness, distractibility, and impatience were highly correlated with a correlation coefficient of greater than .5. The sum of the score for these items could indicate negative affects and was found to be higher in the group treated with denicotinized cigarettes plus nicotine patch during the 2 weeks before the quit date. This finding is compatible with other instances of nonrewarding drug administration used for treatment of addiction that are likely to lead to frustration (Conklin & Tiffany, 2002).

The present study had some limitations. The 3- and 6-month quit rates are based on self-reported information and may overestimate actual quit rates. Both treatment arms received active treatment and clinical monitoring during the study period, which limits the extent to which group differences may exist in misreporting of smoking status. Those who self-reported no cigarettes smoked in the 7 days prior to the 6-month follow-up interview were asked to mail a saliva sample to the investigators for cotinine testing. Of the 21 samples requested, 13 were received (62%), and of these, 10 had salivary cotinine levels $<$15 ng/ml (77%). The fraction of received samples that had high cotinine values was not statistically different between the two treatment arms (p=.70).

Second, the study was not blinded for either the participants or the investigators, raising the possibility of information bias. As noted earlier, both groups received some form of active treatment and the clinical data collection for craving and other measures was based on self-reported information, which should reduce the potential for bias. Information from phone interviews was collected by an independent group of survey interviewers who were not familiar with the specifics of the study design or hypotheses being tested, which limits the likelihood of this type of bias as well.

Third, the present study does not provide information on other precessation treatments such as nicotine patch only, and further research is needed to determine the specific added value of denicotinized cigarettes as a precessation treatment over other possible treatments.

In summary, the findings from this research add to an increasing volume of research that suggests that use of the combination of denicotinized cigarettes plus nicotine patch before quitting can reduce craving for smoking in the immediate postcessation period, which may lead to higher long-term quit rates. However, whether this treatment combination can prevent relapse, thereby increasing long-term quit rates, still remains to be tested in a well-designed clinical trial.

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Summary of Quest smoking-cessation study, conducted at Duke University

Principal Investigator: Jed E. Rose, Ph.D.

Prepared September 16, 2003

Overview

A study was conducted to provide a preliminary evaluation of Quest nicotine-free cigarettes in aiding smoking cessation among regular (nonmenthol) smokers. A total of 45 subjects took part in the study, which has been completed in terms of primary outcome assessments (four-week continuous smoking abstinence). Results thus far show that Quest has considerable promise as a cessation aid---the biochemically confirmed four-week abstinence rate in the subgroup performing the best (of the three groups tested) was approximately 33% (5/15), and the overall success rate across all groups was approximately 15% (7/45). Additionally, the success rate was much higher (54%, or 7/13) in those subjects who complied with using Quest to the exclusion of nicotine-containing cigarettes prior to their target quit-smoking dates. Comparing these results to historical data from our program suggests that a weaning program entailing Quest cigarettes, even without concurrent use of nicotine replacement, may provide success rates comparable to the nicotine patch or other nicotine replacement treatments. Moreover, identification of a promising subgroup of smokers who comply with using Quest may provide the basis for even higher success rates.

Based on these results, a direct comparison of Quest 1-2-3 against standard nicotine patch treatment and against conventional brand fading is recommended, which would help pave the way to being able to make cessation claims for Quest. However, although Quest alone may prove efficacious, previous results from our laboratory strongly suggest that concurrent use of Quest with the nicotine patch (patch administered before and after the quit-smoking date) may further boost efficacy. Therefore, a study design is proposed that will rigorously evaluate Quest, with and without nicotine patch therapy, with respect to standard treatment.

Current Study Design and Results

The current study (see design Fig. 1), which enrolled 45 subject smokers (demographic and smoking characteristics shown in Table 1), was designed to address several key questions, and to provide preliminary data on different methods of employing Quest:

1. What are the quit-smoking rates using Quest?

Figure 2 depicts the success rates in each group. Group 2, which gradually introduced Quest 3 while reducing the number of their usual-brand cigarettes smoked, performed the best--the abstinence rate for this group was 5/15, or approximately 33%. The success rate among smokers using Quest 1-2-3 (Group 3) was 2/15 or approximately 15%. Group 1, which received nicotine skin patches prior to the quit-smoking date, had the lowest success rate (0/15 or 0%). We attribute the low success rate in this group to the premature withdrawal from the patch (see point #3 below). Hence, in making projections of likely success rates using Quest cigarettes as they would be used in practice, based on this knowledge, we would base the estimates on the performance of Group 2 or Group 3. These estimates are subject to statistical uncertainty, and the true rate in the population, based on the 33% sample estimate for Group 2, could lie in a broad range of approximately 10-60% (this is the 95% confidence interval).

2. Can predictors of success using Quest be identified?

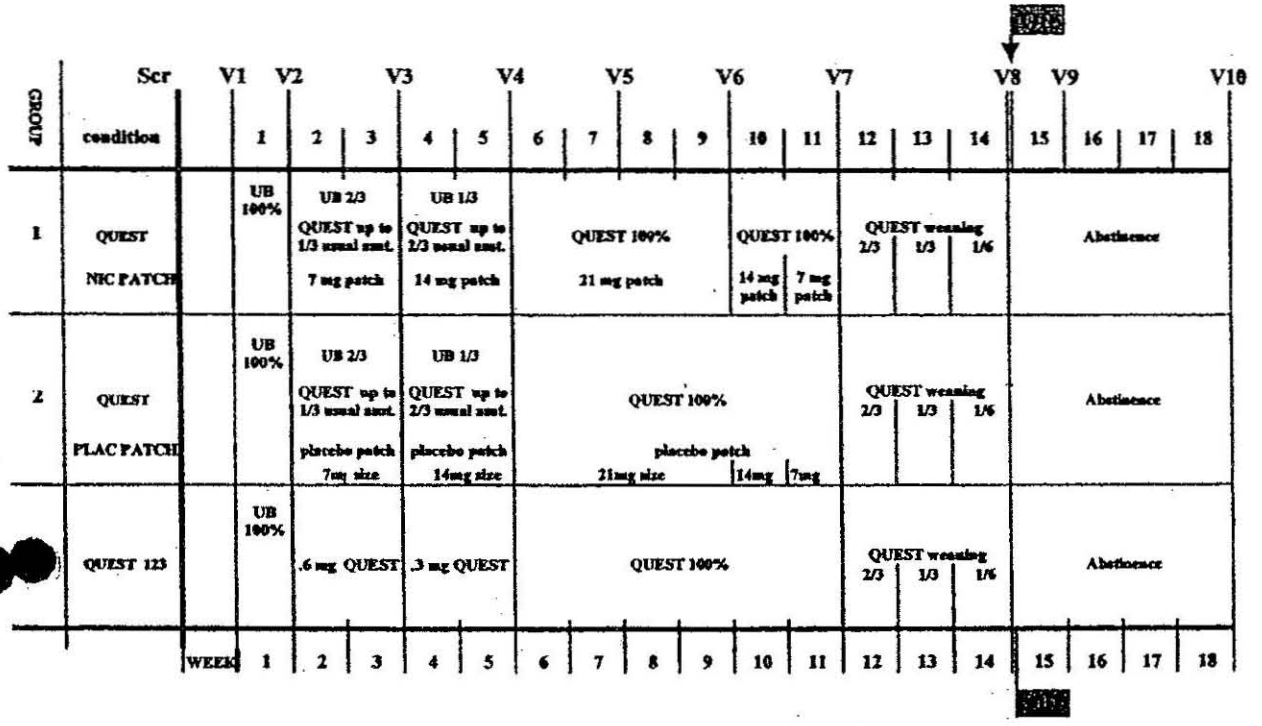
We calculated the correlations between cessation outcome and baseline demographic/smoking characteristics, including sex, age, cigarettes/day, nicotine delivery of usual brand, and nicotine dependence (FTND questionnaire). None of these variables emerged as a significant predictor of four-week abstinence.

However, a strong predictor of successful smoking cessation was compliance with use of Quest in the weeks immediately prior to the target quit-smoking date. Specifically, those smokers who achieved four-week continuous abstinence reported virtually *no* use of their usual brand, whereas failures reported on average 5 nicotine cigarettes per day in the weeks leading up to their quit dates (see Fig. 3). Thus, even a low-level use of nicotine-containing cigarettes markedly reduces the probability of success. Conversely, of those

Figure 1

CURRENT STUDY

Nicotine-free Cigarettes as a Smoking Cessation Aid -- TIMELINE



Table

DEMOGRAPHIC INFORMATION

	MEAN	*S.D.*
AGE	44	*14.2*
GENDER	21 M/24 F	
FTND	5.4	*2.11*
CIGARETTES/DAY	22.6	*6.21*
FTC NICOTINE	0.7	*0.2*
YEARS SMOKED	23.8	*11.91*
# OF QUIT ATTEMPTS	6.9	*15.18*

Figure 2

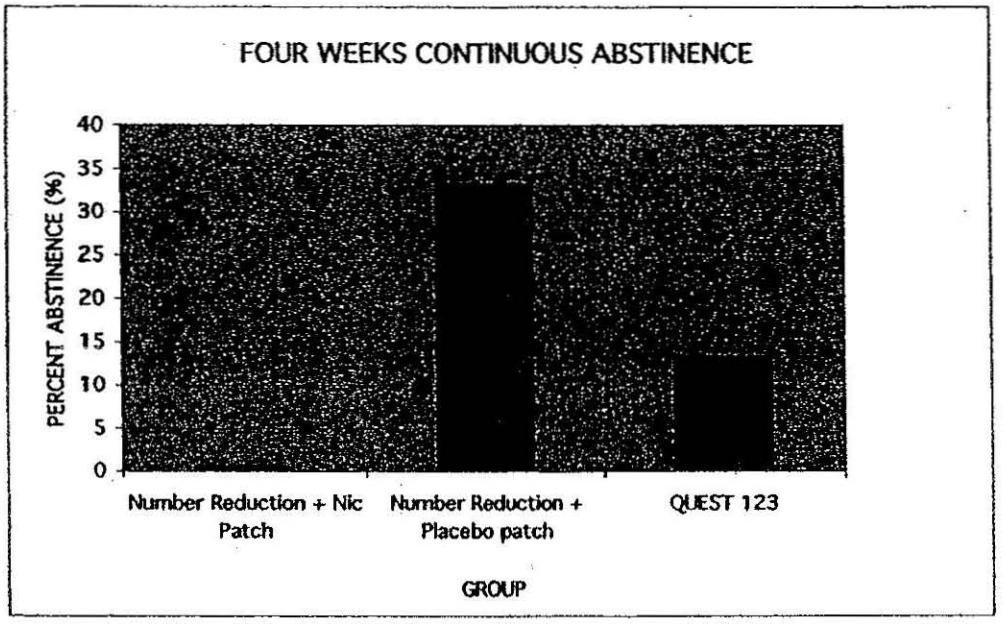
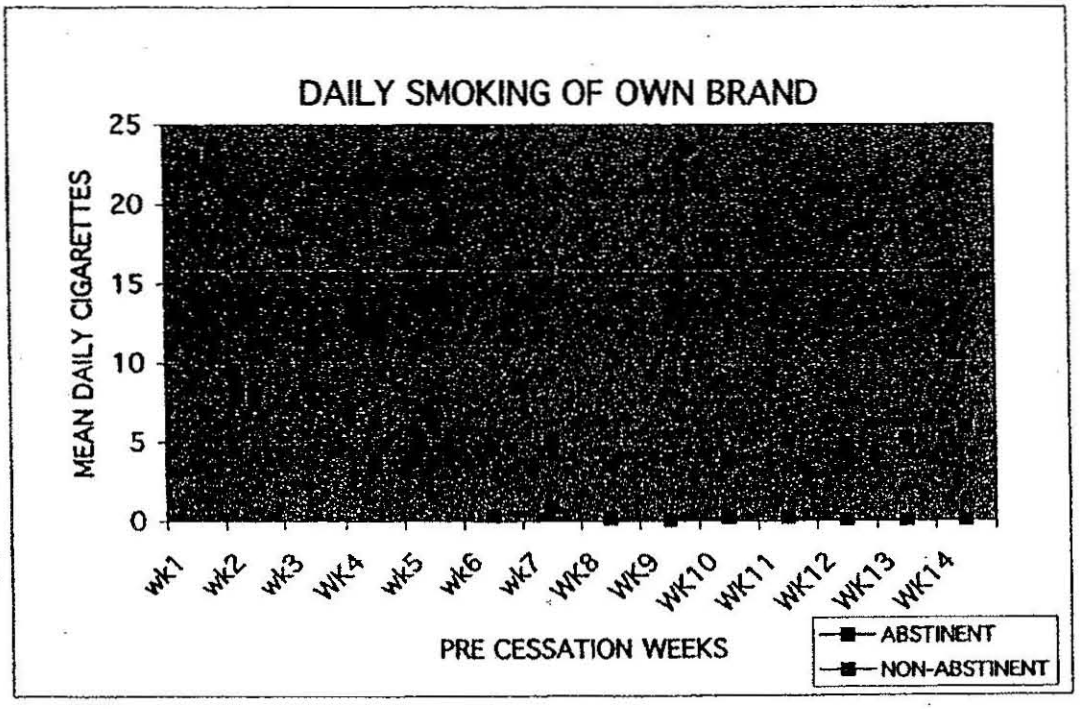

FOUR WEEKS CONTINUOUS ABSTINENCE
PERCENT ABSTINENCE (%)
40
35
30
25
20
15
10
5
0
Number Reduction + Nic Patch
Number Reduction + Placebo patch
QUEST 123
GROUP

Figure 3


DAILY SMOKING OF OWN BRAND
MEAN DAILY CIGARETTES
25
20
15
10
5
0
wk1
wk2
wk3
WK4
wk5
wk6
wk7
WK8
WK9
WK10
WK11
WK12
WK13
WK14
PRE CESSATION WEEKS
ABSTINENT
NON-ABSTINENT

subjects who reported no use of nicotine-containing cigarettes, 55% achieved four-week continuous abstinence.

This finding has potentially great clinical significance in that reported use of nicotine-containing cigarettes could be incorporated into clinical monitoring in order to avert failures before they occur. That is, smokers who report less than complete transition to Quest 3 cigarettes might be instructed not to attempt to quit until they do so, or to use an alternative pharmacologic or behavioral treatment. Use of Quest may serve as an effective "holding pattern" to keep subjects motivated, and in treatment, until the right type of treatment can be provided. By averting a failed quit-smoking attempt, some smokers may be rescued and entered into efficacious treatment programs before becoming disheartened by a failed quit-attempt.

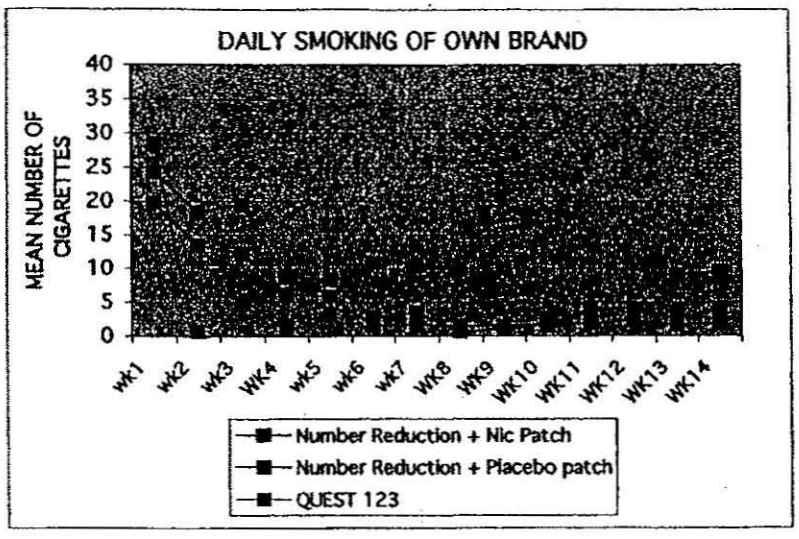
3. Does use of nicotine patches before the quit-smoking date (but not after), in conjunction with Quest, help smokers switch from their usual brand to Quest 3?

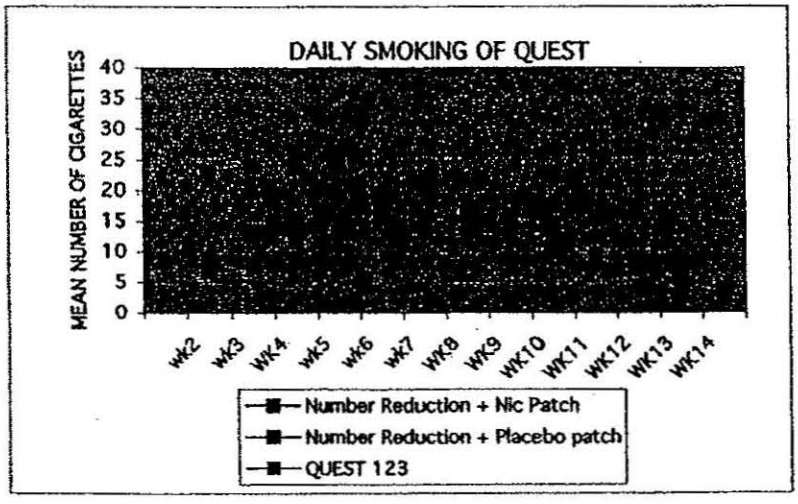
The answer to this question was a definitive "no"; in fact, success rates were significantly lower in the nicotine patch group (0%) than the matched placebo patch condition (33%). It appeared that premature *withdrawal* from the patch before the target quit-smoking date precipitated noncompliance with smoking Quest 3 cigarettes (i.e. more smoking of nicotine-containing cigarettes), which then impeded successful quitting (see Fig. 4). Thus, if nicotine patch therapy is to be combined with Quest, the patch should be worn through the quit date and beyond. Using a pre- and post-cessation use of patch in a previous study of denicotinized cigarettes, we indeed observed very high success rates (50%).

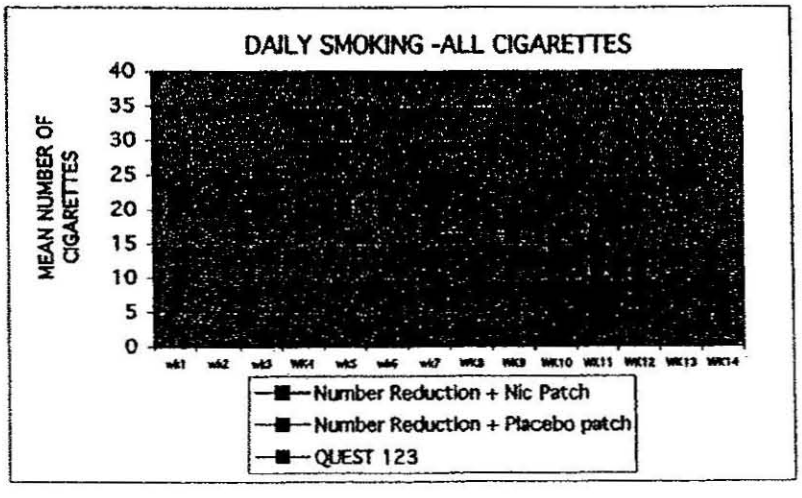
4. Is the Quest 1-2-3 regimen more acceptable than switching gradually from the usual-brand cigarettes to Quest 3 in a number-fading regimen (i.e. reducing the number of usual-brand cigarettes smoked daily to 2/3 of baseline for 2 weeks (while smoking 1/3 Quest 3), and then to 1/3 of baseline for 2 weeks (while smoking 2/3 Quest 3), followed by smoking Quest 3 only)?

There was no advantage in terms of abstinence rates between the Quest 1-2-3 approach (Group 3) and the group fading out the number of usual-brand cigarettes over four weeks while concurrently increasing the number of Quest 3 cigarettes (Group 2). In fact, there was a trend for the Quest 1-2-3 condition to perform *less* well (2/15 successes vs. 5/15 successes in the number-fading

Figure 4







1123

condition). While this difference could be attributed to statistical variability in the small samples studied, some results indicated that there was a genuine superiority of the number-reduction procedure. For example, in the Quest 1-2-3 condition, there was poorer compliance with using Quest 3 than in the number-reduction condition, and a sharp rise in craving for cigarettes when switched to Quest 3 (see Fig. 5). This result was supported by anecdotal reports of subjects indicating that they tended to "hit a wall" when they reached Quest 3 after switching from Quest 2. Thus, it might be advantageous to introduce Quest 3 while subjects are still smoking their usual brands of cigarettes, as was done in the number-reduction regimen (Group 2).

5. Do smokers compensate during the transition to Quest 3 in an attempt to maintain nicotine intake, by smoking more cigarettes or inhaling more smoke from each cigarette?

No--our results showed that participants did not compensate--self-reported smoking rates and expired air CO did not increase during use of Quest 1, 2 or 3, and in general rates of smoking declined over the 6 weeks during which smokers had free access to Quest 3 (see Figs. 4 and 6).

6. How do Quest cigarettes compare in satisfaction and other subjective ratings to subjects' usual brands of cigarettes?

Quest cigarettes were generally rated significantly less satisfying than subjects' usual brands. Subjects also reported much lower craving for Quest than their usual brands. While consumer acceptance may be important to a certain extent, it may also be beneficial that smokers do not crave Quest, in order to facilitate cessation (and to deter youth from acquiring the smoking habit using Quest).

7. Do smokers using Quest 3 for 6 weeks show a high rate of spontaneous quitting?

Spontaneous quitting did not occur at a high rate; smokers generally kept using Quest 3 as long as they were supplied (only one individual quit smoking prior to his scheduled quit date).

Figure 5

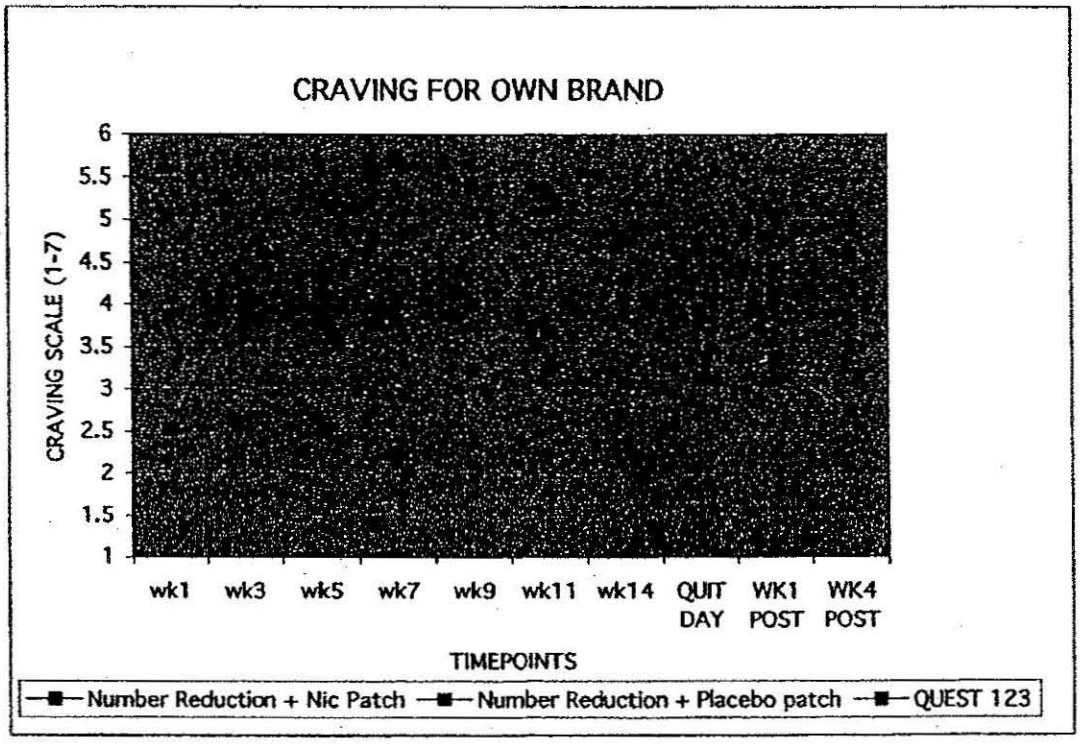
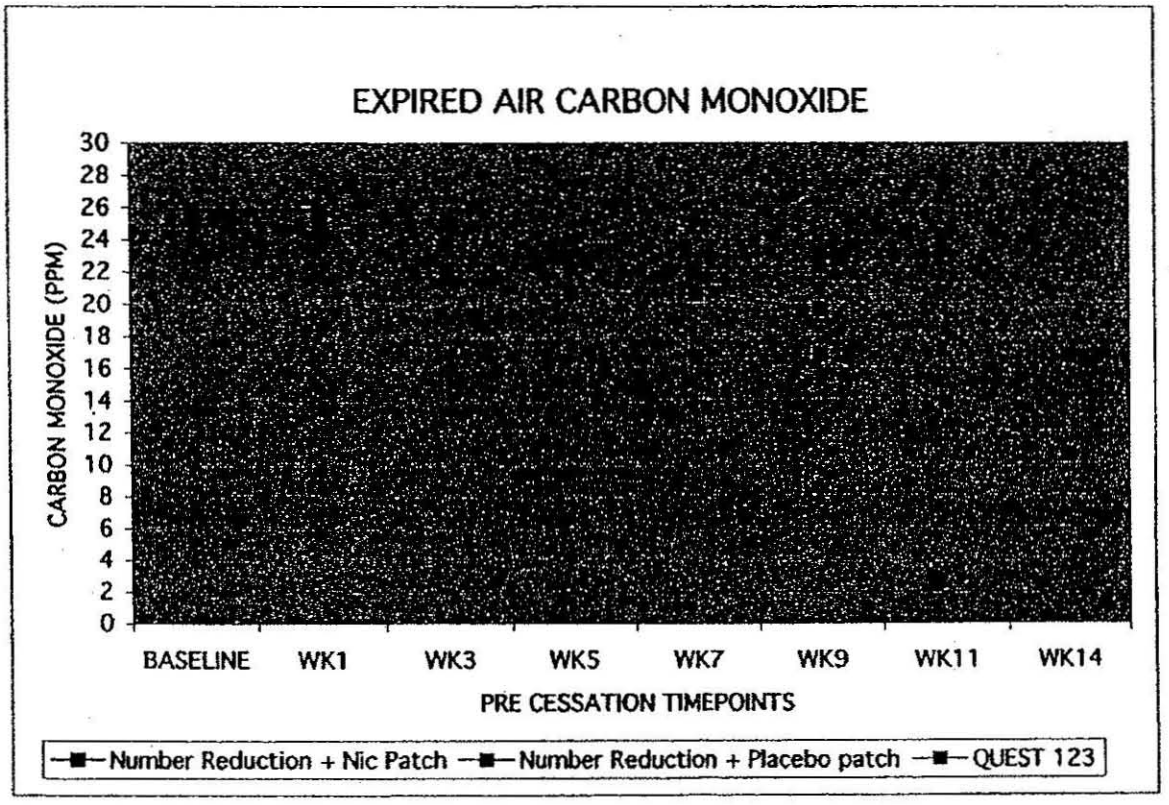

CRAVING FOR OWN BRAND
CRAVING SCALE (1-7)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
wk1
wk3
wk5
wk7
wk9
wk11
wk14
QUIT DAY
WK1 POST
WK4 POST
TIMEPOINTS
Number Reduction + Nic Patch
Number Reduction + Placebo patch
QUEST 123

Figure 6


EXPIRED AIR CARBON MONOXIDE
CARBON MONOXIDE (PPM)
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
0
BASELINE
WK1
WK3
WK5
WK7
WK9
WK11
WK14
PRE CESSATION TIMEPOINTS
Number Reduction + Nic Patch
Number Reduction + Placebo patch
QUEST 123

8. What withdrawal symptoms are experienced upon quitting Quest?

It was very interesting that by the time subjects reached their quit-dates, there appeared to be little in the way of smoking withdrawal symptoms (e.g. craving, see Fig. 5). Based on historical data, ratings of craving and other symptoms typically rise sharply on the quit-smoking date. No such rise was observed in the present study, even among subjects who were indeed judged to be abstinent. Even though many smokers relapsed to nicotine-containing cigarettes after withdrawing from Quest, there may nonetheless be substantial benefit in minimizing the withdrawal discomfort associated with the quitting process.

Recommendations

Based on the promising results obtained in this preliminary study, a controlled clinical trial is proposed (see design Fig. 7). The following take-home lessons from the completed trial were built into the proposed design:

1. The time-line has been shortened by approximately half (from 18 weeks to 10 weeks). There was no evidence that prolonged use of Quest 3 led to a high rate of spontaneous quitting; on the contrary, it is possible that becoming fully habituated to Quest 3 may make it more difficult to relinquish cigarettes altogether. Therefore we recommend evaluation of a relatively brief course of therapy, comprising six weeks of fading, with the last two weeks entailing exclusive use of Quest 3. Some subjects will receive 2 weeks each of Quest 1, 2, and 3. Other subjects will implement the number-fading regimen using Quest 3 from the outset, which was the treatment that appeared to be so successful in the completed study.

2. Nicotine patch therapy, in the one subgroup that receives it prior to the quit-smoking date, will be continued after the quit-smoking date. Withdrawal of nicotine patch therapy before the quit-smoking date, as was done in the completed study, appears to be counterproductive, by inducing use of nicotine-containing cigarettes. On the other hand, previous work conducted in our program showed that use of a denicotinized cigarette (Next) in conjunction with nicotine patch treatment, and continuing use of the nicotine patch beyond the quit date, yielded 50% abstinence rates. Therefore, we recommend including in the experimental design a group of subjects (Group 2) that uses 21 mg/day nicotine patches concurrently with Quest 3. Although the use of 21 mg/day nicotine might seem counterintuitive in view of the fact that subjects will have reduced their daily intake of nicotine (from Quest 2) to approximately 7 mg/day by that point, we feel there may be a significant advantage to using the 21 mg/day patch. Our rationale is that 21 mg/day nicotine dosing generally falls short of matching the high levels of nicotine in arterial blood attained after smoking, which limits the efficacy of the patch in substituting for the nicotine effect sought by smokers. In contrast, a 21 mg daily dose may achieve this end in smokers who have already reduced their peak arterial nicotine levels by having switched to Quest 2. The 21 mg/day nicotine patch would be expected to facilitate compliance upon subsequently switching to Quest 3. Tolerance to the effects of nicotine is long-lasting in smokers, so we do not expect any adverse symptoms to be experienced from use of the 21 mg/day dose.

3. To lay the groundwork for claims with regard to smoking cessation efficacy, it will be necessary to provide a rigorous comparison to control groups. The previous study did not include control groups presenting standard treatment, because the aim was to select the most promising means of employing Quest for further evaluation. However, in the next clinical trial, we recommend that two comparison groups be included:

1) a conventional brand-fading condition (Group 5), which uses a self-help manual and instructions to switch to lower tar and nicotine cigarettes leading up to a quit-smoking date. Inclusion of this group will be critical in demonstrating efficacy of Quest- 1-2-3 as a valid non-drug cessation aid in its own right;

2) standard nicotine patch (post-quit only) treatment (Group 4). By showing at least equivalence to standard nicotine patch treatment, Quest will possibly be seen as an attractive alternative to those smokers seeking to avoid use of any drug, including nicotine. By showing superiority over nicotine patch treatment, which we predict will be observed in the Quest + patch condition, and possibly in the Quest 3 cigarette number-reduction group, a significant advance in smoking cessation treatment will have been achieved. In either case, Quest may be accepted as a first-line smoking cessation treatment if it is more efficacious than conventional brand-fading and equivalent in efficacy to nicotine replacement therapy.

We also recommend that active treatment groups entailing Quest will include both Quest 1-2-3 and number-reduction of usual-brand cigarettes while introducing Quest 3 cigarettes (i.e. no use of Quest 1 and Quest 2). As was noted above, the results of the completed trial showed a strong trend for the number-fading regimen to work better than Quest 1-2-3; on the other hand, Quest 1-2-3 has the appeal of simplicity.

The potential value of monitoring compliance with Quest, and possibly offering alternative treatments to avert a failed quit-attempt, was also mentioned above. Evaluation of this promising clinical strategy is recommended in future studies, but not in the next proposed study, for two main reasons: 1) inclusion of a "rescue" treatment for subjects who fail to relinquish nicotine cigarettes entirely on switching to Quest 3, would significantly lengthen the timeline and delay validation of Quest as an efficacious treatment. Moreover, if Zyban were used as the rescue treatment (a logical choice for smokers whose nicotine levels have already been reduced through use of Quest), greater physician

Figure 7

QUEST CESSATION STUDY SEPTEMBER 2003

N= 40 PER GROUP
TOTAL N=200

GROUP	CONDITION	V1 WK1	WK2	V2 WK3	WK4	V3 WK5	WK6	V4 WK7	V5 WK8	WK9	V6 WK10
1	QUEST 123	QUEST 1		QUEST 2		QUEST 3		NO PATCH			
2	QUEST 123 + NIC PATCH	QUEST 1		QUEST 2		QUEST 3 + 21MG NIC PATCH		21MG NIC PATCH			
3	QUEST 3	2/3 OWN BRAND 1/3 QUEST 3		1/3 OWN BRAND 2/3 QUEST 3		QUEST 3		NO PATCH			
4	PATCH ONLY POST	OWN BRAND		OWN BRAND		OWN BRAND		21MG NIC PATCH			
5	CONVENTIONAL BRAND FADING (LOW TAR & NICOTINE CIGARETTES)							NO PATCH			
		WK1	WK2	WK3	WK4	WK5	WK6	WK7	WK8	WK9	WK10

NOTE: AT V6, GROUP 2 & 4 WILL BE GIVEN A SUPPLY OF 14MG PATCH FOR 2 WEEKS AND A SUPPLY OF 7MG FOR ANOTHER 2 WEEKS

THERE WILL BE A 6 MONTH FOLLOW UP (V7)

involvement and more health exclusions would be required, hampering subject recruitment; 2) It is not known what the psychological effect on smokers would be if they were informed in advance that a second treatment was "waiting in the wings" should they fail to quit using the primary treatment (i.e. Quest). Conceivably this effect could distort the outcome data on the primary treatment.

Conclusion

Our work to date supports Quest as a viable strategy that should be investigated further as a smoking cessation strategy. We have answered critical questions about the duration of use, compensatory smoking, timing of patch use and predictors of efficacy. We have provided a study design that will help further pave the way toward the ultimate validation of Quest as an effective smoking cessation aid.

The effects of nicotine, denicotinized tobacco, and nicotine-containing tobacco on cigarette craving, withdrawal, and self-administration in male and female smokers

Sean P. Barrett

The effects of the acute administration of nicotine [through nicotine inhalers (NI) and placebo inhalers (PI)], nicotine-containing tobacco (NT), and denicotinized tobacco (DT), on smokers' subjective responses and motivation to smoke were examined in 22 smokers (12 male, 10 female; 11 low dependent, 11 high dependent). During four randomized blinded sessions, participants self-administered NI, PI, NT, or DT, and assessed their effects using Visual Analogue Scales and the Brief Questionnaire of Smoking Urges. They could then self-administer their preferred brand of cigarettes using a progressive ratio task. NT and DT were each associated with increased satisfaction and relaxation as well as decreased craving relative to the inhalers and NT increased ratings of stimulation relative to each of the other products. Both NT and DT delayed the onset of preferred tobacco self-administration relative to NI and PI but only NT reduced the total amount self-administered. Sex differences were evident in the effects of DT on withdrawal-related cravings with women experiencing greater DT-induced craving relief than men. Findings suggest that DT is effective in acutely reducing many smoking abstinence symptoms, especially in women, but a combination of nicotine and non-nicotine tobacco ingredients may be necessary to suppress smoking behavior. *Behavioural Pharmacology* 00:000–000 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Keywords: craving, human, nicotine, nicotine replacement therapy, progressive ratio schedule, tobacco

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Introduction

Despite decreases in smoking rates over the past two decades, tobacco use remains prevalent and pervasive (World Health Organization, 2008). Although a majority of smokers report that they intend to quit within 6 months, most quit attempts fail (e.g. Hughes *et al.*, 2004; Brown *et al.*, 2005), even when assistance is sought and current best practice treatments are used (Cook *et al.*, 1995; Hughes *et al.*, 2003). Many tobacco cessation programs routinely use nicotine replacement therapies (NRTs) as a primary means of aiding cessation. The use of NRTs has been shown to increase overall quit rates but in the majority of cases NRTs do not prevent relapse to smoking (e.g. Hughes *et al.*, 2003) and many smokers seem to be resistant to NRT-based treatments even when repeated NRT-assisted quit attempts are made (Fagerstrom, 1999; Hughes *et al.*, 2000). Individual differences in smoking cessation outcomes using NRTs seem to be in part mediated by the degree to which NRTs are able to suppress cigarette craving and other tobacco abstinence symptoms (e.g. Evans *et al.*, 2006). NRT-induced suppression of abstinence symptoms is well established (e.g. Hughes *et al.*, 2003; Evans *et al.*, 2006) but smokers are highly heterogeneous (e.g. Piasecki *et al.*, 2003; Furberg *et al.*, 2005) and individual differences have been documented in the degree of subjective craving and withdrawal symptom relief provided by NRTs (e.g. Killen *et al.*, 1990; Wetter *et al.*, 1999). Such findings suggest that non-nicotine factors may be important for the maintenance of tobacco addiction in many smokers.

Although the addictive properties of tobacco are often attributed exclusively to nicotine (e.g. US Department of Health and Human Services, 1988), the reinforcing effects of nicotine in the absence of tobacco have not been conclusively shown using adequately blinded protocols (Dar and Frenk, 2004; Fulton and Barrett, 2008). Moreover, tobacco smoke contains several thousand compounds in addition to nicotine (Hoffmann and Hoffmann, 1998), and many of these might contribute to tobacco's addictive properties either independently (Rodd-Henricks *et al.*, 2002) or in combination with nicotine (e.g. Talhout *et al.*, 2007; Villegier *et al.*, 2007). To date, little research focus has been directed toward examining the role of non-nicotine tobacco ingredients for the addictive properties of tobacco. However, growing evidence suggests that the replacement of non-nicotine smoking-related stimuli through denicotinized tobacco (DT) may relieve many symptoms of craving and smoking withdrawal at least in some smokers. DTs have been found to provide a significant degree of subjective satisfaction and immediate craving reduction (Baldinger *et al.*, 1995; Butschky *et al.*, 1995; Westman *et al.*, 1996; Gross *et al.*, 1997; Pickworth *et al.*, 1999; Rose *et al.*, 2000;

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Buchhalter *et al.*, 2001; Donny *et al.*, 2007) but, as with NRTs, there seems to be considerable variability in responses to DT (e.g. Finnegan *et al.*, 1945; Brauer *et al.*, 2001; Barrett *et al.*, 2006) and smokers seem to vary in the degree to which they miss the nicotine (e.g. Finnegan *et al.*, 1945).

A number of individual difference variables seem to have some utility for predicting the response to NRTs (e.g. Yudkin *et al.*, 1996; D'Angelo *et al.*, 2001) and/or non-nicotine smoking stimuli (e.g. Brauer *et al.*, 2001; Rose, 2006). For example, while male sex (D'Angelo *et al.*, 2001) and low levels of tobacco dependence (Yudkin *et al.*, 1996) tend to predict more favorable treatment outcomes with NRTs, female sex (Rose, 2006) and high levels of tobacco dependence (Brauer *et al.*, 2001) seem to be associated with more positive responses to the administration of non-nicotine smoking stimuli. Although the extent to which these aggregate results have clinical implications for the individual smokers remains unknown, such findings are consistent with the notion that there may be individual differences in the relative roles of nicotine and non-nicotine tobacco constituents for the maintenance of smoking behavior.

To begin to clarify the relative importance of nicotine and non-nicotine tobacco constituents for tobacco use in different smokers, this study examined the acute subjective effects of nicotine in the absence of tobacco [through nicotine inhalers (NI) and placebo inhalers (PI)], tobacco in the absence of nicotine (through DT), and nicotine-containing tobacco (NT), and their effects on subsequent smoking behavior in a heterogeneous sample of adult smokers.

Methods

Participants

Nontreatment-seeking male and female smokers were recruited from the Halifax Nova Scotia community. Participants were recruited on the basis of either being highly dependent on tobacco [Fagerström Test for Nicotine Dependence (FTND) ≥ 5] or having very low levels of tobacco dependence (FTND ≤ 2). Potential participants were told that the study would involve four experimental test sessions, plus one nonexperimental session to complete screening measures and to provide a nonabstinent carbon monoxide (CO) breath sample, and all were informed that they would be required to remain abstinent from all illicit and prescription drugs for the duration of the study. A total of 26 individuals were invited to participate in the study. All of the participants were medically healthy, free from current or previous mental illness including past or present substance use disorders (excluding nicotine dependence), all had reached the minimum age to legally consume tobacco in Canada and none intended to quit smoking over the subsequent 6 months. The study was carried out in accordance with the Declaration of Helsinki and all participants provided written informed consent.

Tobacco

Two types of tobacco that differ in nicotine content (nicotine-containing Quest 1, Vector Tobacco; and denicotinized Quest 3, Vector Tobacco, Mebane, North Carolina, USA) were used in the experiments. The manufacturer-reported maximum nicotine yield of the DT was 0.05 mg while the NT had an average reported yield of 0.6 mg. Both types of tobacco had reported tar yields of 10 mg.

Inhalers

In sessions using an acute nicotine challenge, a NI (10 mg; 4 mg deliverable, Pharmacia, Mississauga, Ontario Canada) was used. Nicotine administration through an inhaler was selected owing to its tolerability relative to other forms of nicotine administration (Schneider *et al.*, 2004) and to inhalers' similarity to cigarettes across several sensory motor and administration parameters. In the placebo condition, an inhaler identical in appearance but containing a pharmacologically inert solution was used, and the inhalation regimen was identical to that of the nicotine condition.

Blinding

The NT and NI were prepared to appear identical to the DT and PI, respectively, and both the experimenter administering the session and the participant were blinded to their contents. To minimize demand characteristics associated with having a priori knowledge of the possible test conditions (Fulton and Barrett, 2008), participants were informed that the inhalers and tobacco could vary in some ingredients, but not that they would vary in their nicotine contents specifically.

Carbon monoxide measurement

To verify tobacco abstinence, a breath CO analyzer (Vitalograph, Lenexa, Kansas, USA) was used. This instrument enables a quantification of CO exposure ranging from 0 to 500 parts per million and CO measurement is considered to be a valid and reliable index of degree of recent tobacco smoke exposure (SRNT Subcommittee on Biochemical Verification, 2002).

Subjective measures

The following subjective measures were administered at baseline and following the mandatory tobacco/inhaler administration.

Visual Analogue Scale (VAS)

The VAS consisted of 14 subjective mood descriptors (relaxed, satisfied, pleasant, anxious, stimulated, high,

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sedated, jittery, alert, frustrated, dizzy, trouble concentrating, irritable, and head rush). Each item was rated on a 10 cm horizontal line labeled with the integers 1–10 and anchored with the endpoints 'Not at all' and 'Extremely'. Similar scales have been widely used to collect information about subjective drug effects in humans and this method of data collection has been shown to be reliable, valid, and sensitive to participants' subjective experiences (Bond and Ladder, 1972).

The Questionnaire of Smoking Urges-Brief version

The QSU-B is a 10-item, psychometrically sound self-report measure that assesses cravings across two dimensions (factor 1: intention to smoke; factor 2: withdrawal/negative affect relief). The QSU-B has been shown to be sensitive for measuring nicotine, tobacco and abstinence-related effects (Toll *et al.*, 2006).

Design

The research protocol comprised four experimental test sessions. All sessions were conducted between 09:00 and 16:00 h, a minimum of 2 and a maximum of 14 days apart, were double blind, and given in a randomized order. Participants arrived for each testing session having abstained from cigarettes for a minimum of 12 h, alcohol for a minimum of 24 h, and food and caffeine for a minimum of 4 h (caffeine-free fluid intake was not restricted before the sessions). Abstinence from tobacco was confirmed with a breath CO analyzer, using a maximum cutoff of 15 parts per million and a 50% minimum reduction relative to their nonabstinent breath sample. After completing baseline measures, participants were comfortably seated in a chair in front of a computer. They were then required to administer either an inhaler or two cigarettes over a 20-min period. During inhaler sessions participants were instructed to take one deep inhalation every 10 s throughout the entire administration period. This inhalation regimen was selected to ensure significantly elevated plasma nicotine concentrations at the time of the subjective and behavioral assessments (Schneider *et al.*, 2001). In the cigarette conditions participants were instructed to inhale the smoke and to complete the cigarette to the filter but the pace and duration of the 'puffs' were self-determined by the participants. Participants were given their first cigarette at the beginning of the administration period and second cigarette 10 min later. Following the tobacco/inhaler challenge participants completed a second subjective assessment (VAS, QSU-B) and provided another breath sample. They could then begin using a computerized progressive ratio (PR) task to earn puffs of their preferred brand of tobacco. For each puff participants were required to repeatedly press keys on a keyboard a predetermined number of times. The first puff required 10 key presses and for each subsequent puff the number of required key presses increased by one-and-a-third times (i.e. 13, 17, 22, etc.). Similar PR tasks have been shown to be sensitive to pharmacological manipulations (Barrett *et al.*, 2006, 2008) as well as to changes in mood and craving (Willner *et al.*, 2005; Willner and Jones, 2006).

Analyses

All data were analyzed using SPSS Version 15 (Chicago, Illinois, USA). The main behavioral outcomes were the number of button presses during the PR task to earn tobacco puffs during each session and the latency (time in seconds) to initiate this task. Because these data appeared skewed, they were screened for normality using the Kolmogorov–Smirnov method and it was determined that logarithmic transformations were necessary for each variable to satisfy the normality assumption. All data were analyzed using mixed modeling with challenge condition (NT, DT, NI, PI) entered as a fixed and repeated factor, sex, and dependence levels (FNTD ≤ 2 vs. FTND ≥ 5) as fixed factors and subject as a random factor. Covariance structure was selected on the basis of model simplicity and use of the likelihood ratio test (SPSS, 2002). For the VAS and QSU-B data, time (pre-challenge vs. postchallenge) was entered into the model as a fixed and repeated factor and the outcomes of interest were interactions of time with condition, sex, and/or dependence.

For all analyses, tests of simple main effects were conducted on the linearly independent pairwise comparisons among the estimated marginal means. For interactions, the simple effects of variables within each level combination of the other variable(s) were tested. To account for multiple testing, the threshold for statistical significance for all main effects and interactions was set at $P \leq 0.01$.

Results

Participants

Twenty-two participants (12 male, 10 female; 11 high dependence, 11 low dependence) enrolled in the study; 20 completed all four experimental sessions, one completed three sessions (NT, DT, NI) but withdrew owing to experiencing a trauma unrelated to the study, and one completed only two sessions (NI, PI) before relocating to another city. Because mixed models accommodate for missing data by simultaneously considering individual and group effects, the data from all 22 participants were retained for the analyses (post-hoc analyses revealed that the direction and nature of all main effects and interactions were the same when the sample was restricted to those completing all four experimental sessions). An additional four participants (one male, three females) were invited to participate after screening but did not complete any experimental sessions. Characteristics of participants included in the final analyses are presented in Table 1.

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Table 1 Demographic and tobacco-use characteristics

	Age, mean (SD)	FTND score, mean (SD)	Cigarettes per day, mean (SD)	Nonabstinent CO (ppm), mean (SD)
Overall ($N=22$)	26.8 (9.0)	3.6 (2.9)	11.9 (9.3)	17.5 (10.8)
Male ($n=12$)	27.0 (6.4)	3.7 (2.6)	11.3 (5.2)	17.5 (11.1)
5 low FTND				
Female ($n=10$)	26.6 (11.0)	3.4 (3.3)	12.7 (13.0)	17.5 (11.0)
6 low FTND				
High FTND ($n=11$)	31.5 (10.5)	6.2 (1.3)	17.6 (10.3)	25.5 (9.2)
7 male				
Low FTND ($n=11$)	22.2 (2.8)	0.9 (0.8)	6.2 (2.0)	9.5 (4.7)
5 Male				

CO, carbon monoxide; FTND, Fagerström Test for Nicotine Dependence; SD, standard deviation.

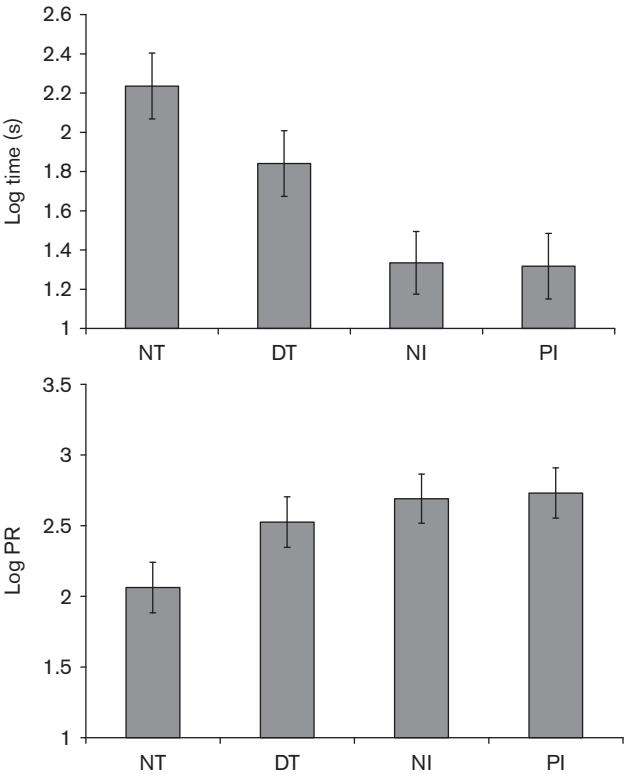
Self-administration

Two self-administration variables were assessed: the log PR values to earn preferred cigarette puffs and the latency (log time in seconds) to earn the first puff. There was a significant main effect of condition on latency to self-administration, [$F(3,49.0) = 7.1$, $P < 0.001$], reflecting a delay in the onset of self-administration in the NT and DT conditions relative to the NI and PI conditions (Ps < 0.01) (Fig. 1; upper). Analyses also revealed a significant main effect of condition on log PR values for cigarette puffs earned, [$F(3,52.0) = 6.7$, $P < 0.01$], reflecting significantly less self administration in the NT condition, relative to the DT, NI, or PI conditions (Ps < 0.01) (Fig. 1; lower). Self administration PR values did not reliably vary between DT, NI, and PI. There were no significant main effects or interactions involving sex or level of dependence for any of the self-administration variables.

Craving

Two craving-related variables were assessed using the QSU-B: intention to smoke and withdrawal/negative affect relief. There was a significant condition × time interaction on factor 1 craving (intention to smoke), [$F(6,120.8) = 13.9$, $P < 0.01$], reflecting significantly decreased craving after NT and DT administration relative to the administration of NI and PI (Ps < 0.01). There was also a significant condition × time interaction on factor 2 craving (withdrawal/negative affect relief), [$F(6,120.5) = 5.3$, $P < 0.01$], reflecting decreased postchallenge craving in the NT condition relative to the NI and PI conditions ($P < 0.01$), as well as decreased craving in the DT condition relative to PI ($P < 0.01$) (Fig. 2; upper). Analyses also revealed a significant sex × condition × time interaction, [$F(6,120.5) = 3.0$, $P < 0.01$] (Fig. 2; lower). However, because there was significant variability in female prechallenge scores ($P < 0.01$), a post-hoc analysis was conducted to examine a potential sex × condition interaction in postchallenge scores when using prechallenge scores as a repeated measures covariate. Again, the analysis revealed a significant condition × sex interaction [$F(7,52.4) = 6.4$, $P < 0.01$]. Among women there was a significant main effect of condition [$F(3,60.4) = 11.4$, $P < 0.001$], reflecting decreased withdrawal-related craving after the administration of either NT or DT relative to NI and PI (Ps < 0.001). In men condition differences were evident only at a trend level, [$F(3,57.8) = 2.9$, $P = 0.04$] with decreased withdrawal-related craving after NT relative to PI only ($P < 0.01$) and no differences between any of the other conditions ($P > 0.1$). Moreover DT tended to decrease factor 2 craving in women relative to men [$F(1,74.0) = 4.9$, $P = 0.03$] and no other postchallenge sex differences were evident (Ps > 0.1).

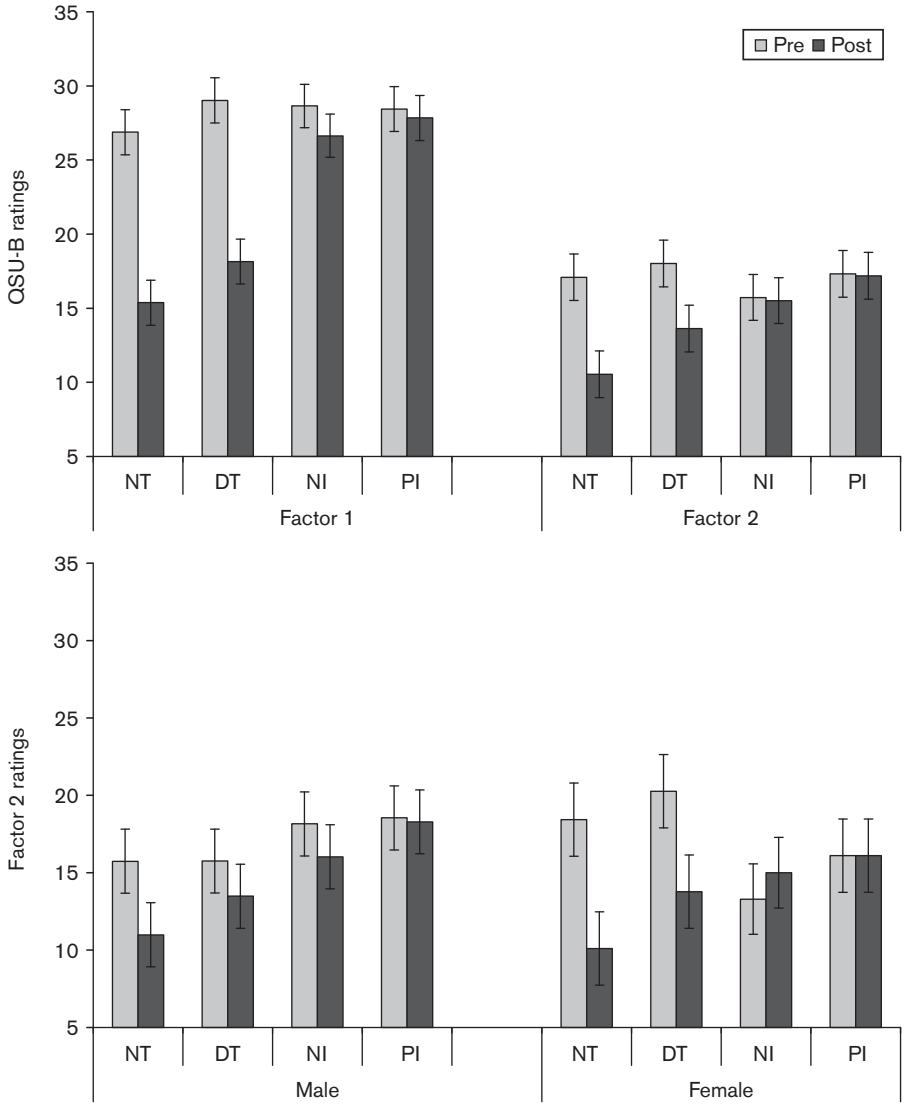
Fig. 1



Mean (±SE) latency to self-administer preferred tobacco in log transformed seconds for each experimental session (upper). There was a significant delay in self-administration after the nicotine-containing tobacco (NT) and denicotinized tobacco (DT) challenges relative to each of the inhaler challenges. Mean (±SE) log transformed progressive ratio (PR) values for each experimental condition (lower). Participants earned significantly fewer preferred tobacco puffs after the NT challenge relative to each of the other conditions. NI, nicotine inhalers; PI, placebo inhalers.

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Fig. 2



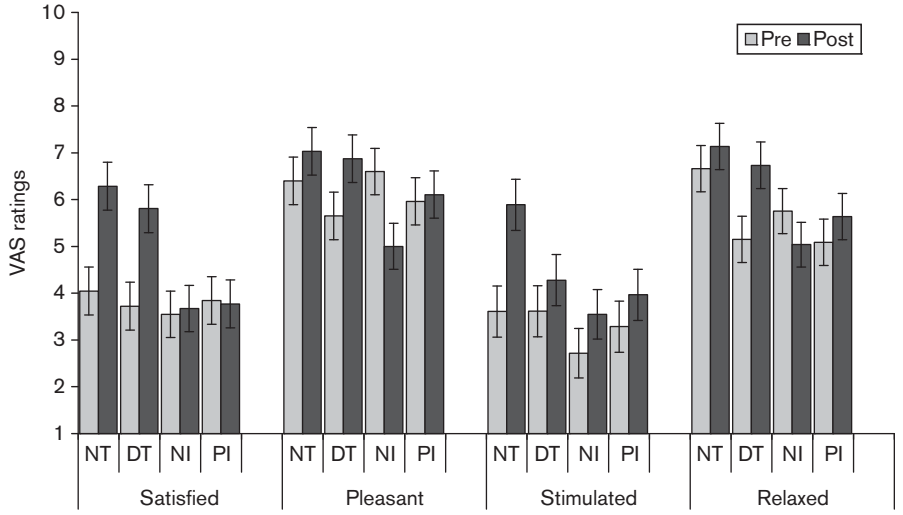
Mean (±SE) factor 1 and factor 2 Questionnaire of Smoking Urges-Brief (QSU-B) subscores before and after each experimental challenge (upper). Administration of both nicotine-containing tobacco (NT) and denicotinized tobacco (DT) significantly reduced factor 1 craving relative to each of the inhaler challenges while NT significantly reduced factor 2 craving relative to each of the other conditions and DT reduced it relative to each of the inhalers. A sex × condition × time interaction revealed that administration of DT reduced factor 2 craving relative to nicotine inhalers (NI) and placebo inhalers (PI) in women but not in men (lower).

Mood

There was a significant interaction of condition × time on ratings of 'satisfied' [$F(6,121.3) = 5.3$, $P < 0.001$], reflecting greater satisfaction after the administration of NT and DT relative to either of the inhaler conditions (Ps < 0.001). There was also a significant condition × time interaction on ratings of 'pleasant' [$F(6,120.4) = 4.8$, $P < 0.01$], with participants rating NI as being less pleasant than each of the other products (Ps ≤ 0.01), as well a significant condition × time interaction on ratings of 'stimulated' [$F(6,121.0) = 4.8$, $P < 0.01$], with participants reporting greater stimulation after NT relative to DT, NI, and PI (Ps ≤ 0.01) (Fig. 3). Analyses also revealed a significant condition × time interaction on ratings of 'relaxed' [$F(6,120.9) = 5.7$, $P < 0.01$] but because prechallenge differences in ratings of relaxed were evident [$F(3,121.0) = 4.1$, $P < 0.01$], a post-hoc analysis was conducted to determine whether there were postchallenge differences between the conditions when considering prechallenge scores as a covariate. There was a significant effect of condition on postchallenge ratings [$F(3,56.4) = 7.7$, $P < 0.001$], with the administration of either NT or DT being associated with significantly greater relaxation than the administration of NI (Ps < 0.001).

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Fig. 3



Mean (±SE) ratings of 'satisfied', 'pleasant', 'stimulated' and 'relaxed' before and after each experimental challenge. Nicotine-containing tobacco (NT) and denicotinized tobacco (DT) were rated as more satisfying than each of the inhalers; nicotine inhalers (NI) as being less pleasant than each of the other products, NT as producing more stimulation than each of the other products, and ratings of 'relaxed' were significantly greater after NT and DT administration relative to NI administration. PI, placebo inhalers; VAS, Visual Analog Scale.

There were no other significant main effects or interactions involving condition for any of the remaining variables. There were, however, significant sex × dependence × time interactions for ratings of 'sedated' $F(2,31.0) = 5.3$, 'irritable' $F(2,31.0) = 7.0$, and 'head rush' $F(2,28.7) = 7.9$ ($P < 0.01$) with greater increases in ratings in high dependence women and low dependence men, relative to other participants, for both 'irritable' and 'head rush', as well as in low dependence males for 'sedated' ($P < 0.01$). There was also a significant sex × time interaction for ratings of 'dizzy' [$F(2,30.3) = 6.9$, $P < 0.01$], reflecting relatively increased post-challenge dizziness in men.

Discussion

The present results suggest that non-nicotine tobacco smoking factors may significantly contribute to the addictive properties of smoking. Across smokers, DT was found to significantly increase subjective satisfaction and reduce intentions to smoke as well as to delay the onset of preferred tobacco self-administration, and among women DT also significantly reduced withdrawal-related craving. Interestingly, in addition to its effects on craving and tobacco use, DT was found to be associated with increased feelings of relaxation across smokers relative to NI (Fig. 3). These findings raise the possibility that non-nicotine tobacco smoking factors may be especially important for the calming effects of tobacco smoking; effects that are often considered paradoxical in light of the stimulant properties of nicotine (Nesbitt, 1973). Collectively these results suggest that, for many smokers, targeting non-nicotine smoking factors may be an effective means of reducing abstinence symptoms as well as suppressing preferred tobacco use. It should be noted, however, that NT was found to reduce preferred tobacco self-administration as well as produce greater subjective stimulation relative to DT. Such findings suggest that a combination of nicotine and non-nicotine smoking factors may be important to the reinforcing value of cigarettes.

Although this study did not assess the mechanisms by which DT suppressed tobacco craving and use, or increased subjective reports of relaxation and satisfaction, it is possible that non-nicotine tobacco constituents contributed to these effects through a neuropharmacological action. A number of ingredients found in tobacco smoke, apart from nicotine, are known to have central actions and to interact with mechanisms that mediate reinforcement (e.g. Fowler *et al.*, 1996; Rodd-Henricks *et al.*, 2002; Talhout *et al.*, 2007; Villegier *et al.*, 2007; Clemens *et al.*, 2009). Despite this, the extent to which various non-nicotine tobacco constituents contribute to tobacco craving and withdrawal remains largely uninvestigated. Alternatively, it is possible that the sensory properties of DT, independent of any neuropharmacological effect, account for the observed effects. Attenuating smoking-related sensory cues has been associated with a reduction in smoking reinforcement (Perkins *et al.*, 2001; Rose *et al.*, 1984, 1985) and it is possible that the replacement of such sensory cues in the absence of any pharmacological action is sufficient to attenuate tobacco craving and smoking (Rose, 2006; Donny and Jones, 2009). However, it is important to note that in this study, the use of NI and PI would be expected to control for many of the sensory aspects of smoking (Schneider *et al.*, 2001).

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DT was found to reduce withdrawal-related craving in women but not in men (Fig. 2; lower). It is possible that this sex difference may result from a differential involvement of nicotine and non-nicotine tobacco constituents in the addictive properties of tobacco in men and women. Nicotine-specific therapies seem to be more effective in promoting smoking cessation in men than in women (Perkins *et al.*, 1999) and although little is currently known about individual differences in response to pharmacological effects of non-nicotine tobacco constituents, evidence suggests that women may have a greater susceptibility to smoking-related expectancy effects than men (Perkins *et al.*, 2006). Irrespective of the mechanism, the ability of DT to attenuate tobacco withdrawal in women suggests that it may be important to target non-nicotine smoking factors for smoking cessation in many smokers. However, it is also important to note that across smokers only NT was effective in significantly reducing the total amount of preferred tobacco self administered. Thus, it may be appropriate to target a combination of nicotine and non-nicotine tobacco constituents to suppress tobacco use maximally in both men and women.

A relatively unexpected result of this study was an adverse response to NI, as reflected by significantly decreased ratings of 'pleasant'. Although in past research participants have shown a preference for NI relative to other forms of NRT (Schneider *et al.*, 2004), it is important to note that smokers often rate nicotine apart from tobacco as having unpleasant effects across modes of administration (e.g. Dar and Frenk, 2004; Rose, 2006), and in this study NI were compared with tobacco and with an inert placebo, not with other forms of nicotine. Moreover, in contrast to most other investigations, participants in this study were not informed that they would be receiving nicotine during one of the inhaler conditions. Earlier research suggests that the expectation that one is receiving nicotine increases the likeability and clinical efficacy of NRTs and that expectancies interact with pharmacological factors to produce overall subjective and behavioral responses (e.g. Hughes *et al.*, 1989; Perkins *et al.*, 2009). Thus, the nonpharmacological aspects of nicotine administration would be expected to be de-emphasized in this study and this may account for some of the effects (and lack thereof) associated with NI administration. It is also important to note that the experimental protocol required participants to administer 120 inhalations over 20 min during each of the inhaler conditions. Although this inhalation regimen was selected to ensure maximum nicotine concentrations at the time of the subjective and behavioral assessments, it is possible that it decreased the likeability of the NI. In past research, inhalation regimens requiring 50% fewer inhalations of NI over the same time period have been well tolerated (Schneider *et al.*, 2005) and resulted in plasma nicotine concentrations similar to those achieved by other NRTs (Schneider *et al.*, 2001). Although the present investigation required higher levels of NI intake to maximize nicotine exposure, participants readily accepted this dosing procedure and there was no evidence of toxicity or other adverse events.

The present results should be interpreted in light of the following methodological considerations. First, because the participants were not treatment-seeking and were not attempting to remain abstinent during the experimental sessions, it is not clear to what extent the findings can extend to those attempting to quit smoking. Although a number of recent trials have examined the use of DT as an adjunct to NRT for smoking cessation (Rezaishiraz *et al.*, 2007; Becker *et al.*, 2008), to my knowledge the acute effects of DT on tobacco reinforcement have not been examined in samples attempting to quit smoking. Additional research should be directed toward investigating non-nicotine smoking factors in those attempting to achieve abstinence. Second, the DT used in this study contains trace amounts of nicotine (Vector Tobacco), and as a result a potential nicotinic contribution to DT-related effects cannot be excluded. However, because the nicotine yield of the NT is approximately 12 times higher than that of the DT, and DT administration has been shown to result in substantially less nicotinic receptor occupancy than NT (Brody *et al.*, 2009), it is unlikely that similarities observed between NT and DT in this study can be solely attributed to their nicotinic effects. Third, although the sample size was well within the norms for assessing within-subjects drug effects, it may have been inadequate to detect certain interaction effects involving sex and/or dependence, and further study will be required to fully evaluate the effects of nicotine and non-nicotine smoking factors on tobacco craving and administration in different subsets of smokers. Finally, because NT and NI have different delivery kinetics, it is possible that pharmacokinetic differences between the products may account for some of the differences in their subjective and behavioral effects. Conclusive evidence linking the delivery kinetics of nicotine to tobacco reinforcement is currently lacking (Dar and Frenk, 2007), and evidence suggests that DT may be preferred to nicotine in the absence of tobacco, even when the delivery kinetics of nicotine are identical to those achieved by smoking (Rose, 2006). Moreover, explanations based on nicotine kinetics are unlikely to account for similarities observed between NT and DT. Nevertheless, it will be important to examine nicotine and non-nicotine smoking factors under a variety of conditions to understand more fully their relative contributions to tobacco addiction.

In conclusion, DT administration seems to provide significant subjective satisfaction as well as to reduce tobacco abstinence symptoms. Moreover, although NT was superior to DT in suppressing smoking behavior, the administration of NI tended to have little effect on

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acute tobacco use relative to placebo. These findings suggest that non-nicotine smoking factors may be important to smoking-related reinforcement and may be an appropriate target for smoking cessation. Increased attention should be directed toward identifying the pharmacological and/or nonpharmacological elements of DT that are effective in suppressing tobacco craving and administration.

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Acute Negative Affect Relief from Smoking Depends on the Affect Situation and Measure but Not on Nicotine

Kenneth A. Perkins, Joshua L. Karelitz, Cynthia A. Conklin, Michael A. Sayette, and Grace E. Giedgowd

Background: Smoking acutely relieves negative affect (NA) due to smoking abstinence but may not relieve NA from other sources, such as stressors.

Methods: Dependent smokers ($n = 104$) randomly assigned to one of three smoking conditions (nicotine or denicotinized cigarettes, or no smoking) completed four negative mood induction procedures (one per session): 1) overnight smoking abstinence, 2) challenging computer task, 3) public speech preparation, and 4) watching negative mood slides. A fifth session involved a neutral mood control. The two smoking groups took four puffs on their assigned cigarette and then smoked those same cigarettes ad libitum during continued mood induction. All subjects rated their level of NA and positive affect on several measures (Mood Form, Positive and Negative Affect Scale, Stress-Arousal Checklist, and State-Trait Anxiety Inventory-state). They also rated craving and withdrawal.

Results: Negative affect relief from smoking depended on the NA source (i.e., mood induction procedure) and the affect measure. Smoking robustly relieved NA due to abstinence on all four measures but only modestly relieved NA due to the other sources and typically on only some measures. Smoking's effects on positive affect and withdrawal were similar to effects on NA, but relief of craving depended less on NA source. Smoking reinforcement only partly matched the pattern of NA relief. Few responses differed between the nicotine and denicotinized smoking groups.

Conclusions: Acute NA relief from smoking depends on the situation and the affect measure used but may not depend on nicotine intake. These results challenge the common assumption that smoking, and nicotine in particular, broadly alleviates NA.

Key Words: Negative affect, negative reinforcement, nicotine, nicotine dependence, smoking, stress, withdrawal

Smokers and clinicians, as well as researchers, assume that a critical reinforcing effect of smoking, in general, and nicotine, in particular, is relief of negative affect (1–3). Clinical research clearly shows that negative affect promotes smoking relapse (3,4), and smoking reliably relieves negative affect due to smoking abstinence (i.e., withdrawal) (5). However, controlled studies rarely show that smoking relieves negative affect due to other sources, such as stressors (2,6). Thus, smoking may actually relieve negative affect only under very limited conditions. Knowing under what conditions smoking does or does not relieve negative affect is important to guide research into the mechanisms of nicotine's actions and smoking reinforcement.

Several explanations of when smoking may relieve negative affect have been proposed, with the range of these explanations perhaps best captured by two competing models. In one, the Nicotine Resource Model, smoking relieves negative affect from virtually all sources (1), even in the absence of withdrawal. The other view, the Deprivation Reversal Model, posits that smoking only relieves negative affect that arises from withdrawal and not from other sources (7,8). Intermediate views suggest that smoking may robustly relieve negative affect only in some vulnerable smokers or only during some challenges depending on characteristics such as their immediacy, vagueness, demand for active coping, and opportunity for distraction (1,2,9).

Determining from the literature the conditions under which smoking relieves negative affect is complicated by procedural differences across studies. With very few exceptions (10), prior laboratory research examined smoking's influence on affect during just one task, and different studies used different tasks (2). Moreover, different studies often used different measures of negative affect (2). Watson *et al.* (11) define negative affect as a "general dimension of subjective distress and unpleasurable engagement that subsumes a variety of aversive mood states, including anger, contempt, disgust, guilt, fear, and nervousness" (p. 1063). Such a broad concept cannot be easily captured with one measure, and no single measure of affect is considered the gold standard. Relief of negative affect due to smoking may depend on the affect measure, as well as its source, as suggested by rodent studies of nicotine's effects on antinociception (12) and anxiety (13). As a result, these procedural differences may prevent clear interpretation of findings aggregated across studies.

This study examined the effects of smoking on negative affect relief as functions of the affect source and measure. Tasks varying in response demands were administered in separate sessions for comparison with smoking effects after overnight abstinence. Subjects were randomized to nicotine versus denicotinized cigarette smoking versus no smoking, to determine the influence of nicotine per se, and smoking in general, on negative affect relief (14). Multiple common affect measures were employed to determine the consistency of findings across measures. We also assessed craving, withdrawal, cigarette reward (i.e., "liking"), and smoking behavior in response to these situations. A pattern of increase in smoking reinforcement coinciding with the pattern of negative affect relief could support a negative reinforcement explanation of greater smoking due to negative mood.

Methods and Materials

Study Design

Participants were randomly assigned to one of three groups varying in the smoking condition for all sessions: nicotine cigarettes, denicotinized cigarettes, and no smoking control. All participated in five experimental sessions, one per mood induc-

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tion procedure (four negative, one neutral). Thus, this study involved one between-subjects factor, smoking condition (three levels), and one main within-subjects factor, mood induction procedure (five levels). This study was approved by the University of Pittsburgh Institutional Review Board.

Participants

Participants (n = 104; 62 male, 42 female) were adult smokers (≥10 cigarettes/day) recruited via ads posted in the surrounding community. Excluded were those admitting to treatment for psychiatric problems in the last year or who scored above 13 on the Beck Depression Inventory II (15). Most were Caucasian (84.8%), with 14.2% African American and 1% Asian. Mean ± SE sample characteristics were age of 27.2 ± .9 years, nicotine yield of preferred brand of 1.04 ± .02 mg, daily smoking rate of 19.3 ± .5 cigarettes/day for 11.0 ± .9 years, and Fagerstrom Test of Nicotine Dependence (16) score of 4.7 ± .2, indicating moderate dependence.

Smoking Conditions

Subjects in the smoking conditions received either nicotine (Quest 1, .6 mg nicotine, 9 mg tar) (n = 37) or denicotinized cigarettes (Quest 3, .05 mg nicotine, 9 mg tar) (n = 34) widely used in smoking research (e.g., 17,18) and obtained commercially from Vector Group, Ltd. (Miami, Florida). All were blind to brand. The no smoking control subjects (n = 33) were not given any cigarettes. These three groups did not differ in age, ethnicity, or smoking characteristics.

Mood Induction Procedures

Subjects were presented with five different procedures to induce negative (four) or neutral (one) mood, with one procedure per session. Neutral mood was a control procedure. Overnight abstinence provided an assessment of the well-established effects of smoking on negative affect due to abstinence. The remaining three were nonabstinence negative mood induction procedures designed to induce a level of negative affect comparable with that due to overnight abstinence. These varied in active versus passive coping (i.e., explicit behavior that subjects believed could reduce the degree of challenge posed by the task) and between immediate and clear versus anticipatory and vague response demands (1).

Neutral Mood Control. Subjects watched slides from the International Affective Picture System (19) judged neutral in emotional content. Slides were shown in a dark room for 12 seconds each, one after the other, to sustain the intended mood. A similar procedure produces no increase in either positive or negative affect (6). This condition was intended as a control procedure, to determine effects of smoking and nicotine in the absence of elevated negative affect.

Tobacco Abstinence. Subjects abstained overnight from smoking for >12 hours before this session, verified by expired air carbon monoxide ≤10 ppm (20). A half day of abstinence reliably produces increased negative affect due to withdrawal (5). To maintain subject attention during the session, they engaged in the control task (above), in the neutral mood condition, although different neutral slides were presented to eliminate repetition of stimuli. Abstinence-induced negative affect, which is widely shown to be alleviated by smoking and nicotine, provided a reference for the magnitude of negative affect relief due to smoking during the other mood procedures.

Computer Challenge. Subjects were presented with a sequence of the digits 1 to 4 in random order on a computer monitor and were instructed to repeat the sequence in either the same or the reverse order on command using a computer keypad (similar to the commercial game Simon). Responding required the use of just one hand, allowing the other hand for smoking. To standardize the degree of challenge, task difficulty (i.e., number of digits to remember) was continuously adjusted by the computer to ensure comparable success (40% correct) for all subjects, regardless of ability. Correct responses earned $0.50 and errors resulted in a loss of $0.25. This task, which requires active coping and presents a clear and immediate challenge, increases self-reported negative affect (21).

Negative Mood Slides. This procedure altered an existing technique (22), involving high arousal negative affect pictorial slides from the International Affective Picture System (19), and was similar to the neutral mood procedure described above except for the mood valence of the slides. This task allowed no active coping (i.e., only passive viewing was possible) and presented a clear and immediate challenge. A similar procedure has been used to increase negative affect in prior smoking studies (6,17).

Subjects correctly identified 86.1% of the content of the slides shown to them during the negative and neutral mood slide procedures, with no differences between mood valence, verifying that they attended to these procedures that lacked explicit response requirements.

Speech Preparation. Subjects were instructed to prepare and then give two 3-minute speeches, one at a time, on what they like and dislike (in that order) about their body. Subjects were unaware they would have to give a second speech (dislike) when they received instructions for the first (like). Requiring two speeches allowed for extended periods of active speech preparation, to match the duration of the other mood induction procedures. A staff member dressed in a white coat was introduced and described as a professional observer who would rate the speech on psychological characteristics. Subjects also were shown a video camera and told it would be used to record the speech for later evaluation, although speeches were not actually recorded. Affect ratings were obtained before each speech (see below), so that affect during speech preparation was assessed. This task, which required active coping and presented a vague and anticipatory challenge, elicited robust negative affect in similar research (e.g., 23,24).

Self-Report Measures

Affect. The four measures of negative affect were selected for their diversity of items and frequency of use in acute mood induction studies. These measures were the Diener and Emmons (25) Mood Form (converted from 0–6 to a 0–100 visual analogue scale [VAS]), the Positive and Negative Affect Scale (PANAS) (11), the stress items of the Stress-Arousal Checklist (SACL) (26), and an abbreviated version of the State-Trait Anxiety Inventory (STAI)-state (27). This STAI version (STAI-brief) contains 6 of the 20 items, three negative affect items (upset, worried, frightened) and three reverse-scored items (calm, secure, self-confident), and has been shown to be sensitive to acute laboratory challenges (24).

Craving and Withdrawal. The brief 10-item version of the Questionnaire on Smoking Urges (28) provides two separate craving factors, one reflecting a strong intention and desire to smoke (factor 1) and the other reflecting anticipation of relief from negative affect (factor 2). Withdrawal was assessed by the Minnesota Nicotine Withdrawal Scale (29), with items rated on a 0 ("not at all") to 100 ("extremely") VAS and averaged for a total score.

Smoking Reward and Perception. Two items from the Cigarette Evaluation Scale (30), "How much do you like this

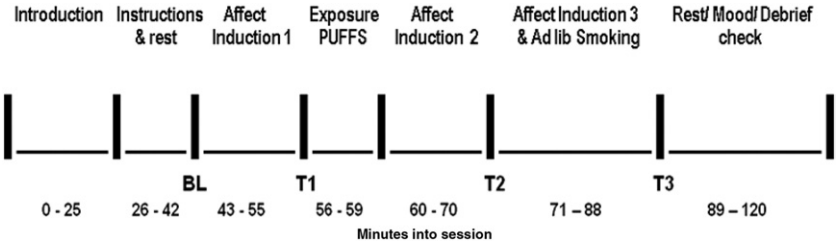


Figure 1. Timeline of each session. BL, T1, T2, and T3 indicate the points at which affect, craving, and withdrawal measures were obtained. Cigarette ratings were obtained at the unlabeled point just after the exposure puffs. The time intervals between points indicate the time between the start of the completion of the measures and not necessarily the duration of mood induction procedures (see text for details of mood induction timing). BL, baseline.

cigarette?" and "How strong is this cigarette?," assessed smoking reward (liking) (31) and perception, respectively. Each was rated on a 0 to 100 VAS.

Session Procedures

Subjects participated in five sessions, one per mood induction procedure, over an average of 15.9 days (mean of 2.8 ± .1 days between sessions). The session timeline is shown in Figure 1. Except for the smoking abstinence session, participants smoked ad libitum before each session and smoked one cigarette of their own brand upon arrival. We wanted to ensure that negative affect later in those sessions was due to the specific mood induction procedure assigned for that day and not to withdrawal. Participants then rested quietly to obtain baseline measures, were introduced to the mood procedure for that session, and engaged in it for 5 minutes to induce mood (followed by T1 measures). The nicotine and denicotinized smoking groups were given their designated cigarette; they took four standard puffs (see below) and rated it for reward and perception. The no smoking control group simply rested. Four puffs were selected to provide sufficient basis to rate the cigarette but not enough to induce satiation, which would interfere with the subsequent assessment of ad libitum smoking. The mood induction procedure resumed for another 3 minutes (T2 measures). Subsequently, mood induction continued, and the two smoking groups were allowed to smoke their assigned cigarettes ad libitum over the last 10 minutes (T3 measures). Affect, craving, and withdrawal were assessed in all three groups at each time point (baseline, T1, T2, T3). The order of mood induction procedures across sessions was randomized between subjects, but the same sequence of orders was used for each smoking group, stratified by sex.

All smoking was done via the Clinical Research Support System (Borgwaldt KC, Inc., Richmond Virginia; http://www.plowshare.com), which assesses puff volume. Smoke inhalation during the four standard puffs was successfully controlled by computer instructions guiding the timing and amount of puff inhalation (17,21), as shown by the lack of differences in total volume from the four puffs across the five mood procedures (ranging from 235 ± 8 mL to 248 ± 9 mL) and between the nicotine and denicotinized smoking groups (238 ± 11 mL vs. 243 ± 11 mL, respectively). Smoking during the ad libitum period was not controlled by instructions.

Data Analyses

Order of mood procedures across sessions was not analyzed because of the large number of orders. No interactions involving sex were found in analyses, so results were collapsed across men and women. For each set of affect analyses, the four negative affect measures and the two positive affect measures were first

Table 1. MANOVA and Follow-Up ANOVA Results (*F* Ratios) for Negative and Positive Affect, Craving, and Withdrawal Across Time (T1, T2, T3) as Functions of Mood Induction Procedure, Smoking Group, and, for Affect, Affect Measure

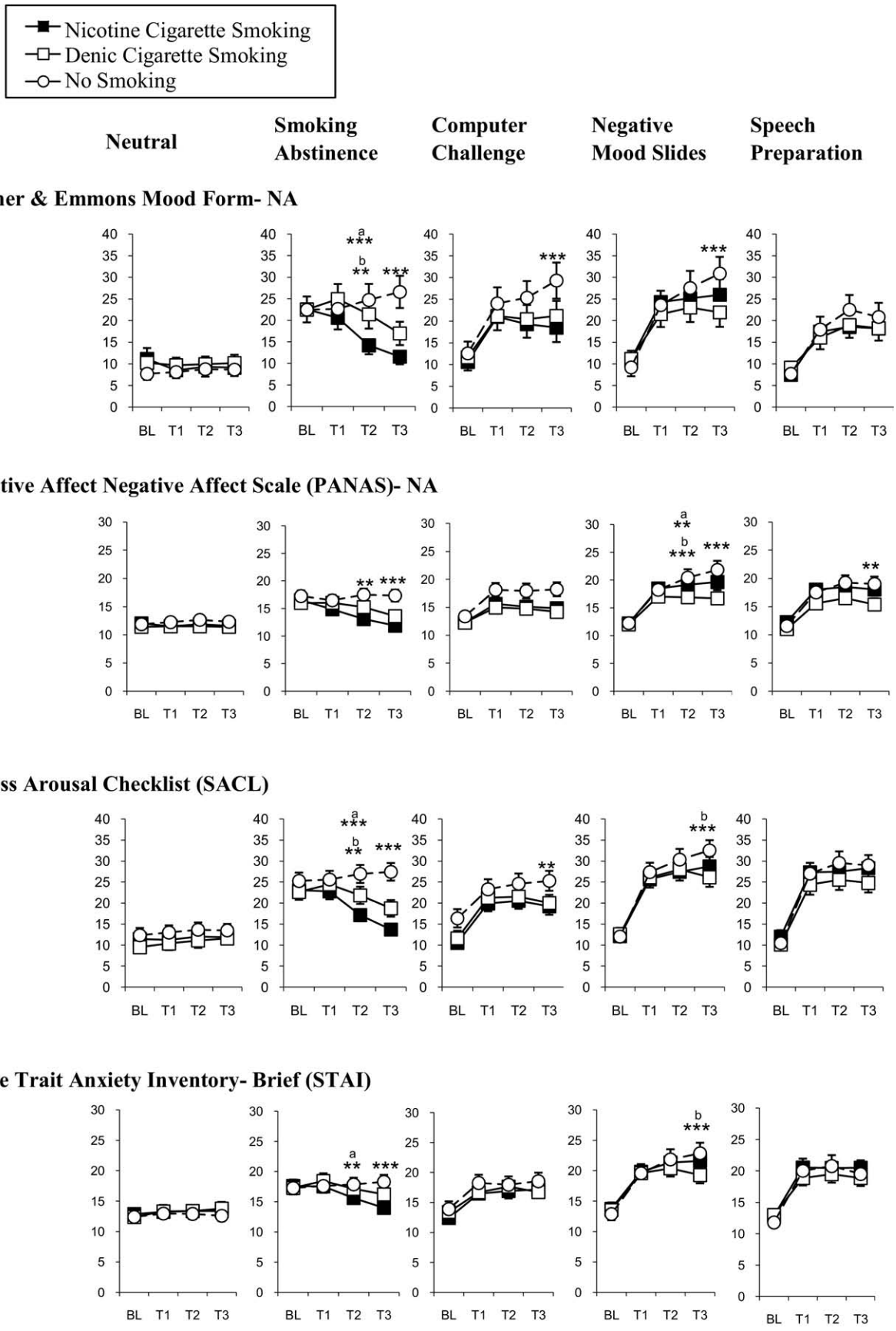
Measure	Time × Smoking Group	Time × Mood Induction Procedure	Time × Smoking Group × Mood Induction Procedure
Negative Affect			
MANOVA	$F(16, 782) = 3.15^{b}$	$F(32, 3182) = 3.48^{c}$	$F(64, 3182) = 1.24^{b}$
Univariate ANOVAs	*F*(4, 200)s of:	*F*(8, 800)s of:	*F*(16, 800)s of:
Mood Form	10.02^{b}	5.82^{c}	1.87^{a}
PANAS	9.21^{c}	6.08^{c}	1.40
SACL-stress	6.16^{c}	8.41^{c}	2.29^{a}
STAI	2.35	5.80^{c}	2.27^{b}
Positive Affect			
MANOVA	$F(16, 796) = 1.14$, ns	$F(16, 1596) = 3.29^{c}$	$F(32, 1596) = 1.64^{a}$
Univariate ANOVAs	—	*F*(8, 800)s of:	*F*(16, 800)s of:
Mood Form	—	5.07^{c}	1.78^{a}
PANAS	—	2.20^{a}	1.30
Craving and Withdrawal	*F*(4, 200)s of:	*F*(8, 800)s of:	*F*(16, 800)s of:
QSU-Factor 1	53.83^{c}	10.95^{c}	1.30
QSU-Factor 2	34.24^{c}	17.75^{c}	2.38^{b}
MNWS withdrawal	13.48^{c}	11.26^{c}	2.44^{c}

ANOVA, analysis of variance; MANOVA, multivariate analysis of variance; MNWS, Minnesota Nicotine Withdrawal Scale; PANAS, Positive and Negative Affect Scale; QSU, Questionnaire on Smoking Urges; SACL, Stress-Arousal Checklist; STAI, State-Trait Anxiety Inventory.

$^{a}p < .05$.

$^{b}p < .01$.

$^{c}p < .001$.


Nicotine Cigarette Smoking
Denic Cigarette Smoking
No Smoking
Neutral
Smoking Abstinence
Computer Challenge
Negative Mood Slides
Speech Preparation
Diener & Emmons Mood Form- NA
Positive Affect Negative Affect Scale (PANAS)- NA
Stress Arousal Checklist (SACL)
State Trait Anxiety Inventory- Brief (STAI)
BL T1 T2 T3

analyzed by multivariate analyses of variance (MANOVAs; Hotelling's Trace), with follow-up analyses of variance (ANOVAs) and least significant difference t tests (32) where significant. The validity of the negative mood induction procedures was determined by comparing affect at T1, after induction but before any smoking, between the four negative mood procedures versus the neutral control procedure.

Affect in response to smoking was determined by the change across time from T1 to T2 and T3. Only interactions involving time were of interest. We hypothesized that negative affect over time would be influenced by the interaction of smoking group by mood procedure, such that smoking would relieve negative affect due to abstinence but not during neutral mood and some of the other negative mood procedures. Affect relief during all four negative mood procedures would support the Nicotine Resource Model, while affect relief during abstinence but not the other procedures would support the Deprivation Reversal Model (1,7). Differences between the nicotine and denicotinized smoking groups tested the effects of nicotine per se, while differences between the denicotinized smoking and no smoking groups indicated effects of the act of smoking, in the absence of significant nicotine. In follow-ups, the change from T1 (presmoking) to T2 examined the influence of a fixed amount of smoke intake (four controlled puffs), while the change from T1 to T3 examined the influence of a greater (but variable, i.e., ad libitum) amount of smoke intake. Because of the large number of these comparisons, significance was set at $p < .01$. Similar analyses were conducted for craving and withdrawal, except no initial MANOVAs were conducted because each involved a single measure.

Smoking reward and perception, rated after the four standard puffs (between T1 and T2), were analyzed by ANOVA, with task and nicotine/denicotinized smoking groups as factors. Smoking reinforcement in the two smoking groups, determined by total puff volume during the ad libitum smoking period (between T2 and T3), was analyzed in the same manner.

Results

Validation of Mood Induction

Negative affect upon initial mood induction (T1) was similarly elevated during each of the four negative mood induction procedures, compared with the neutral control procedure, as intended. The main effect of procedure, but no other effects, was highly significant both in the MANOVA, $F(16,1598) = 17.89$, $p < .001$, and in the individual ANOVAs for each of the four negative affect measures, $F(4,404)$s of 21.79 to 46.24, all $p < .001$. Similar results were seen for the reduction in positive affect due to the negative mood induction procedures, as only the main effect of procedure was significant in the MANOVA, $F(8,804) = 31.23$, $p < .001$, and the ANOVAs for the Mood Form and PANAS, $F(4,404)$s of 38.72 and 20.38, respectively, both $p < .001$.

Affect, Craving, and Withdrawal in Response to Smoking

Negative Affect. Effects of smoking were determined by the change in affect across time (T1, T2, T3), by smoking group and mood procedure. As shown in Table 1, MANOVA results and most of the follow-up ANOVAs for each affect measure revealed significant interaction effects for time by smoking group, time by mood procedure, and time by smoking group by mood procedure.

The magnitude of negative affect relief from smoking varied strongly by mood procedure. Smoking robustly relieved negative affect after abstinence, regardless of the affect measure. As shown in Figure 2, this relief was observed after the four standard puffs (T2), and only after abstinence did ad libitum smoking (T3) fully relieve negative affect to the level observed in the neutral mood procedure. However, the effects of smoking during the other sources of negative affect were modest and depended on the affect measure. Smoking modestly attenuated negative affect on the Mood Form and SACL during the computer challenge, on all four measures during the negative mood slides, and on only the PANAS during speech preparation (Figure 2). Negative affect varied little between the nicotine and denicotinized smoking groups, although negative affect on the SACL and STAI was attenuated during negative mood slides by smoking denicotinized cigarettes, not nicotine cigarettes.

Positive Affect. Significant effects in the MANOVA and most of the follow-up ANOVAs were the interactions of time by mood procedure and of time by smoking group by mood procedure (Table 1). Consistent with the results for negative affect, smoking nicotine or denicotinized cigarettes enhanced both measures of positive affect during abstinence but had minimal effects during the other mood induction procedures (Figure S1 in Supplement 1).

Craving and Withdrawal. Analysis of variance results for Questionnaire on Smoking Urges factors 1 and 2 and Minnesota Nicotine Withdrawal Scale withdrawal were similar to those for affect (Table 1). As with negative affect, smoking relieved withdrawal more robustly after abstinence than during the other procedures, although, as expected, withdrawal was elevated only after abstinence. However, relief of craving by smoking was substantial and generally similar across mood induction procedures (Figure S2 in Supplement 1). The nicotine content of cigarettes again had little influence on these responses to smoking.

Smoking Reward, Perception, and Reinforcement

Reward and Perception. Cigarette liking was influenced by the main effects of procedure, $F(4,188) = 3.33$, $p < .02$, and smoking group, $F(1,47) = 5.97$, $p < .02$, but not by the interaction of smoking group by procedure, $F(4,188) = 1.14$. As shown in Figure 3, liking was greater for the nicotine versus denicotinized cigarette and after abstinence versus neutral mood; liking did not differ between the other negative mood procedures and neutral mood. "How strong" was influenced only by the main effect of smoking group, $F(1,47) = 13.61$, $p < .001$, as ratings were higher for the nicotine cigarette (not shown).

Reinforcement. Total puff volume during the ad libitum smoking period was significantly influenced only by mood procedure, $F(4,256) = 14.21$, $p < .001$. As also shown in Figure

Figure 2. Negative affect (NA) across time points as functions of mood induction procedure, smoking condition, and affect measure. The five mood conditions are arrayed across the top in different columns, while results for the specific measures are presented in different rows. Results for neutral mood are presented in the first column, followed by abstinence, to show the effects of smoking after abstinence versus neutral mood. Effects of smoking under the three nonabstinence negative mood procedures are then presented to facilitate comparison with smoking effects after abstinence. $^{**}p < .01$; $^{***}p < .001$ for comparisons between the no smoking versus the nicotine or denicotinized cigarette smoking groups in the change from T1 to T2 or from T1 to T3. Asterisks apply to both comparisons unless otherwise specified by superscripts (a, comparison between No Smoking and Nicotine Cigarette Smoking Groups only; b, comparison between No Smoking and Denic Cigarette Smoking Groups only). BL, baseline.

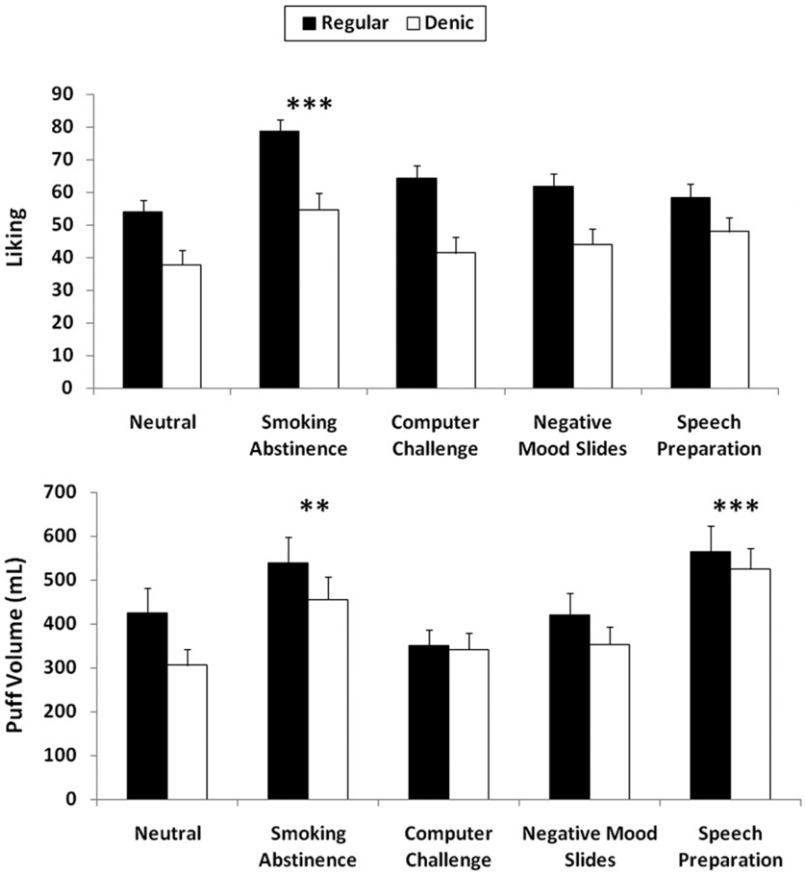


Figure 3. Smoking reward (top) and puff volume during the ad libitum smoking period (bottom) as functions of mood induction procedure and nicotine versus denicotinized smoking group. The main effect of nicotine was significant for reward but not for puff volume. $^{**}p < .01$; $^{***}p < .001$ for the difference from neutral mood control procedure. Denic, denicotinized.

3, smoke intake was greater than neutral control during the speech preparation and smoking abstinence procedures but not during the computer challenge and negative mood slides. Effects of smoking group, $F(1,64) = 2.44$, $p > .10$, and the interaction of mood procedure by smoking group, $F(4,256) < 1$, were not significant, as no differences in smoking behavior were seen between the nicotine and denicotinized cigarettes.

Discussion

Rather consistent with the Deprivation Reversal Model (7,8), negative affect relief by cigarette smoking was robust after smoking abstinence but much more modest or absent during other negative mood procedures. Only with abstinence was negative affect relief reliably seen after the four standard puffs and virtually complete after the ad libitum smoking period (Figure 2). The influence of situation was also apparent for enhancement of positive affect, although the effects of smoking on craving were far less influenced by situation. We know of no other human study clearly demonstrating the situation specificity of smoking's influence on relieving negative affect.

A second novel finding was that even the limited relief of negative affect during some sources other than abstinence depended on the affect measure. During the computer challenge task, smoking attenuated negative affect measured by the Mood Form and SACL but not the PANAS and STAI-brief. During the public speech, smoking attenuated negative affect assessed only by the PANAS. We are not aware of any other controlled study clearly demonstrating that negative affect relief from smoking may be specific to the affect measure used, in addition to the source of the negative affect. Future studies of acute negative affect relief due to smoking should employ multiple affect measures or risk the likelihood that their results may be specific only to the single measure used.

Another important, if less novel, finding is that the acute influences of smoking on affect, craving, and withdrawal were largely unrelated to nicotine intake, consistent with prior studies by us (17) and others (14,18,33–35). These results further emphasize that nonpharmacological, perhaps conditioned, influences are critical to these acute effects of smoking. Alternatively, constituents in tobacco other than nicotine may be psychoactive in ways that relieve negative affect or even the tiny amounts of nicotine in denicotinized cigarettes may be sufficient to alleviate many effects of abstinence (36).

The increase in smoking behavior (reinforcement) did not completely parallel the influence of smoking on relieving negative affect across the five procedures. Smoking was increased by abstinence, the source of negative affect robustly relieved by smoking, but smoking was increased similarly by speech preparation, where smoking did not clearly relieve negative affect. Smoking was not increased during the other negative mood procedures, where negative affect was a bit more consistently relieved by smoking, depending on the measure. These observations question any straightforward negative reinforcement explanation for the relationship between negative mood and smoking behavior (2,6,17). Yet, the lack of influence of smoking on enhancing positive affect, other than during abstinence, may also be contrary to the notion that negative mood situations, or "stressors," increase smoking behavior by enhancing smoking's positive reinforcing effects.

The situational specificity of negative affect relief by smoking makes it important to understand mediators of negative affect in these situations. The emergence of negative affect during abstinence may be due to resensitization of upregulated nicotine receptors, following the end of chronic nicotine effects in desensitizing the receptors (37,38). Nicotine intake would then desensitize receptors, providing a clear mechanism for smoking's relief of negative affect due to abstinence that is likely not relevant to the origin and relief of negative affect due to situational challenges. Ventral striatal dopamine release may also be important (39). However, the conditioning of nicotine effects via smoking also likely plays a substantial role. Withdrawal symptoms, including negative affect, may be behavioral as well as pharmacological in nature (40), accounting for why smoking either nicotine or denicotinized cigarettes fully relieved negative affect during abstinence but not the other conditions of this study. Other study findings do not appear to help clarify the situations in which negative affect is relieved by smoking. Although the nonabstinence negative mood procedures varied in terms of active coping, immediacy, and vagueness of the challenge, these characteristics appeared to have little impact in moderating relief of negative affect due to smoking because such relief was minimal.

Our results also may have implications for clinical research. Several times per day, smokers may experience mild withdrawal and, upon smoking, subsequent relief of negative affect related to withdrawal. Over time, the association of frequent complete negative affect relief from smoking during withdrawal could foster conditioning of smoking behavior in response to any experience of negative affect (4,7). Moreover, broad expectations of negative affect relief by smoking may also be promoted when smokers experience this relief during situational challenges that occur concomitant with brief smoking abstinence; the resulting relief of abstinence-induced negative affect may be interpreted as relief of negative affect arising from the situational challenges. Our findings may also be relevant to clinical research on the influence of other drug use, such as alcohol, on negative affect relief (41).

Study limitations include the uncertain generalizability of the mood procedures to common causes of negative affect in the natural environment, exclusion of smokers with current depression, use of cigarettes unfamiliar to subjects, the relatively brief period of mood induction and observation of smoking behavior, and the absence of nonself-report measures of negative affect. Our results should be replicated in studies involving other sources of negative affect, the smokers' preferred brand of cigarettes, longer periods of mood induction and smoking access, and psychophysiological or other measures of negative affect (42,43). It is possible that measures capturing other dimensions of negative affect not assessed here may reveal relief due to smoking during nonabstinence challenges. Other moderating or mediating factors of the situation not manipulated here, such as distraction, may also show that smoking relieves negative affect during such challenges (23). Individual differences in magnitude of negative affect relief may also be important (1,44,45). If our findings are replicated, smoking's influence on negative affect may be far more modest and narrow than typically assumed, guiding future research on mechanisms of smoking reinforcement.

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Supplementary material cited in this article is available online.

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Evaluating the acute effects of oral, non-combustible potential reduced exposure products marketed to smokers

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ABSTRACT

Background Non-combustible potential reduced exposure products (PREPs; eg, Star Scientific's Ariva; a variety of other smokeless tobacco products) are marketed to reduce the harm associated with smoking. This marketing occurs despite an absence of objective data concerning the toxicant exposure and effects of these PREPs. Methods used to examine combustible PREPs were adapted to assess the acute effects of non-combustible PREPs for smokers.

Methods 28 overnight abstinent cigarette smokers (17 men, 14 non-white) each completed seven, Latin-squared ordered, approximately 2.5 h laboratory sessions that differed by product administered: Ariva, Marlboro Snus (Philip Morris, USA), Camel Snus (RJ Reynolds, Winston-Salem, North Carolina, USA), Commit nicotine lozenge (GlaxoSmithKline; 2 mg), own brand cigarettes, Quest cigarettes (Vector Tobacco; delivers very low levels of nicotine) and sham smoking (ie, puffing on an unlit cigarette). In each session, the product was administered twice (separated by 60 min), and plasma nicotine levels, expired air CO and subjective effects were assessed regularly.

Results Non-combustible products delivered less nicotine than own brand cigarettes, did not expose smokers to CO and failed to suppress tobacco abstinence symptoms as effectively as combustible products.

Conclusions While decreased toxicant exposure is a potential indicator of harm reduction potential, a failure to suppress abstinence symptoms suggests that currently marketed non-combustible PREPs may not be a viable harm reduction strategy for US smokers. This study demonstrates how clinical laboratory methods can be used to evaluate the short-term effects of non-combustible PREPs for smokers.

Chronic inhalation of tobacco cigarette smoke toxicants such as carbon monoxide (CO) and carcinogens causes disease and death.[1 2] Quitting smoking is the most effective way to avoid smoking-related diseases[3 4]; however, this is challenging because many smokers are nicotine dependent.[5] When these dependent smokers abstain, aversive abstinence symptoms (eg, anxiety, restlessness[6]) increase the likelihood of relapse.[7 8] Because relapse rates are high,[9 10] there is a growing interest in harm reduction approaches to preventing smoking-related diseases and death (eg, Stratton *et al*[11]).

For cigarette smokers, tobacco harm reduction may involve reducing exposure to cigarette smoke toxicants, perhaps with potential reduced exposure products (PREPs[12]). Currently marketed PREPs for smokers include cigarette-like combustible tobacco products, non-combustible tobacco products (ie, smokeless tobacco) and non-combustible, non-tobacco products that deliver pharmaceutically pure nicotine (eg, nicotine patch or gum). While PREPs are often marketed to reduce toxicant exposure, decades of experience with the so-called low-yield cigarettes, which decrease neither toxicant exposure nor smoking-related diseases, highlights the importance of measuring smokers' PREP-associated toxicant exposure.[11 13] Abstinence symptom suppression is also relevant: if a PREP cannot suppress aversive abstinence symptoms, smokers who try the PREP may relapse to cigarette use as they often do during a quit attempt.[14 15] Thus, tobacco toxicant exposure and abstinence symptom suppression are key PREP evaluation outcomes.

A growing body of literature demonstrates, at least for combustible PREPs, that the clinical laboratory methods are valuable for PREP evaluation. Clinical laboratory outcomes include nicotine and CO exposure, cardiovascular response and tobacco abstinence symptom suppression (eg, Breland and colleagues[16–18]). However, there have been no published clinical laboratory evaluations of the toxicant exposure and abstinence symptom suppressing effects of non-combustible PREPs in cigarette smokers. The present study was designed to explore the extent to which controlled clinical laboratory methods can be used to investigate the acute effects of non-combustible PREPs. We hypothesised that, relative to own brand cigarettes, non-combustible PREPs would expose users to lower levels of nicotine and CO, and suppress tobacco abstinence symptoms less effectively.

METHOD

Participants

Forty-eight participants provided informed consent and attended at least one session in this institutional review board-approved study. Seven of these participants were discontinued because venous access could not be maintained (n=4), a previously undisclosed chronic health condition was revealed during the first session (n=1), non-compliance with protocol occurred during session (n=1) or study recruitment goals had been reached (n=1). In addition, eight participants withdrew (ie, did not attend scheduled sessions; failed to respond to subsequent contact attempts): inspection of demographic characteristics and other information did not suggest a factor predictive of withdrawal

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from the protocol. Thirty-three individuals completed the study, and, through administrative error, five duplicated already-completed Latin square condition orders. Data from these five individuals were excluded from further analyses.

The 28 study participants (17 men, 14 non-white) were included if they were healthy, aged 18–55 years (mean 32.2 years (SD 10.1)), provided an afternoon screening expired air CO level of at least 15 ppm (mean 25.7 ppm (SD 10.7)), had a urine cotinine analysis result of at least 4 on a seven-point (0–6; NicAlert, Nymox Corp., Maywood, New Jersey, USA[19];) scale (mean 6 (SD 0)), and reported smoking at least 15 cigarettes/day (mean 22.4 (SD 7.5)) for at least 1 year (mean 10.8 years (SD 9.9)). Exclusion criteria consisted of history of chronic health problems; current pregnancy or breastfeeding; active menopause; current use of smokeless tobacco; currently attempting to quit smoking; or self-reported use of Marlboro Snus, Camel Snus or Ariva (ie, >1 pack).

Materials

Non-combustible products

Star Scientific Inc.'s Ariva (ARIVA) is a compressed tobacco tablet purported to be low in tobacco-specific nitrosamines (approximate weight 0.2 g). "Mint" was chosen arbitrarily as the flavour for this study. One tablet produces a maximal plasma nicotine concentration of approximately 2.7 ng/ml nicotine.[20 21]

R.J. Reynolds's Camel Snus (CS) is a pouch of pasteurised tobacco (approximate weight 0.3 g). "Original" was chosen arbitrarily for this study. CS was first marketed in select US cities in summer 2006, and R.J. Reynolds Tobacco Company provided product at that time (stored at −4°C). In spring 2008, a new version of CS was marketed. Relative to the 2006 version, the 2008 version is packaged differently and weighs more (approximate weight 0.4 g). Because the two CS versions may differ in other ways, this study compared them: 14 participants used the 2006 version and 14 used the 2008 version (obtained through retail outlets). The 2006 CS contains 28.2 mg/g dry weight total nicotine and 6.1 mg/g unprotonated nicotine (nicotine data for the 2008 CS are not yet available).[22]

Philip Morris Inc.'s Marlboro Snus (MS) is also a pouch of pasteurised tobacco (approximate weight 0.2 g). "Mild" was chosen arbitrarily for this study. MS contains 12.8 mg/g dry weight total nicotine and 0.35 mg/g unprotonated nicotine.[22] MS was obtained through retail outlets.

GlaxoSmithKline's Commit lozenge (COMMIT) is a pharmaceutical product marketed as a smoking cessation aid. The 2 mg (original flavour) lozenge was chosen for this study (maximal plasma nicotine concentration approximately 4.4 ng/ml[23]).

Combustible products

Participants' self-reported own brand of cigarettes were used in the own brand (OWN) and sham (SHAM; puffing on an unlit cigarette) conditions. According to the Federal Trade Commission method, on average, OWN yielded 1.1 mg nicotine (SD 0.3), 15.5 mg tar (SD 3.3) and 15.2 mg CO (SD 2.8). Vector Tobacco Inc.'s Quest (QUEST) is made from genetically modified low nicotine tobacco (Quest 3, nicotine=0.05 mg, menthol flavour if own brand was menthol).

Procedure

Participants completed seven, approximately 2.5 h sessions that differed by product used: ARIVA, CS, MS, COMMIT, OWN, SHAM, QUEST. Before a session could begin, overnight cigarette abstinence was verified (ie, expired air CO ≤10 ppm; see Breland *et al*[16]). Then, a catheter was inserted into a forearm vein and the session began (at time 0) with continuous physiological recording. Thirty minutes after session onset (time +30), participants responded to all subjective effect measures, 7 ml of blood was sampled and session-specific product was administered. Participants then responded to subjective measures, and 7 ml blood was sampled at 5, 15, 30 and 45 min post administration. CO was measured 15, 30 and 45 min post administration (reliable CO measurement occurs at least 5 min post smoking[24]). Sixty minutes after the first product administration (time +90), participants responded to the subjective measures, blood was sampled, product was administered again and the postadministration measurement/sampling schedule was repeated. The catheter was removed, participants were paid for their time and, if necessary, another session scheduled. Payment for completing the study was US$450.

Administration instructions

Opaque paper was used to mask brand identifiers on OWN, QUEST and SHAM. OWN and QUEST were smoked ad libitum. For SHAM, participants were asked to take 10 puffs approximately 20 s apart (similar to Breland *et al*[16]). For the non-combustible products, no brand information was revealed to participants; products were administered in unmarked containers. For CS and MS, participants were asked to place the pouch between their lip and gum for 15 min. For ARIVA and COMMIT, participants allowed the product to dissolve in their mouth without chewing or swallowing it.

Outcome measures

Physiological measures

Blood samples were centrifuged, plasma stored at −70°C and analysed for nicotine level (as in Breland *et al*[18]; limit of quantitation (LOQ)=2.0 ng/ml). Heart rate was measured every 20 s (Model 506, Criticare Systems, Waukesha, Wisconsin, USA). Expired air CO was assessed with a BreathCO monitor (Vitalograph, Lenexa, Kansas, USA).

Subjective measures

The Tiffany–Drobes Questionnaire of Smoking Urges Brief (QSU Brief) consists of 10 smoking-related items and has been empirically validated.[25] Participants rated each item on a seven-point scale ranging from 0 (strongly disagree) to 6 (strongly agree). The items form two factors: factor 1 (intention to smoke) and factor 2 (anticipation of relief from withdrawal). In addition, 35 items used a computerised visual analogue scale (VAS) to assess various subjective effects. A word or phrase was centred above a horizontal line anchored on the left with "Not at all" and on the right with "Extremely". Participants responded by moving a cursor to any point on the line and clicking, producing a vertical mark that could be adjusted if necessary. The score for each scale was the distance of the vertical mark from the left anchor, expressed as a percentage of total line length (see Breland *et al*[16]). Tobacco abstinence symptoms[6] were used to form 11 VAS items, which are presented verbatim in table 1. The 10 VAS items of the Direct Effects of Nicotine Scale are also presented in table 1 (see Evans *et al*[26]). The 14 VAS items of the Direct Effects of Tobacco scale were adapted from items reported in studies of smoking's subjective effects (eg, Foulds, Pickworth and colleagues[27 28]) by substituting the word "product" for "cigarette". The 14 items were "Was the product satisfying?", "Was the product pleasant?", "Did the product taste good?", "Did the product make you dizzy?", "Did the product calm you down?", "Did the product help you concentrate?", "Did the product make you feel more awake?", "[illegible] the

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Table 1 Statistical analyses results for all measures

	Condition (C)		Use (U)		Time (T)		C×U		C×T		U×T		C×U×T	
	F	p	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p	*F*	p
Physiological effects														
Plasma nicotine*	48.5	<0.001	44.6	<0.001	56.6	<0.001	10.3	<0.001	47.6	<0.001	3.5	<0.05	1.7	NS
Heart rate*	7.0	<0.001	35.8	<0.001	62.9	<0.001	2.3	<0.05	24.3	<0.001	18.0	<0.001	2.9	<0.001
Expired air CO†	59.7	<0.001	38.8	<0.001	116.4	<0.001	147.8	<0.001	156.4	<0.001	2.1	NS	1.9	NS
Subjective effects QSU brief*														
Factor 1	11.4	<0.001	11.8	<0.01	31.3	<0.001	3.1	<0.05	10.7	<0.001	2.1	NS	1.0	NS
Factor 2	4.9	<0.01	1.1	NS	8.7	<0.01	2.2	NS	4.4	<0.001	2.0	NS	1.4	NS
Hughes–Hatsukami*														
Urges to smoke	10.4	<0.001	6.1	<0.05	37.2	<0.001	1.2	NS	6.7	<0.001	4.3	<0.05	1.0	NS
Irritability/frustration/anger	2.6	<0.05	1.9	NS	7.2	<0.001	1.9	NS	1.6	NS	3.2	<0.05	1.5	NS
Anxious	2.5	<0.05	0.1	NS	7.4	<0.001	0.5	NS	0.8	NS	6.8	<0.01	0.7	NS
Difficulty concentrating	1.6	NS	0.0	NS	3.7	<0.05	1.5	NS	0.9	NS	1.4	NS	1.3	NS
Restlessness	1.4	NS	1.2	NS	6.6	<0.01	2.2	NS	1.1	NS	3.9	<0.05	0.9	NS
Hunger	1.3	NS	7.9	<0.01	5.8	<0.01	1.8	NS	1.5	NS	0.8	NS	1.2	NS
Impatient	3.2	<0.05	0.2	NS	6.2	<0.01	1.4	NS	1.5	NS	4.1	<0.01	1.2	NS
Craving a cigarette/nicotine	11.3	<0.001	9.6	<0.01	29.2	<0.001	2.9	<0.05	6.9	<0.001	5.8	<0.01	1.4	NS
Drowsiness	0.4	NS	0.1	NS	4.9	<0.01	1.5	NS	1.0	NS	3.6	<0.05	1.5	NS
Depression/feeling blue	0.8	NS	0.4	NS	0.8	NS	1.1	NS	1.1	NS	0.6	NS	1.2	NS
Desire for sweets	1.8	NS	0.5	NS	2.7	NS	2.0	NS	1.3	NS	2.8	<0.05	0.8	NS
Direct effects of nicotine*														
Nauseous	2.9	<0.05	0.0	NS	4.7	<0.05	1.8	NS	2.5	<0.05	1.9	NS	1.4	NS
Dizzy	2.1	NS	0.1	NS	4.4	<0.01	1.1	NS	1.6	NS	1.5	NS	2.3	<0.05
Lightheaded	1.7	NS	0.1	NS	5.0	<0.01	1.0	NS	2.3	<0.05	0.5	NS	1.2	NS
Nervous	0.7	NS	0.4	NS	1.1	NS	1.9	NS	1.5	NS	1.3	NS	1.1	NS
Sweaty	0.7	NS	1.9	NS	1.7	NS	1.1	NS	2.0	NS	1.1	NS	1.0	NS
Headache	1.6	NS	4.1	NS	2.2	NS	1.3	NS	2.1	<0.05	1.9	NS	1.0	NS
Excessive salivation	5.9	<0.001	0.8	NS	17.0	<0.001	1.5	NS	4.8	<0.001	2.8	<0.05	1.6	NS
Heart pounding	3.2	<0.05	0.9	NS	1.2	NS	0.5	NS	0.9	NS	1.1	NS	1.2	NS
Confused	2.4	NS	2.0	NS	0.7	NS	1.0	NS	0.9	NS	1.1	NS	0.6	NS
Weak	1.3	NS	2.9	NS	0.9	NS	0.7	NS	1.0	NS	1.0	NS	0.8	NS
Direct effects of tobacco*														
Satisfy	35.9	<0.001	31.1	<0.001	54.5	<0.001	4.4	<0.01	15.5	<0.001	30.7	<0.001	7.7	<0.001
Pleasant	30.9	<0.001	16.6	<0.001	59.2	<0.001	3.7	<0.01	13.5	<0.001	34.4	<0.001	8.7	<0.001
Taste good	24.9	<0.001	16.4	<0.001	50.6	<0.001	6.6	<0.001	9.5	<0.001	38.4	<0.001	6.6	<0.001
Dizzy	6.3	<0.001	0.5	NS	8.2	<0.001	2.5	<0.05	1.8	NS	11.0	<0.001	3.6	<0.01
Calm	11.3	<0.001	1.3	NS	26.0	<0.001	2.5	<0.05	5.1	<0.001	27.7	<0.001	3.7	<0.001
Help concentrate	9.5	<0.001	5.6	<0.05	13.8	<0.001	1.3	NS	3.2	<0.01	10.4	<0.001	2.4	<0.01
Feel awake	11.5	<0.001	1.8	NS	21.3	<0.001	0.6	NS	3.4	<0.001	17.5	<0.001	3.2	<0.01
Reduce hunger	3.3	<0.01	9.0	<0.01	14.6	<0.001	0.2	NS	1.7	NS	17.9	<0.001	2.1	<0.05
Feel sick	5.2	<0.01	1.3	NS	11.4	<0.001	0.6	NS	2.7	<0.05	6.8	<0.01	1.8	NS
Taste like own brand	55.8	<0.001	24.1	<0.001	32.1	<0.001	12.8	<0.001	22.9	<0.001	19.5	<0.001	16.3	<0.001
Feel like own brand	48.4	<0.001	22.3	<0.001	36.9	<0.001	8.3	<0.001	22.2	<0.001	23.3	<0.001	13.7	<0.001
Harsh as own brand	13.9	<0.001	11.9	<0.01	22.9	<0.001	4.1	<0.01	5.3	<0.001	17.5	<0.001	4.0	<0.001
Mild as own brand	34.6	<0.001	8.8	<0.01	33.6	<0.001	4.7	<0.01	11.1	<0.001	21.7	<0.001	9.8	<0.001
More of product right now	17.4	<0.001	0.7	NS	12.6	<0.001	0.8	NS	3.4	<0.001	40.1	<0.001	10.9	<0.001

*df_C=(6162); df_U=(1,27); df_T=(4108); $df_{C\times U}$=(6162); $df_{C\times T}$=(24 648); $df_{U\times T}$=(4108); $df_{C\times U\times T}$=(24 648).
†df_C=(6162); df_U=(1,27); df_T=(2,54); $df_{C\times U}$=(6162); $df_{C\times T}$=(12 324); $df_{U\times T}$=(2,54); $df_{C\times U\times T}$=(12 324).

product reduce your hunger for food?", "Did the product make you sick?", "Did the product taste like your own brand of cigarette?", "Did the product feel like your own brand of cigarette?", "Did the product feel as harsh as your own brand of cigarette?", "Did the product feel as mild as your own brand of cigarette?" and "Would you like more of the product RIGHT NOW?" (see Breland *et al*[18]).

Data analyses

Heart rate values were averaged for 5 min periods before each product administration or any blood sampling procedures. For plasma nicotine, results below the LOQ were replaced with the LOQ. In the event of missing data, an average of the value before and after the missing value was used (less than 0.07% of data were missing).

Plasma nicotine, subjective and physiological data were analysed initially using a mixed repeated measures analysis of variance where CS version (2006 or 2008) was entered as a between-subjects factor, and the three within-subjects factors were condition (seven levels; ARIVA, CS, MS, COMMIT, OWN, SHAM, QUEST) by use episode (two levels; first, second) by time (number of levels depended upon outcome measure). Of 320 main effects and interactions involving the between-subjects factor (CS version), only 6 were significant ($p<0.05$). Because these few significant results may reflect chance rather than real differences, the between-subjects factor was dropped and analyses were repeated using the within-subject factors

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only. Huynh–Feldt corrections were used to account for violations of sphericity,[29] and Tukey's Honestly Significant Difference[30] was used to explore differences between means ($p<0.05$).

RESULTS

Statistical analyses (main effects and interactions) for all measures are displayed in table 1. Interactions that involve the condition factor are most relevant as they indicate that the results observed differed across products and at least one other factor.

Physiological measures

As table 1 shows, significant condition by use episode and condition by time interactions were observed for plasma nicotine ($F>3.5$; $p<0.05$). As seen in figure 1A, relative to baseline (collapsed across all conditions, 2.4 ng/ml (SEM 0.2)), OWN was associated with significant increases in plasma nicotine level at nearly every time point. The greatest mean increase was observed 5 min after the first (mean 20.7 ng/ml (SEM 2.8)) and second (mean 20.6 ng/ml (SEM 2.0)) product administration. Neither QUEST nor SHAM increased plasma nicotine level reliably. Relative to baseline, mean CS plasma nicotine level was significantly greater 15 min after the second product administration (7.6 ng/ml (SEM 1.1)). At that same time point, mean plasma nicotine level for MS was 2.9 ng/ml (SEM 0.3), for ARIVA was 3.4 ng/ml (SEM 0.3) and for COMMIT was 4.6 ng/ml (SEM 0.5; all NS). Relative to mean plasma nicotine levels in the OWN condition, levels observed in all other conditions were significantly lower 5 and 15 min after the first, and 5, 15 and 30 min after the second product administration.

For heart rate, a significant condition by use episode by time interaction was observed ($p<0.001$). For OWN, mean baseline heart rate was 67.8 bpm (SEM 1.9), which increased significantly to 82.3 bpm (SEM 2.3) 5 min after the first product administration; it remained significantly elevated 15 and 30 min post administration. Significant increases relative to baseline also

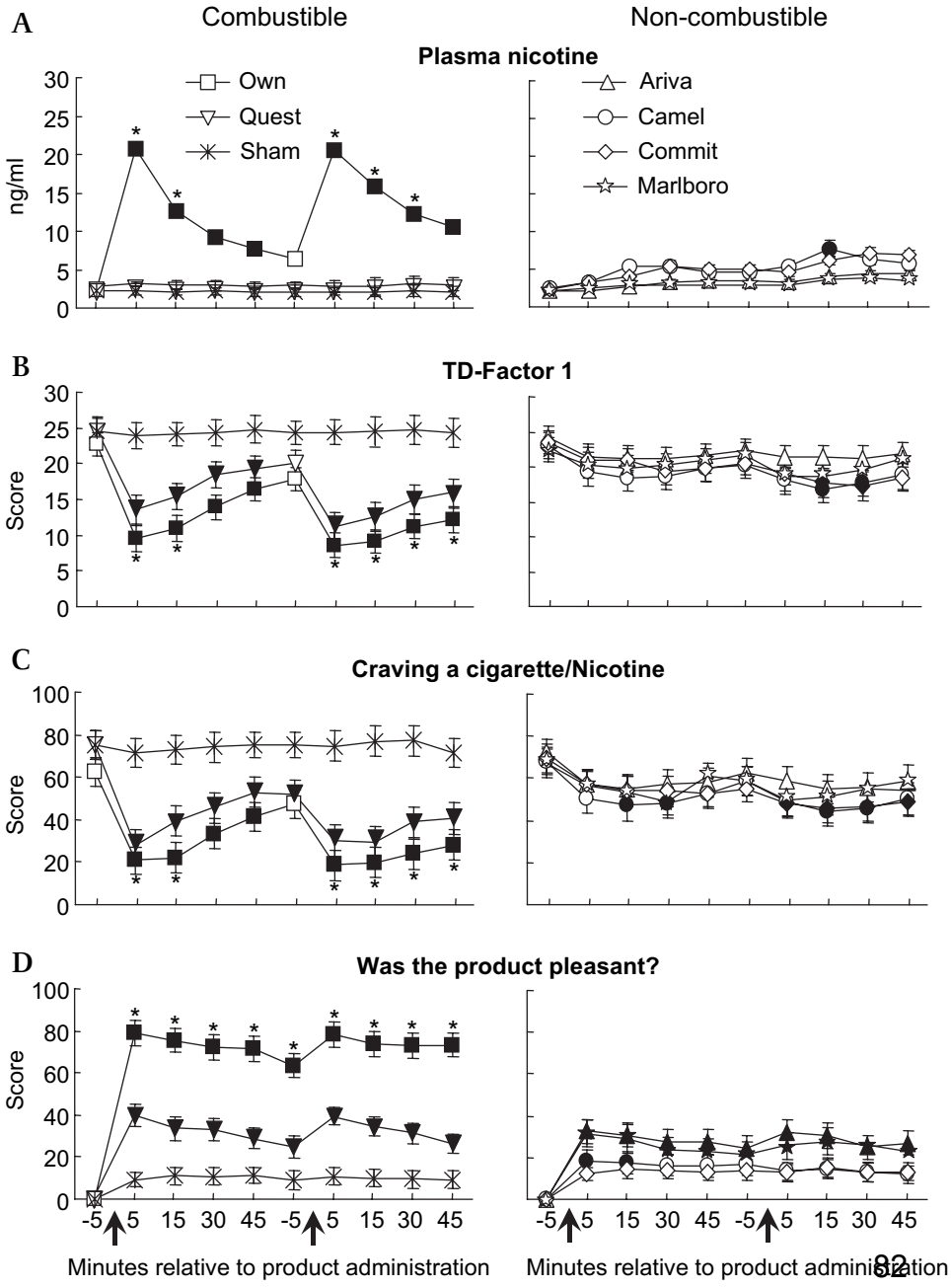


Figure 1 Mean data (±1 SEM) for plasma nicotine (A), factor 1 from the Tiffany Drobes QSU Brief (B), "Craving a cigarette/nicotine" from the Hughes–Hatsukami Withdrawal Scale (C) and "Was the product pleasant?" from the Direct Effects of Tobacco Scale (D) across conditions (n=28). Arrows indicate product administration, filled symbols indicate a significant difference relative to baseline, and asterisks (*) indicate a significant difference of OWN mean relative to all non-combustible product means at that time point ($p<0.05$, Tukey's Honestly Significant Difference).

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were observed 5, 15 and 30 min after the second administration. For QUEST, mean baseline heart rate was 68.7 bpm (SEM 2.1), which increased significantly to 73.1 bpm (SEM 2.1) 5 min after the first but not the second product administration. Mean heart rate in the SHAM condition was stable or decreased over both administration periods. For the non-combustible conditions, a significant increase in heart rate was observed during the CS condition; heart rate at baseline was 67.8 bpm (SEM 2.3), which increased significantly to 72.0 bpm (SEM 2.4) 15 min after the first product administration. No significant increases were observed for ARIVA, COMMIT or MS.

For CO, a significant condition by use episode interaction was observed ($p<0.001$). Relative to baseline (collapsed across all conditions, mean 6.5 ppm (SEM 0.5)), expired air CO increased in the OWN and QUEST conditions after the first (OWN, mean 12.8 ppm (SEM 0.7); QUEST, 11.0 ppm (SEM 0.6)) and second product administration (OWN, mean 17.5 ppm (SEM 1.0); QUEST, 14.4 ppm (SEM 0.8)). There were no significant changes in any other condition.

Abstinence symptom suppression and direct effects of nicotine

A significant condition by time interaction was observed for both factors of the QSU Brief ($F>4.4$, $p<0.001$). Figure 1B displays data for factor 1 (intention to smoke), which had the higher condition by time F value. Relative to baseline, OWN was associated with significant decreases at nearly every time point. A similar pattern was observed for QUEST. For SHAM, scores remained relatively high and stable. Factor 1 scores during non-combustible conditions tended to decrease after the second product administration only. Relative to baseline, scores in the CS condition were significantly decreased 15 min (mean decrease 6.3 (SEM 2.3)) and 30 min (mean decrease 5.4 (SEM 2.2)) after the second product administration. Similarly, for COMMIT, scores were decreased significantly relative to baseline at 15 min (mean decrease 5.9 (SEM 1.9)) and 30 min (mean decrease 6.3 (SEM 1.9)) after the second product administration ($p<0.05$). No significant differences were observed after ARIVA or MS. During all non-combustible conditions, factor 1 scores were significantly higher relative to OWN at 5 and 15 min after the first product administration and at 5, 15, 30 and 45 min after the second product administration.

Significant condition by time interactions were observed for "Urges to smoke" and "Craving a cigarette/nicotine" (see table 1; $F>6.7$, $p<0.001$). Figure 1C shows the results for "Craving a cigarette/nicotine", the item with the larger F value. For this item, OWN was associated with significant decreases in craving scores relative to baseline at nearly every time point. QUEST was associated with a similar pattern, while mean craving scores during SHAM were relatively high and stable over both administrations. For the non-combustible products, relative to baseline, scores in the CS condition were significantly decreased 15 min (mean decrease 20.9 (SEM 6.5)) and 30 min (mean decrease 20.0 (SEM 5.5)) after the first product administration and 5 min (mean decrease 19.0 (SEM 6.0)), 15 min (mean decrease 23.9 (SEM 6.1)) and 30 min (mean decrease 22.1 (SEM 6.0)) after the second product administration. For COMMIT, mean craving score decreased significantly at every time point after the second product administration. For MS, craving decreased significantly only once, 30 min after the first product administration, while ratings in the ARIVA condition never decreased significantly. During all non-combustible conditions, craving scores were significantly higher relative to OWN at 5 and 15 min after the first product administration and at 5, 15, 30 and 45 min after the second product administration.

A significant condition by use episode by time interaction was observed for the Direct Effects of Nicotine item assessing "Dizzy", while significant condition by time interactions were observed on items assessing salivation, nausea, headache and heart pounding ($F>2.1$, $p<0.05$). The three-way interaction for the "Dizzy" item is explained by an increase from baseline in the OWN condition at the 5 min time point after the first administration (mean increase 12.3 (SEM 5.5); NS) that was not observed after the second administration and was also not observed in any other condition. For salivation, all non-combustible products (but no combustible products) produced a significant increase relative to baseline. For nausea, no products produced significant increases relative to baseline; however, after the first administration of ARIVA or CS, there was a trend towards increased ratings 5 min after product administration (ARIVA mean increase 11.9 (SEM 5.5); CS mean increase 14.5 (SEM 5.2); NS).

Direct effects of tobacco

Significant condition by use episode by time interactions were observed for most Direct Effects of Tobacco items (table 1; $F>2.1$, $p<0.05$). Four of these items related to own brand cigarette smoking (eg, "Did the cigarette taste like your own brand of cigarette?"). Across these items, scores during the OWN condition were consistently higher than other conditions. For example, for the item "Does the product taste like your own brand?" in the OWN condition, the mean score at baseline was 0.3 (SEM 0.3), which increased significantly to 67.0 (SEM 6.9) 5 min after the first product administration. For QUEST, the mean baseline score was 0.1 (SEM 0.1), which increased to 11.1 (SEM 3.4) 5 min after the first product administration (NS). Among non-combustible products, no significant increases were observed for this item at any time point after the first product administration.

The other items on this scale concerned perceptions on a variety of dimensions (eg, taste, satisfaction). Figure 1D presents the responses to one such item, "Was the product pleasant?". Relative to baseline, scores on this item increased significantly for all time points after the first product administration in the OWN, QUEST, ARIVA and MS conditions, with significant increases in the CS condition at 5 and 15 min after the first product administration and no significant increases observed in SHAM or COMMIT. Relative to OWN, mean scores on this item were significantly lower for all other conditions across all postadministration time points. This general pattern of results was also observed on items assessing "Was the product satisfying?" and "Did the product taste good?". For items assessing product effects on ratings of "calm you down", "help you concentrate", "reduce hunger", "more awake" and "more product right now", significant increases were observed for OWN, with fewer or no significant increases relative to baseline observed in any other non-combustible conditions.

DISCUSSION

The clinical laboratory methods used to evaluate the toxicant exposure and subjective effects associated with acute exposure to combustible PREPs (eg, Breland, Strasser and colleagues[16 17 31]) were adapted in this study to evaluate non-combustible PREPs for smokers. This study demonstrated the importance of controlled laboratory evaluation. For example, including an own brand condition demonstrated the dose of nicotine to which smokers are accustomed as well as the rapidity of its delivery: on average, 5 min after beginning to smoke a single own brand cigarette, participants' mean plasma nicotine level

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increased by approximately 18 ng/ml. Non-combustible PREPs included in this study delivered a nicotine dose an order of magnitude less, and significant increases were observed later, if at all (ie, 15 min after the second product administration; see figure 1A). The own brand condition also revealed the usual level of CO exposure (about 6 ppm on average) and, as might be expected, no non-combustible PREP increased CO.

This study also highlighted the importance of assessing the PREP subjective effects and compared them to positive (OWN) and negative (SHAM) controls. For example, on measures of tobacco abstinence effects, OWN demonstrated the magnitude of suppression a smoker might expect a PREP to deliver, while SHAM demonstrated a failure to suppress abstinence (see figure 1B and C). Several non-combustible PREPs produced reliable suppression at some time points among several measures of abstinence symptomology (eg, QSU Brief factor 1, "Craving a cigarette/nicotine", "Urges to smoke"), although OWN always produced suppression of greater magnitude. PREPs that fail to suppress abstinence effectively are unlikely to substitute for normally marketed cigarettes completely, and thus may have limited harm reduction potential.[15 18]

Another factor that may limit the likelihood that a PREP will substitute completely for normally marketed cigarettes involves acceptability—the extent to which the product provides sensory characteristics that are pleasant and/or match those provided by a smoker's usual brand of cigarettes. In this study, in virtually every measure of acceptability, non-combustible PREPs were closer to the lower limit (ie, SHAM) than the upper limit (ie, OWN). When PREPs are unable to deliver nicotine, suppress abstinence symptoms and satisfy sensory demands as effectively as a smoker's usual brand of cigarettes, their potential as instruments of harm reduction for tobacco users is very uncertain.

In addition to offering experimental control and a variety of relevant outcome measures, clinical laboratory methods for PREP evaluation are reliable and adaptable. The reliability of these methods has been noted elsewhere[17] and is evidenced here by the similarity of results across studies that used the same outcome measures and control conditions.[14 16 17] The adaptability of these methods is evidenced by the fact that they are demonstrably useful for evaluating combustible PREPs for smokers,[14 16–18] and non-combustible PREPs for smokeless tobacco users[32] and smokers (the present study). In addition, our ability to test two versions of CS in this study demonstrates how the methods might be used to respond to changes in PREP design, although a within-subjects manipulation would likely have greater power than the relatively insensitive between-subjects factor reported here.

An acute study limits assessment of how a longer PREP experience influences study outcomes as well as PREP-induced changes in carcinogen exposure. However, evaluating the short-term effects of PREP exposure remains relevant because, after trying a particular PREP a few times, smokers must choose to purchase that PREP or their usual brand of cigarettes. That choice may be guided by the knowledge that, in the short-term, the PREP failed to suppress abstinence symptoms and satisfy sensory factors.

In conclusion, this study demonstrates how clinical laboratory methods can be used to evaluate the short-term effects of non-combustible PREPs for smokers. Results suggest that while these non-combustible products do not expose smokers to CO, they also deliver less nicotine than own brand cigarettes and fail to suppress tobacco abstinence symptoms effectively. Indeed, the subjective effects observed in this study do not support the notion that, as presently formulated, non-combustible PREPs for smokers will be a viable harm reduction strategy for the population from which this sample was drawn (ie, US tobacco cigarette smokers). Comprehensive premarket PREP evaluation using established methods and representative samples in the context of a regulated and iterative process designed to minimise toxicant exposure and maximise abstinence symptom suppression may be the most productive method for realising the public health potential of PREPs for tobacco users.

What this paper adds

- The tobacco industry has begun marketing to smokers orally administered, non-combustible potential reduced exposure products (PREPs; eg, compressed tobacco tablets; small pouches of tobacco). Few studies describe the effects of these PREPs in smokers. Careful evaluation of these and other PREPs is essential, given that previous industry-sponsored PREPs (ie, low-yield cigarettes) reduced neither smokers' exposure to tobacco-delivered toxicants nor tobacco-associated mortality.
- This study adapted methods used to examine combustible PREPs to assess the acute effects of these non-combustible PREPs for smokers. The non-combustible products delivered less nicotine than own brand cigarettes, did not expose smokers to CO and failed to suppress tobacco abstinence symptoms as effectively as combustible products. While decreased toxicant exposure is a potential indicator of harm reduction potential, a failure to suppress abstinence symptoms suggests that currently marketed non-combustible PREPs may not be a viable harm reduction strategy. This study demonstrates how clinical laboratory methods can be used to evaluate the short-term effects of non-combustible PREPs for smokers.

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Short Communication

Digital Image Analysis of Cigarette Filter Stains as an Indicator of Compensatory Smoking

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Abstract

Objective: **Cigarette filters trap a significant portion of carcinogen-containing smoke particulate and may provide an indication of cigarette constituent exposure. A technique for quantifying filter tar staining with digital imaging has shown predictive value between typical total puff volume and filter tar stain intensity. The current study uses smoking topography data acquired during an examination of compensatory smoking of Quest cigarettes and digital analyses of the tar stains of the spent Quest cigarette filters. Due to reduced nicotine levels, we hypothesized compensatory smoking to occur. The purposes of the current study were to describe the physical characteristics of the Quest cigarettes, to further validate the effect of puff volume on filter tar staining, and to examine the effect of compensatory smoking on changes in filter tar staining, hypothesizing that compensatory smoking would result in more intense tar staining.**

Methods: **Physical characteristics of the Quest cigarettes were measured to characterize the product. Spent cigarette filters were digitally analyzed for color intensity features, matched to smoking topography measures, and examined in regression models. Difference scores for digital imaging and smoking topography were used in a regression model to identify the effect of compensatory smoking on tar stain.**

Results: **Total puff volume was a significant predictor of a*center (redness) [β = 0.003 (SE, 0.0004), R^2 = 0.42, t = 7.87, P < 0.001] and L*center (lightness) [β = −0.015 (SE, 0.002), R^2 = 0.45, t = −8.18, P < 0.001] for Quest cigarettes and a significant predictor of a*center [β = 0.003 (SE, 0.0005), R^2 = 0.37, t = 5.27, P < 0.001] and L*center [β = −0.009 (SE, 0.002), R^2 = 0.35, t = −5.05, P < 0.001] for own preferred brand. Regression models indicate total puff volume difference was a significant predictor of L*center difference [β = −0.01 (SE, 0.003), R^2 = 0.171, t = −3.15, P = 0.003] and approached significance for a*center difference [β = 0.002 (SE, 0.001), R^2 = 0.057, t = 1.99, P = 0.053].**

Conclusion: **Results suggest that compensatory smoking may be detectable by darkening and reddening of the tar stain. This type of measure could be useful in quantifying the extent to which human smokers smoke differently than standard testing protocols and in assessing the prevalence of compensatory smoking in population samples.** (Cancer Epidemiol Biomarkers Prev 2006;15(12):2565–9)

Introduction

There is substantial variation in how people smoke cigarettes (1), including the type of cigarettes smoked, the number of cigarettes smoked daily, and how each individual cigarette is smoked, including number of puffs, puff volume, duration, and velocity (collectively referred to as smoking topography). Nicotine delivery drives dependent smokers' smoking topography (2, 3), but topography also affects exposure to the harmful compounds in cigarette smoke (4-7). Evidence of nicotine-driving smoking behavior is seen in brand-switching studies, where smokers are switched to lower nicotine cigarettes (8), and in cigarette reduction studies, where smokers smoke fewer allotted daily cigarettes more intensely to obtain nicotine (4).

The effect of smoking behavior on cigarette smoke toxin exposure has been shown by measuring carbon monoxide boost (9, 10) and carcinogen levels (6, 7, 11). A limitation of some biomarkers, particularly those measured in urine and saliva, is that they are not sensitive to the smoking of individual cigarettes. Knowing the effect of smoking behavior at the per-cigarette level is important because, as Harris (12) showed, smoke constituent compounds do not increase at the same rates during compensatory smoking. Although Bernstein (13) reported that puff volume has no effect on size of particulate matter capable of reaching the lung, others have suggested that puffing can affect which products are generated within the tobacco rod (14, 15). Furthermore, Hecht et al. (6, 7) have shown that when number of cigarettes smoked per day is restricted, carcinogen biomarkers do not decrease in a manner proportionate with the reduced number of cigarettes, showing that compensatory smoking at the per-cigarette level can minimize any perceived harm reduction in smoking fewer cigarettes. It is therefore reasonable to postulate that smoking more intensely may not only expose a smoker to greater quantities of toxin but also to different proportions and types of toxicants.

Cigarette filters trap a significant portion of the smoke particulate matter during smoking and, therefore, may provide an indication of particulate exposure to the smoker (16, 17). Kozlowski (16) first reported a strong positive correlation between number of puffs and intensity of tar stain using a visual scoring mechanism. Others have reported on more subtle smoking behaviors and tar stains (18, 19) as well as on the effect of blocking filter vents on tar staining (20).

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O'Connor et al. have refined a technique for quantifying filter tar staining with digital imaging (21, 22) that has shown predictive value discriminating between blocked and unblocked filter vents. Recently, O'Connor et al. showed a relationship between smokers' typical total puff volume and filter tar stain intensity (23) using the CIELAB color system (24). A limitation noted by the authors was the inability to link smoking topography measures to specific cigarette filters. Mean smoking topography measures were compared with mean tar stain measures. In addition, variability in cigarette type and ventilation levels required analyses of both the edge and core of the filter stain.

A new type of potential reduced exposure product, Quest, uses genetically modified tobacco to provide three ''steps'' of gradually reduced nicotine. Unlike Light cigarettes, because the nicotine content of the rod itself is reduced, smoking Quest cigarettes more intensely should not allow the smoker to extract additional nicotine. Yet, smokers are often unaware of the complexities of cigarette design and may continue to attempt to extract nicotine by increased puffing behavior. A recent publication has reported that smokers initially increase their total puff volume when smoking the lowest-nicotine (0.05 mg) Quest cigarette compared with the highest-nicotine (0.6 mg) Quest cigarette and, subsequently, have a significant increase in carbon monoxide boost (25). The current study uses the smoking topography data from the Quest cigarettes and digital analyses of the tar stains of the spent Quest cigarette filters. The purposes of the current study were to (*a*) describe the physical characteristics of the Quest cigarettes; (*b*) to further validate the effect of puff volume on filter tar staining, by linking smoking topography data to specific cigarette filters; and (*c*) to examine the effect of compensatory smoking on changes in filter tar staining, hypothesizing that compensatory smoking would result in more intense tar staining.

Materials and Methods

Participants and Procedures. Participants were recruited via community-based flyers, and eligibility was determined by telephone. Eligibility requirements included being over age 18, report smoking at least 10 daily cigarettes, smoking for a minimum 5 years, not currently trying to quit smoking, report inhaling when smoking, not currently using nicotine replacement therapies, and not consuming >25 alcohol-containing drinks per week. Procedures are provided in detail elsewhere (25), but briefly, participants smoked four cigarettes using the Clinical Research Support System (CReSS) desktop smoking topography machine (Plowshare, Baltimore, MD). The first cigarette was always their own preferred brand cigarette followed by Quest 1 (0.6 mg nicotine), Quest 2 (0.3 mg nicotine), and Quest 3 (0.05 mg nicotine) cigarettes. Quest cigarette nicotine level was blinded to researcher and participant, and order of presentation was randomized and counterbalanced across participants to minimize order effects. All cigarettes were smoked 30 minutes apart. Used cigarette filters were stored at −80°C in labeled plastic re-sealable bags until analysis. Fifty participants was determined as an appropriate sample size based on effect sizes (ES = 0.4) from previous smoking topography studies (5, 9), with α set to 0.05 for a two-tailed test, and power >80% to detect between-cigarette nicotine level differences in total puff volume. The study was approved by the University Institutional Review Board.

Measures

Smoking Topography. Cigarettes were smoked using the CReSS smoking topography machine (Plowshare). The smoking topography device employs a pressure transducer that measures pressure changes during puffing. Pressure changes are amplified, digitized, and sampled at 1,000 Hz, and software converts signal to airflow (mL/s) in real time (s), which is subsequently converted to data. Cigarettes are placed in a sterilized mouthpiece during smoking. This device has been previously used in our laboratory to assess smoking behavior (5, 9), has been shown to be a valid and reliable means to measure smoking behavior (26), and was calibrated before each session.

Imaging Techniques. The specifics of the imaging system and technique are described in greater detail elsewhere (22, 23). In brief, the system involves using a color CCD digital camera (DFW-x710, Sony USA, New York, NY) to capture a 1024 × 768 pixel resolution image of a cigarette butt (mouth end) and then processing that image to extract color information from the center of the spent filter, as well as a 7-pixel-wide ring around the outside of the filter. All image processing and analysis is done using MATLAB (The Mathworks, Natick, MA). Red, Green, Blue (RGB) values captured by the camera are converted to the CIELAB color space (L* or lightness, a* or red-green, and b* or yellow-blue) using standard formulas (see ref. 24). Each filter imaged then receives six imaging values: L*, a*, and b* each at the center and the edge of the filter.

Physical Characteristics of Cigarettes. New Quest cigarettes were assessed for physical characteristics, including total cigarette length, diameter, tobacco rod length, filter length, tipping paper length, tobacco mass, filter mass, filter components length and mass, pressure drop, and ventilation. Lengths were measured using digital calipers (VWR, Philadelphia, PA); mass was assessed using a digital analytic balance (AB-104s, Mettler-Toledo, Columbus, OH); and pressure drop and ventilation was measured using a KC-3 apparatus (Borgwaldt-KC, Richmond, VA). Results are in Table 1.

Analytic Plan. Participants, Quest cigarettes, smoking topography measures, and CIELAB data were characterized using descriptive statistics. We hypothesized that L*center score, a measure of lightness, and a*center, a measure of redness to greenness, would be most responsive to total puff volume, as observed previously (17, 23). We addressed these questions using (linear) regression methods, using the robust variance estimate to account for the repeated measures. Model 1 examined the effect of total puff volume on L*center and a*center which first examined for the Quest cigarettes only, controlling for cigarette nicotine level. Model 2 examined the effect of total puff volume on L*center and a*center using the participant own brand cigarettes, which varied substantially in standard tar and nicotine yield, as well as design

Table 1. Descriptive characteristics of Quest cigarettes

	Quest 1	Quest 2	Quest 3
Reported nicotine level (mg)	0.6	0.3	0.05
Reported tar level (mg)	10.0	10.0	10.0
Cigarette length (mm)	82.5	82.8	82.2
Cigarette diameter (mm)	7.6	7.8	7.9
Tobacco rod length (mm)	57.6	57.8	57.5
Tobacco mass (g)	0.60	0.57	0.57
Tipping paper length (mm)	30.0	30.1	30.0
Filter length (mm)	24.9	24.8	24.7
Filer length, mouth end (mm)	12.1	12.0	12.5
Filter length, rod end (mm)	8.0	7.9	7.6
Total filter mass (g)	0.23	0.22	0.24
Filter mass, mouth end (g)	0.071	0.073	0.072
Filter mass, rod end (g)	0.068	0.068	0.065
Filter carbon mass (g)	0.100	0.102	0.108
Pressure drop (mm H_2O)	111.1 (1.1)	101.5 (1.4)	115.8 (0.9)
Ventilation (%)*	1.1 (0.1)	1.4 (0.1)	1.3 (0.04)

NOTE: Pressure drop and ventilation are measured on 20 sticks of a freshly opened pack. Physicals are measured on five rods from freshly opened pack. Weights derived from combined mass of five rods per filter divided by five.
*The readability of the ventilation tester is ± 1%; thus, these are essentially 0.

Table 2. Smoking topography and tar stain image values by cigarette type and overall

	Quest 1	Quest 2	Quest 3	Own brand	Overall
Total puff volume (mL)	540.6 (20.5)	518.1 (20.6)	570.5 (22.2)	827.1 (50.9)	615.4 (17.5)
Mean puff velocity (mL/s)	34.5 (1.3)	34.4 (1.3)	35.1 (1.4)	35.6 (1.2)	34.9 (0.63)
a*center	0.51 (0.1)	0.56 (0.1)	0.99 (0.1)	1.92 (0.2)	0.98 (0.08)
a*edge	−0.03 (0.1)	0.17 (0.1)	0.27 (0.1)	0.13 (0.1)	0.134 (0.04)
b*center	20.7 (0.3)	20.0 (0.3)	21.0 (0.3)	21.6 (0.2)	20.8 (0.14)
b*edge	18.3 (0.3)	18.3 (0.3)	18.7 (0.3)	16.3 (0.6)	17.9 (0.20)
L*center	74.4 (0.4)	74.4 (0.5)	72.3 (0.5)	69.2 (0.7)	72.6 (0.3)
L*edge	76.1 (0.4)	75.7 (0.5)	74.6 (0.5)	75.8 (0.7)	75.6 (0.3)

NOTE: Data are presented as mean (SE). Total puff volume refers to the sum of all puffs taken; mean puff velocity is defined as the mean of all velocities obtained for each puff. a* refers to redness/greenness color space measure; b* refers to the yellowness/blueness color space measure; L* refers to lightness color space measure. Center refers to the central core of the cigarette filter and edge refers to the outer edge of the cigarette filter.

characteristics, such as ventilation. Analyses were repeated (models 3 and 4) using mean puff velocity as the predictor for tar staining. Puff velocity has predicted tar staining during machine-based smoking simulation (17) and with other biomarkers (9). Finally, difference scores were calculated between the highest and lowest nicotine Quest cigarettes for total puff volume, L*center (lightness) and a*center (redness/greenness), to be used as measures of compensatory smoking. Models 5 and 6 used puff volume changes to predict tar stain changes.

Results

Descriptive Statistics. Fifty participants (54% male) completed the laboratory session, thus providing 200 cigarette filters. Three filters of participant own brand could not be digitally analyzed due to filter damage. Participant characteristics are presented elsewhere in extensive detail (25), but briefly, participants reported smoking about a pack per day (21.3; SD, 8.1; range, 10-40) and had moderate nicotine dependence scores 5.5 (SD, 1.9; range, 2-10; FTND; ref. 27). Fifty percent of participants usually smoked a Regular brand cigarette, 48% smoked Light brand cigarettes, and 2% Ultra-Light brand cigarettes.

In general, the three versions of Quest were nearly identical in terms of cigarette construction. Unlike virtually all other Light cigarettes, none of the Quest cigarettes had measurable filter ventilation (indeed, no holes are visible under a ×5 magnification lens). This meant that uniform smoke flow through the filter could be expected, and center scores would be sufficient to examine. The ratios of edge scores to center scores tended to be around 1.

Mean total puff volume was 615.43 mL (SE, 17.5), and mean puff velocity was 34.92 mL/s (SE, 0.63). Mean total puff volume and mean puff velocity for each Quest cigarette level and participant own brand are reported in Table 2. Mean L*center (lightness) score was 72.65 (SE, 0.32), and mean a*center (redness/greenness) score was 0.98 (SE, 0.08). L*center and a*center values for each Quest cigarette level and participant own brand are reported in Table 2.

Regression Models: Smoking Topography and Filter Stains

Model 1: Total Puff Volume and Tar Stain (Quest Cigarettes). When controlling for Quest cigarette nicotine level, total puff volume was a significant predictor of a*center (redness/greenness) [β = 0.003 (SE, 0.0004), R^2 = 0.42, t = 7.87, P < 0.001] and L*center (lightness) [β = −0.015 (SE, 0.002), R^2 = 0.45, t = −8.18, P < 0.001].

Model 2: Total Puff Volume and Tar Stain (Own Brand Cigarettes). In a model using own brand cigarette data, total puff volume was a significant predictor of a*center (redness/greenness) [β = 0.003 (SE, 0.0005), R^2 = 0.37, t = 5.27, P < 0.001] and L*center (lightness) [β = −0.009 (SE, 0.002), R^2 = 0.35, t = −5.05, P < 0.001].

Model 3: Mean Puff Velocity and Tar Stain (Quest Cigarettes). When controlling for Quest cigarette nicotine level, mean puff velocity was not a significant predictor of a*center (redness/greenness) [β = −0.012 (SE, 0.012), R^2 = 0.10, t = −1.04, P = 0.303] or L*center (lightness) [β = 0.074 (SE, 0.054), R^2 = 0.11, t = 1.39, P = 0.171].

Model 4: Mean Puff Velocity and Tar Stain (Own Brand Cigarettes). In a model using own brand cigarette data, mean puff velocity was not a significant predictor of a*center (redness/greenness) [β = 0.006 (SE, 0.027), R^2 = 0.02, t = 0.22, P = 0.831] or L*center (lightness) [β = 0.020 (SE, 0.090), R^2 = 0.02, t = 0.22, P = 0.828].

Models 5 and 6: Compensatory Smoking. We assessed compensatory smoking by first creating difference scores between the greatest-nicotine level Quest cigarette and the lowest-nicotine level Quest cigarette. Total puff volume difference was defined as the total puff volume obtained during smoking of the 0.6 mg nicotine cigarette minus total puff volume obtained during smoking of the 0.05 mg nicotine cigarette, whereby negative values would indicate compensatory smoking for the lower nicotine level cigarette. L*center difference was defined as L*center score for 0.6 mg nicotine cigarette minus L*center score for 0.05 mg nicotine cigarette, whereby a positive value would indicate a darkening of the tar stain. a*center difference was defined as a*center score for 0.6 mg nicotine cigarette minus a*center score for 0.05 mg nicotine cigarette, whereby a positive value would indicate a reddening of the tar stain.[4] Regression models indicate total puff volume difference was a significant predictor of L*center (lightness) difference [β = −0.01 (SE, 0.003), R^2 = 0.171, t = −3.15, P = 0.003] and approached significance for a*center (redness/greenness) difference [β = 0.002 (SE, 0.001), R^2 = 0.057, t = 1.99, P = 0.053). These results suggest that compensatory smoking may be detectable by darkening (L*center) and reddening (a*center) of the tar stain (Fig. 1).

Discussion

This study is the first to match participant smoking topography to tar stain patterns that have been digitally analyzed. Furthermore, this study used filters that seem to be nearly identical on several physical characteristics that have been shown to affect smoking topography and the pattern of tar stains (5, 22). Total puff volume was a significant predictor of a*center (redness/greenness) and L*center (lightness), such that increases in total puff volume lead to increased reddening

[4] Because a*center scores range from −128 to +128, a*center (0.6 mg) and a*center (0.5 mg) scores were rescaled by adding 128.

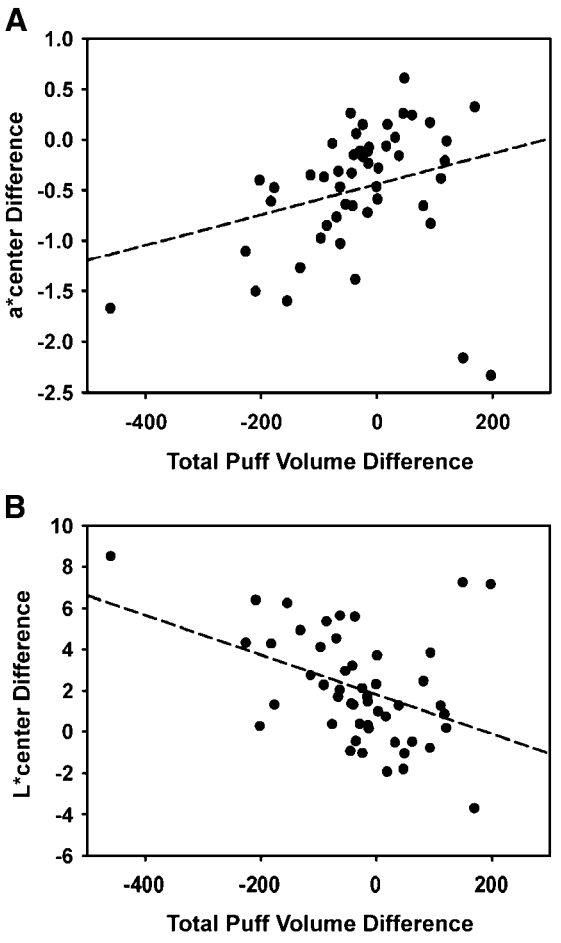


Figure 1. The effect of compensatory smoking on a*center (redness/greenness) difference score (**A**) and L*center (lightness) difference score (**B**).

and darkening of the tar stain. However, mean puff velocity was not a significant predictor of any of the digital imaging values. Because previous research of varying puffing variables, including puff velocity, had been shown to affect tar stain intensity, we had hypothesized to observe similar results. The lack of association may be attributable to the use of mean puff velocity values as opposed to identical, repeating puff velocity values, as used in prior research using machine-driven syringes to mimic puff behavior (17, 28).

The physical characteristics of the Quest cigarettes were similar across the three nicotine levels. Quest cigarette size was most similar to king size (85 mm) cigarettes: none of the filters seem to be ventilated, and all are similarly constructed for each of the three nicotine levels; pressure drop, a measure of draw resistance, was comparable across the three nicotine levels. Thus, the most plausible explanation for observed differences in tar staining would be differential puffing on the cigarettes.

Changes in a*score and L*score were related to changes in total puff volume between the highest-nicotine level Quest cigarette and the lowest-nicotine level Quest cigarette. Results suggest that a darker more red tar stain results from compensatory smoking. Hence, relative changes in the intensity of tar staining (particularly darkening and reddening) may be useful as an indicator of per-cigarette compensatory smoking. A measure such as this could be useful for a number of applications, including quantifying the extent to which human smokers smoke differently than smoking machines. For example, butts from a cigarette smoker could be compared with the same brand smoked under FTC/ISO conditions, and the relative difference in color would serve as a compensation index. This measure is sensitive to relatively small changes in color, by virtue of using the CIELAB system (cf. 24), meaning that the imaging analysis could reveal compensation where the human eye might not discern a color difference.

The value of digital imaging as a technique for assessing compensatory smoking can be illustrated by a few examples, based on the generated regression equations. An a*score difference of −0.441 would be indicative of zero compensation, and any value greater would indicate compensation. An a*score difference of −0.511 would mean compensation of 35 mL occurred, the volume of a puff during standardized cigarette testing. An L*score difference of 1.814 would suggest no compensation, and any value greater would indicate compensation. An L*score difference score of 2.164 would suggest 35 mL greater volume taken when smoking the 0.05 mg nicotine cigarette.

Another application of our results could pertain to reduced-cigarette smoking studies. Some smokers may attempt to reduce harm exposure by smoking fewer daily cigarettes. However, research suggests that the decrease in number of daily cigarettes does not produce equivalent decreases in carcinogen biomarkers (6, 7). The discrepancy between cigarette reduction and reduced harm exposure is most likely due to compensatory smoking, which can allow a smoker to extract more nicotine and, consequently, more tar from low-yield cigarettes. Comparison of tar stain intensity and pattern may provide support for evidence of compensatory smoking. Disease implications from compensatory smoking during reduced smoking are evident in epidemiologic results of lung cancer diagnoses in those who reduce number of daily cigarettes versus those who do not reduce and those who successfully quit smoking (29).

Tar contains many chemicals and has been used as an indicator of general particulate exposure. Some of the components in tar are known carcinogens (30), and the relationship between variations in smoking topography and resultant carcinogen exposure has been well shown (11, 17). However, tar is not homogeneous, and its correlation with carcinogens varies (31). Harris (12) showed that if smokers compensate to obtain equivalent levels of nicotine from a lower-nicotine cigarette, there is increased exposure to several carcinogens relative to the higher-nicotine cigarette. In addition, these carcinogens do not increase uniformly. Therefore, linking smoking topography and tar staining to multiple biomarkers of carcinogens would be preferred, but many biomarkers are not sensitive to single smoking sessions due to long half-lives (32).

Other potential methods for assessing compensatory smoking exist, including flow meter devices such as CReSS (or its portable version CReSSMicro), or filter analysis of solanesol (33). Measurement of puffing variables requires using a device that is not typically used during normal behavior, and their cost precludes use on a large scale for epidemiologic studies. Filter solanesol is a promising method but is technically complicated, expensive, and requires wet laboratory facilities and trained chemists. The imaging technique could be done in most office or laboratory facilities with minimal training and cost. The literature would benefit from comparative studies of topographic, chemical, and image-based measures of compensation to elucidate relative strengths and weaknesses.

A limitation of this study is the inability to link per-cigarette estimates of compensatory smoking to multiple biomarkers of exposure. Further studies are planned to examine the link between average daily smoke intake and average cotinine levels in smokers. In addition, because Quest is a unique cigarette, these results may not replicate across more traditionally designed brands (including those with filter vents). In addition, results are limited by participants smoking only one cigarette at each nicotine level. Ongoing work is examining the measurement of compensatory smoking in popular U.S. cigarette brands using the technique described here.

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Short communication

New lower nicotine cigarettes can produce compensatory smoking and increased carbon monoxide exposure

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Abstract

Potential reduced exposure products (PREPs) are marketed as a means to reduce exposure to tobacco toxicants. Quest® cigarettes, a new type of PREP, use genetically modified tobacco to provide a nicotine step-down approach, and are available as 0.6, 0.3 and 0.05 mg nicotine cigarettes. However, these cigarettes deliver equivalent levels of tar (10 mg). Prior research on low nicotine cigarettes suggests smokers will compensate for lower nicotine delivery by increasing their puffing behavior to extract more nicotine. This study tested the hypothesis that compensatory smoking will occur with this PREP as nicotine levels decrease, increasing exposure to tobacco toxins. Fifty smokers completed a within-subject human laboratory study investigating the effect of cigarette nicotine level on smoking behavior. Cigarette nicotine level was double-blinded and order of presentation counter-balanced. Breath carbon monoxide (CO) boost was used as a biomarker of smoke exposure; total puff volume to assess smoking behavior. Total puff volume was greatest for the 0.05 mg nicotine cigarette and CO boost was moderately greater after smoking the 0.3 and 0.05 mg cigarettes compared to the 0.6 mg nicotine cigarette. These data provide novel behavioral and biochemical evidence of compensatory smoking when smoking lower nicotine cigarettes. Although marketed as a PREP, increases in CO boost suggest this product can potentially be a harm-increasing product.
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Keywords: Nicotine; Puff volume; Topography; Carbon monoxide

1. Introduction

The Institute of Medicine (IOM) report on tobacco harm reduction defines a product as harm reducing if it lowers total tobacco related mortality and morbidity, even though use of that product may involve continued exposure to tobacco-related toxicants (Stratton et al., 2001a,b). The IOM concludes that products that actually reduce harm may be a feasible and justifiable public health policy, particularly for those who cannot or will not quit smoking (Stratton et al., 2001a). However, careful evaluation of these potential reduced exposure products (PREPs) is necessary to characterize reductions in exposure to harmful substances, and to determine if there is an association between reduced exposure and reduced harm to health (Hatsukami et al., 2005; Stratton et al., 2001b).

One type of PREP, called Quest® (Vector Tobacco Inc., Durham, NC), uses genetically modified tobacco with lower nicotine levels to provide a means for smokers to "enjoy smoking by choosing to reduce your level of nicotine" (http://www.questcigs.com). Quest® cigarettes are manufactured with three progressively lower nicotine levels (0.6, 0.3, and 0.05 mg) and marketed as allowing smokers to "step-down" nicotine levels to "nicotine-free smoking" (http://www.questcigs.com). However, each of the three levels of Quest® cigarettes deliver equivalent levels of tar (10 mg) during standardized testing, and thus, are likely to pose health risks (Thun and Burns, 2001).

Nicotine is the primary addictive agent in cigarettes (United States Department of Health and Human Services (USDHHS, 1988)), and previous research has shown that smokers will alter their smoking behavior when switched to "light" cigarettes with lower delivery of nicotine (Benowitz et al., 1983; Zacny and Stitzer, 1988). By taking larger and more frequent puffs, a smoker can extract more nicotine from a "light" cigarette, and is

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consequently exposed to more tar from the smoke particulates that contain 81 known carcinogenic compounds (Hoffmann and Hecht, 1990; Smith et al., 2003). How a cigarette is smoked is referred to as smoking topography, and includes such measures as the number of puffs, puff volume, duration, and velocity (Bridges et al., 1986; Kolonen et al., 1992; USDHHS, 1988).

Altering smoking topography to offset a lower nicotine cigarette is a mechanism called compensation, and includes taking bigger puffs, taking more puffs, and smoking more cigarettes per day (Kozlowski et al., 2000; Scherer, 1999). Studies of "light" cigarettes show that smokers are able to extract more nicotine, tar and carbon monoxide (CO) from a cigarette than the standard levels reported by the Federal Trade Commission (FTC) (Benowitz et al., 2005; FTC Report, 2000, Scherer, 1999). However, unlike "light" cigarettes, smokers who use Quest® cigarettes may not be able to increase their nicotine levels through compensation due to the genetically modified tobacco not containing nicotine. Little is known about whether changes in smoking topography occur when smokers switch to this new low nicotine product. Since smoking topography can determine, in part, overall exposure to CO (Hofer et al., 1992; Zacny et al., 1987) and tobacco carcinogens (Djordjevic et al., 2000) characterizing topography and exposure with these new cigarette products is an important first step toward understanding associated health risks.

We conducted a within-subject human laboratory study to investigate the effect of cigarette nicotine level (0.6, 0.3, and 0.05 mg) on smoking topography and carbon monoxide exposure. We hypothesized that compensatory smoking, specifically greater total puff volume, would be observed as nicotine levels decreased, thereby supporting behavioral compensation. Further, due to increased total puff volume, we hypothesized increases in CO boost as nicotine levels decreased (i.e. biochemical evidence of compensation).

2. Methods

2.1. Participant recruitment

Smokers responding to community-based flyers were screened for eligibility via telephone. Inclusion criteria were: over age 18, smoke at least 10 daily cigarettes, smoking for a minimum 5 years, not currently trying to quit smoking, and report inhaling when smoking. Participants were excluded if they did not meet each of the inclusion criteria; reported trying to quit smoking, including current use of nicotine replacement therapy; or reported consuming more than 25 alcohol drinks per week. No participants had previously used Quest® cigarettes. Participants completed the single session experiment between March and August 2005 and were paid $100.00 for completing the entire study.

Sixty-nine people called the research center and 61 were eligible to participate. Ineligibility was due to smoking too few cigarettes ($n = 4$), trying to quit smoking ($n = 2$) and consuming more than 25 alcohol drinks per week ($n = 2$). Of the 61 eligible participants, eight refused to participate (not interested, time commitment too great); 53 scheduled an appointment. Three people missed their scheduled appointment time and 50 completed the single session.

2.2. Procedures

The study protocol was approved by the University Institutional Review Board. Experimental sessions were run between 8:00 and 16:00 h, and lasted approximately 3.5 h. Participants were required to bring a pack of their own cigarettes and were informed that they would smoke one of their own cigarettes and three other cigarettes with varying levels of nicotine during the session. They were instructed to abstain from cigarettes and caffeinated products for 1 h, and alcohol for 12 h, prior to attending the experimental session. Participants were encouraged to eat prior to attending the session and eating was not permitted between cigarettes.

Upon arrival to the laboratory, participants were seated in a comfortable chair in an observation room equipped with a smoke filtration system, and permitted to read or watch television when not smoking. After providing consent, breath alcohol content and baseline exhaled breath CO samples were collected, along with self-report questionnaires including demographic information, smoking history and general mood measures.

All cigarettes were smoked using the CReSS (Clinical Research Support System) smoking topography machine (Plowshare, Baltimore, MD). This device has been previously used in our laboratory to assess smoking behavior (Strasser et al., 2004, 2005), and has been shown to be a valid and reliable means to measure smoking behavior (Lee et al., 2003). Cigarettes are placed in a mouthpiece attached to an air-filled tube which leads to a pressure transducer. The device converts pressure changes during puffing into a digital signal. Sterilization of mouthpieces and unit calibration were performed prior to each session and by the guidelines of the manufacturer.

Participants had the smoking topography equipment explained to them and then were asked to smoke one of their own brand cigarette through the device. This permitted the participant to get accustomed to the smoking topography and CO devices and to standardize the time since last cigarette.

Thirty minutes after smoking their own brand cigarette, participants smoked the first of three Quest® cigarettes. The researcher instructed participants when to light each cigarette and a 30-min interval between cigarettes was timed from when the cigarette was extinguished to the lighting of the next cigarette. The Quest® cigarettes were presented in randomized order, counter-balanced across participants to minimize potential order effects. Quest® cigarettes were masked and color-coded so that participant and researcher were blind to nicotine level but able to distinguish between cigarettes by color. Color code was randomly generated and a study staff member who did not conduct sessions prepared the cigarettes.

Prior to, and 4 min after smoking each cigarette, participants provided breath CO samples. To obtain consistent readings, participants were instructed to inhale deeply, hold their breath for 2 s, then exhale, inhale again, hold their breath for 15 s and then exhale as long as possible in the CO breath sample device (Vitalograph, Lenexa, KS). The highest reading displayed on the digital screen, in parts per million (ppm), was recorded. Upon extinguishing each cigarette, participants completed a 14-item subjective rating of the cigarette they had just finished smoking.

2.3. Measures

2.3.1. Covariates. Covariates included age, sex, body mass index based on measured height and weight, nicotine level of preferred brand cigarette, and nicotine dependence (Fagerstrom Test for Nicotine Dependence, FTND; Heatherton et al., 1991).

2.3.2. Primary outcome: smoking topography. The smoking topography device employs a pressure transducer that measures pressure changes during puffing. Pressure changes are amplified, digitized and sampled at 1000 Hz and software converts signal to airflow (ml/s) in real time (s), which is subsequently converted to puff number, puff volume, puff duration, puff velocity and interpuff interval. The primary topography measure utilized for the current study was total puff volume, the sum of the volumes of all puffs taken while consuming the cigarette. Total puff volume was selected because it would provide a metric to quantify smoke exposure; permit comparison between nicotine levels and between participants; provide evidence of compensation; while allowing flexibility to account for individual differences in compensatory smoking behavior, such as taking more puffs and/or larger puff volumes. Total puff volume allows participants to compensate in different ways, a necessity demonstrated by Benowitz et al. (2005).

2.3.3. Secondary outcome: carbon monoxide. Participants provided two breath CO samples prior to, and 4 min after smoking each cigarette. Pairs of readings were averaged to determine pre-cigarette CO level and post-cigarette CO level. The difference between the pre- and post-cigarette readings was calculated as the measure of CO boost (Strasser et al., 2005; Zacny et al., 1987).

2.3.4. Subjective cigarette ratings. To assess subjective responses related to taste and flavor, participants completed a rating scale of cigarette features. The measure uses a 100 mm visual analog scale with descriptive anchors and participant instructions are to: place a vertical line at the location that best represents their rating of the cigarette for each characteristic. Items include: strength, harshness, heat, draw, taste (bad/good), satisfaction, burn rate, taste (mildness), too mild, harshness of smoke, after taste, staleness, strength of smoke, and smoke smell (pleasantness). These items have been used previously to assess subjective ratings of cigarettes (Strasser et al., 2005). At the conclusion of the session, participants were asked which color cigarette had the most and least amount of nicotine.

2.4. Statistical methods

Recruitment of 50 participants was determined as an appropriate sample size based on effect sizes (ES = 0.4) from similar smoking topography and CO boost studies (Strasser et al., 2004, 2005), with alpha set to 0.05 for a two-tailed test, and to provide greater than 80% power to detect between-cigarette nicotine level differences in total puff volume and CO boost.

Analyses were conducted using SPSS 12.0 (SPSS, Chicago, IL). Descriptive statistics were used to characterize the participant population in terms of demographic and smoking history variables. Associations between descriptive statistics and baseline CO and CO boost of own cigarette were tested by correlation analysis for continuous variables and analysis of variance (ANOVA) for nominal variables in order to identify potential confounds.

Repeated measures ANOVA was used to test for effects of cigarette nicotine levels on total puff volume, CO boost, and subjective ratings, and to test for potential order effects. Covariates were included in the analyses and those with significance levels of $p > 0.15$ were removed from the model. Bonferroni post hoc analyses with an alpha conservatively set to 0.0167 (0.05/3) were performed to compare differences between specific pairs of nicotine levels only after significant main effects were observed.

3. Results

3.1. Descriptive statistics

All 50 participants (54% male) who entered the study completed the single session. The participant sample was White (72%) and Black (24%), Native American (2%) and Asian American (2%); 4% identified themselves as Hispanic, irrespective of race. Thirty percent of participants were college graduates and 90% had completed high school. Participant mean body mass index (BMI; kg/m^2) was 26.7 (S.D. = 5.4, range 18.9–40.2, BMI $\geq$ 30, $n = 12$). Participants were on average 44.5 (S.D. = 12.1, range 22–72) years of age, reported smoking 21.3 (S.D. = 8.1, range 10–40) daily cigarettes, and have smoked daily for 27.8 (S.D. = 12.6, range 6–59) years. Their mean FTND nicotine dependence score was 5.5 (S.D. = 1.9, range 2–10). Participant preferred brand was varied; the most prevalent included: Marlboro Regular (24%), Marlboro Light (12%), USA (10%), Winston (8%), Camel (4%), Parliament (4%), and GPC (4%). Half of participants preferred cigarette type was Regular; 48% was Light; 2% was Ultra-Light. Mean standard nicotine level was 0.94 mg nicotine (S.D. = 0.3) and mean standard tar was 12.9 mg (S.D. = 8.7; FTC Report, 2000). None of the participants smoked unfiltered cigarettes. All participants had a breath alcohol content reading equal to 0.000 at the beginning of the session.

3.2. Baseline CO and CO boost from preferred brand cigarette

Mean CO level at baseline, prior to smoking own cigarette was 24.4 ppm (S.D. = 12.3, range 1.0–58.0). The participant with baseline CO = 1.0 ppm reported having not smoked for the previous 15 h, but did report typically smoking 10 daily cigarettes and had a nicotine dependence score of 4. Mean CO boost of own cigarette was 5.5 ppm (S.D. = 2.64, range 0.5–10.0). Start time of sessions varied between 08:00 and 16:00 and was not associated with baseline CO ($r = 0.09$, $p = 0.52$) or CO boost of own cigarette ($r = -0.01$, $p = 0.96$).

Associations between participant characteristics and baseline CO levels were explored to identify potential confounds to include in the analyses of nicotine level on total puff volume and CO boost. Self reported number of daily cigarettes smoked was significantly associated with baseline CO ($r = 0.56$, $p < 0.01$). Sex, nicotine dependence, preferred cigarette type, years smoked, and BMI were not associated with baseline CO ($p > 0.15$).

Neither the participant with baseline CO = 1 ppm nor the participant with CO boost of own cigarette equal to 0.5 ppm affected significance for analyses of association when excluded. Therefore both were included in all further analyses. Order of cigarette presentation had no effect on smoking topography or CO boost.

3.3. Outcome measure: smoking topography

Mean total puff volume measures were 570.5 ml (S.D. = 156.9), 518.1 ml (S.D. = 145.6), and 540.3 ml (S.D. = 144.9) for the 0.05 mg nicotine, 0.3 mg nicotine and 0.6 mg nicotine cigarettes, respectively [$F(2, 47) = 5.73$, $p = 0.006$]. Self-reported number of daily cigarettes was the single significant covariate remaining in the model. Post hoc Bonferroni analyses indicate that total puff volume for the 0.05 mg nicotine cigarette was statistically greater than total puff volume of the 0.3 mg nicotine cigarette ($p = 0.0013$), and had a trend toward being significantly greater than the total puff volume of the 0.6 mg nicotine cigarette ($p = 0.049$). Smoking topography measures are presented in Table 1 and (Fig. 1).

3.4. Outcome measure: carbon monoxide boost

Mean CO boost measures were 5.3 ppm (S.D. = 2.5), 5.8 ppm (S.D. = 2.6), and 4.7 ppm (S.D. = 2.7) for the 0.05 mg nicotine, 0.3 mg nicotine and 0.6 mg nicotine cigarettes, respectively [$F(2, 47) = 5.43$, $p = 0.01$]. Self-reported number of daily cigarettes was the single significant covariate remaining in the model. Post hoc Bonferroni analyses indicate that CO boost for the 0.3 mg nicotine cigarette was significantly greater than the 0.6 mg nicotine cigarette ($p = 0.007$). Values for CO boost for own cigarette and Quest® cigarettes are presented in Table 1 and (Fig. 1).

Table 1
CO boost, smoking topography and subjective ratings of own brand and three nicotine levels of Quest® cigarettes

	Cigarette type (mg of nicotine)			
	Own brand (various)	Quest® 1 (0.6 mg)	Quest® 2 (0.3 mg)	Quest® 3 (0.05 mg)
Biochemical measure				
CO boost (ppm)	5.5 (4.8–6.3)	4.7 (4.0–5.5)	5.8 (5.1–6.6)	5.3 (4.6–6.0)
Smoking topography measures				
Total puff volume (ml)	832.0 (737–926)	540.3 (500–580)	518.1 (478–558)	570.5 (527–614)
Number of puffs	14.3 (12.8–15.7)	9.8 (9.0–10.5)	9.9 (8.9–10.9)	10.0 (9.1–10.9)
Puff duration (s)	1.8 (1.6–1.9)	1.8 (1.6–1.9)	1.7 (1.5–1.8)	1.8 (1.6–1.9)
Puff volume (ml)	60.5 (55.2–65.7)	58.1 (53.3–62.8)	55.9 (51.0–60.8)	59.4 (54.6–64.3)
Puff velocity (ml/s)	35.5 (22.3–37.8)	34.5 (32.0–37.0)	34.4 (31.9–37.0)	35.1 (32.5–37.8)
Interpuff interval (s)	21.6 (19.1–24.1)	21.6 (19.0–24.2)	19.6 (16.9–22.3)	18.6 (15.7–21.6)
Subjective ratings				
Strength-very weak/very strong	60.7 (53.7–67.8)	44.4 (38.4–50.5)	40.3 (32.9–47.6)	28.3 (21.1–35.6)
Harshness-very mild/very harsh	29.6 (23.3–35.9)	33.7 (27.3–40.1)	35.9 (28.1–43.7)	27.8 (20.2–35.4)
Heat-no heat/very hot	19.0 (13.7–24.2)	24.5 (18.9–30.1)	27.6 (20.2–35.1)	26.4 (19.6–33.2)
Draw-easy/difficult	46.5 (7.8–85.2)	26.3 (19.9–32.7)	46.9 (8.1–85.6)	28.3 (20.8–35.8)
Taste-very bad/very good	70.0 (62.7–78.0)	44.6 (30.1–43.6)	36.8 (30.1–43.6)	53.3 (14.7–91.8)
Satisfaction from smoking-unsatisfying/satisfying	70.3 (62.7–78.0)	44.3 (36.3–52.2)	36.8 (29.3–44.3)	24.4 (18.3–30.5)
Burned/did not burn too fast in too few puffs	86.7 (49.4–100.0)	26.0 (19.3–32.7)	22.3 (17.0–27.6)	21.8 (15.5–28.2)
Mild taste/not mild taste	46.7 (38.8–54.7)	33.2 (26.3–40.2)	35.5 (27.8–43.2)	33.2 (25.5–41.0)
It was/was not too mild for me	71.4 (64.0–78.8)	42.4 (33.5–51.4)	41.1 (31.7–50.5)	31.4 (23.0–39.8)
Smoke seemed/did not seem harsh	75.3 (68.8–81.8)	56.2 (47.7–64.8)	57.6 (48.9–66.3)	61.6 (52.6–70.5)
Did not leave/left a good aftertaste in my mouth	54.1 (45.5–62.7)	59.7 (21.3–98.1)	35.0 (27.4–42.5)	44.4 (5.6–83.1)
Somehow it seemed/did not seem stale	84.1 (79.1–89.1)	59.4 (51.5–67.3)	58.0 (49.9–66.1)	53.7 (44.4–63.0)
Smoke seemed very weak/very strong	62.4 (55.4–69.4)	44.6 (37.7–51.4)	37.8 (30.8–44.8)	31.9 (24.2–39.5)
Smoke smell-unpleasant/pleasant	65.0 (58.4–71.6)	50.2 (43.1–57.3)	48.4 (41.4–55.3)	46.0 (39.4–52.7)

Data presented as mean (±95% confidence interval).

3.5. Subjective measures and identifying nicotine levels

Strength of cigarette, satisfaction from smoking, and smoke strength differed significantly by nicotine level ($p < 0.01$). The 0.6 mg nicotine cigarette was rated as stronger, more satisfying, and its smoke reported as most strong compared to the other Quest® cigarettes. The 0.05 mg nicotine cigarette was rated the lowest on each of these subjective ratings. Values for all subjective measures for participant own brand and Quest® cigarettes are presented in Table 1.

At the conclusion of the session participants were asked which color cigarette had the most and least nicotine. Fifty four percent correctly identified the 0.6 mg nicotine cigarette as the highest nicotine cigarette. Only 12% stated the 0.05 mg nicotine cigarette had the highest nicotine level. Fifty-eight percent correctly identified the 0.05 mg nicotine cigarette as the least nicotine-containing cigarette; 18% incorrectly identified the 0.6 mg nicotine cigarette as the lowest. Only 38% correctly identified both the 0.6 mg nicotine cigarette and the 0.05 mg nicotine cigarette. Ability to discriminate nicotine levels did not affect total puff volume or CO boost. However, those participants who correctly discriminated cigarette nicotine levels rated the 0.6 mg nicotine cigarette as strongest and most satisfying and the 0.05 mg nicotine as least strong and satisfying, while the non-discriminators reported no subjective differences by nicotine level.

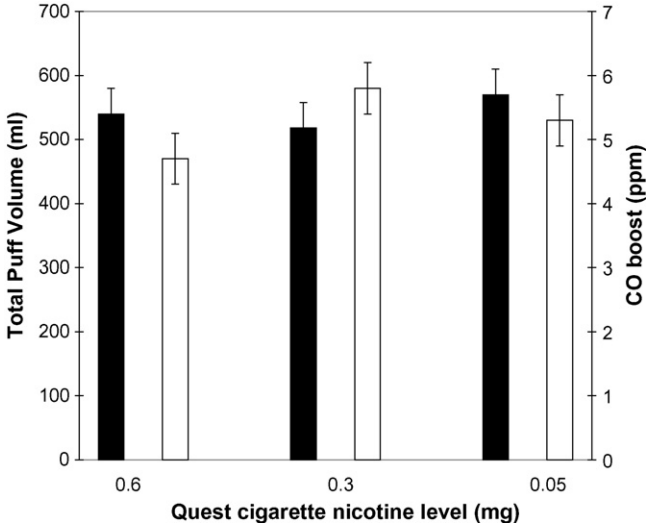


Fig. 1. The effect of cigarette nicotine level on total puff volume [(ml; solid bars; left y-axis); $F(2, 47) = 5.73$, $p = 0.006$, $N = 50$] and mean carbon monoxide boost [(ppm; open bars; right y-axis); $F(2, 47) = 5.43$, $p = 0.01$, $N = 50$]. Data presented as mean (±95% confidence interval).

4. Discussion

This study provides novel behavioral and biochemical evidence for the potential of compensatory smoking with a new low nicotine cigarette product, supporting the potential for increased, rather than reduced, harm from this PREP. As hypothesized, both total puff volume and CO boost per cigarette increased when cigarette nicotine level decreased, although the effect was modest. Subjective ratings of cigarette strength and satisfaction

were significantly lower for the lower nicotine cigarettes, ratings of other cigarette features (e.g., draw and taste) were very similar across nicotine levels, consistent with evidence that tar levels contribute to the ability to discriminate cigarettes (Schuh et al., 2001). The non-significant results for most cigarette rating items are important because most brand-switching studies find several subjective and sensory differences by cigarette type (Pickworth et al., 1999; Rose et al., 1993; Strasser et al., 2005; Zacny et al., 1987), likely attributable, in part, to non-nicotine differences between the cigarettes. A true test of compensatory smoking attributable to nicotine levels should use cigarettes that are identical in tar levels and design characteristics, such as filter ventilation levels, draw and taste (Scherer, 1999).

These findings extend previous investigations of compensation when smokers switched to cigarette brands with lower nicotine and tar yields based on standardized testing (Benowitz, 2001; Zacny et al., 1987). However, the lower nicotine cigarettes tested in previous studies had design features that would permit smokers to extract more nicotine from the cigarette by increasing puffing or by occluding filter ventilation holes (Jarvis et al., 2001; Rickert et al., 1983). Quest® cigarettes contain equivalent levels of tar, do not appear to be ventilated, and nicotine delivery is restricted due to the use of genetically modified, nicotine-free tobacco. However, since smokers are unlikely to be aware of, or fully understand, these design differences, compensatory smoking behavior is still likely to occur.

Compensatory smoking behavior and increased CO boost was not a ubiquitous occurrence, although total puff volume at the 0.3 mg nicotine and 0.05 mg nicotine levels were significantly correlated ($r = 0.60$, $p < 0.001$); as was CO boost ($r = 0.93$, $p < 0.001$). Overall, effect sizes of cigarette nicotine level on total puff volume and CO boost were modest. However, for some participants the effect was notable. Twenty percent of participants exhibited 20% or greater increase in total puff volume [mean 42.5% (S.D. = 20.1)] when smoking the 0.05 mg nicotine cigarette compared to the 0.6 mg nicotine cigarette; 12% had 20% or greater increase in total puff volume [mean 41.5% (S.D. = 14.5)] for the 0.3 mg nicotine cigarette compared to smoking the 0.6 mg nicotine cigarette. Forty two percent of participants had 20% or greater increase in CO boost [mean 290% (S.D. = 452)] at 0.05 mg nicotine cigarette compared to 0.6 mg nicotine cigarette; 44% had a 20% or greater CO boost increase [mean 269% (S.D. = 515)] for 0.3 mg nicotine cigarette compared to 0.6 mg nicotine cigarette. This study was designed to be a preliminary investigation for evidence of compensatory smoking of a new PREP product, and was not designed or powered to identify subgroup differences. Future research on PREPs should attempt to characterize subgroups of those most prone to increased harm exposure from PREP products.

Participants smoked their preferred own brand as the first cigarette in order to permit them to become accustomed to the smoking topography device and to standardize the time since the last cigarette prior to smoking the Quest® cigarettes. Comparison of total puff volume and CO boost between preferred own brand and Quest® cigarettes is problematic due to the variation in participants' cigarette brands. However, it is noteworthy that: all standard nicotine levels of participants' preferred brand was greater than the highest nicotine Quest® (0.6 mg nicotine) cigarette; total puff volume when smoking preferred own brand cigarettes was greater than for any of the Quest® cigarettes; CO boost for preferred brand was similar to the lowest nicotine level Quest® cigarettes. Filter ventilation in the preferred own brand cigarettes can dilute the concentration of smoke, such that similar CO boost is observed although total puff volume of own brand is greater than for Quest® cigarettes. However, due to Quest® having less available nicotine, smokers potentially may use relatively more Quest® cigarettes daily.

Smoking topography has been used as an index of systemic smoke exposure from conventional cigarettes (Zacny et al., 1987) and proposed reduced exposure products (Breland et al., 2002; Buchhalter et al., 2001; Lee et al., 2004). Total puff volume, the metric chosen for the analysis of the present study, is a particularly informative index of systemic exposure (CO or nicotine boost); Burling et al. (1985), Gust and Pickens (1982) and Zacny et al. (1987) identified puff volume as an important determinant of tobacco exposure.

Ample evidence exists for the effect of smoking topography on carbon monoxide exposure (Hofer et al., 1992; USDHHS, 1988; Zacny et al., 1987) and support exists for its effect on carcinogen exposure (Djordjevic et al., 2000). Studies assessing exposure to multiple smoking-attributable biomarkers report significant, positive correlations between carbon monoxide levels and biomarkers of known carcinogens (Hecht et al., 2004; Joseph et al., 2005); specifically, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and 1-hydroxypyrene (1-HOP) are biomarkers of the carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and of polycyclic aromatic hydrocarbons (PAHs), respectively. Further evidence for the relationship between smoking topography and carcinogen exposure is suggested by changes in carcinogen yield per cigarette as a result of compensatory smoking when daily cigarette consumption is reduced (Murphy et al., 2004), and when smokers are switched to a lower nicotine cigarette for an extended duration (Benowitz et al., 2005).

The present line of research should also be extended to include the measurement of additional biomarkers of harm. Research has shown that not all cigarette compounds change at the same rate as a function of compensatory smoking in cigarettes (Harris, 2004) and during PREP usage (Hatsukami et al., 2004). CO boost is a practical measure to use in this study because of its sensitivity to smoking during a single cigarette. However, extended use of Quest® would permit inclusion of other biomarkers of exposure that are better indicators of carcinogen exposure, such as NNK (4-methylnitrosamino-1-(3-pyridyl)-1-butanone) and 1-HOP (1-hydroxypyrene) uptake (Hecht, 1999; Hecht et al., 2004).

Smokers' perceptions of the features of new PREPs may also play an important role in determining smoking behavior with these products. As such, it is therefore important to understand how smokers interpret the explicit and implicit claims made in product marketing (Stratton et al., 2001b). Of particular relevance to the present findings is evidence that a substantial proportion of smokers make false inferences about the safety of Quest® cigarettes based on the Quest® advertise-

ment (Shadel et al., 2006). Smokers who switch to lower tar cigarettes, rather than quitting, often perceive that the cigarettes marketed by such advertisements are safer or healthier than regular cigarettes (Hamilton et al., 2004; Shiffman et al., 2004). Analyses of tobacco industry documents suggest that deliberate attempts have been made to create misunderstanding of health risk (Dunsby and Bero, 2004). If a novel cigarette product is misperceived as harm reducing, it possesses the risk of attracting cigarette smokers who otherwise would have quit or who would have adopted validated less harmful nicotine products.

There are a few limitations to our study. Participants smoked only one cigarette of each nicotine level. Based on the evidence for compensation when smoking a single cigarette, future investigations of smoking topography and CO boost when smoking lower nicotine cigarettes over a longer period of time are warranted. Extended observation of smoking behavior using Quest® cigarettes would also permit assessing multiple biomarkers of harm exposure. It is also possible that smokers who switch to Quest® cigarettes to reduce their nicotine exposure may increase their daily consumption of cigarettes (Benowitz, 2001). As a result, they may administer more daily nicotine when smoking Quest® than when smoking their own brand. Replication of results, characterization of prone subgroups, and investigation of long-term use of Quest® is necessary.

The present study makes an important first step in understanding the behavioral and biochemical effects of new lower nicotine cigarettes that have conventional tar levels. Evidence for behavioral compensation and increased CO exposure with lower nicotine cigarettes is particularly important in light of evidence that smokers infer from marketing claims that these cigarettes are less harmful (Shadel et al., 2006).

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Smoking in the absence of nicotine: behavioral, subjective and physiological effects over 11 days

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ABSTRACT

Aims Sensorimotor stimuli associated with tobacco smoking influence smoking behavior; however, current research has focused almost exclusively on the effects of brief, laboratory-based exposure to smoking-related stimuli. The purpose of this experiment was to characterize the effects of smoking stimuli delivered in the absence of nicotine over an extended (11-day) exposure. **Design, setting and participants** Thirty adult regular smokers participated in an in-patient study. After assessing preferred brand smoking, participants were assigned randomly to one of three groups corresponding to subsequent smoking conditions: nicotine-containing cigarettes, de-nicotinized cigarettes or no smoking. **Measurements** Measures of smoking reinforcement, subjective effects, physiological effects, withdrawal/craving and puff topography were taken repeatedly during both periods of free access and controlled assessments during abstinence. **Findings** Daily de-nicotinized cigarette use declined immediately by 1.7 cigarettes/day compared to the preferred brand baseline and declined by another 3.5 cigarettes over time; participants smoking de-nicotinized cigarettes also demonstrated a 31% decline in the number of puffs earned on a progressive ratio, a measure of the motivation to smoke, during the study. Subjective ratings of smoking were largely negative throughout the study in the de-nicotinized group, while the nicotine-containing condition reported increasingly positive subjective effects with repeated exposure. Acute craving suppression following smoking remained evident throughout the study regardless of nicotine content. **Conclusions** These effects highlight the importance of non-nicotine sensorimotor stimuli as determinants of the maintenance of smoking behavior and suggests that extinction of conditioned reinforcement in the absence of nicotine progresses slowly.

Keywords Cigarettes, conditioning, de-nicotinized, extinction, nicotine, reinforcement, reward.

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INTRODUCTION

It is generally accepted that nicotine is a necessary constituent in tobacco driving smoking [1]. However, converging molecular, neuropharmacological, behavioral and clinical evidence suggests that the primary reinforcing consequences of nicotine are not a sufficient explanation for smoking [2–4]. Much of this research has focused on sensorimotor smoking stimuli as critical determinants of smoking.

The sensory stimuli produced by cigarette smoke are distinct, salient and consistently paired with nicotine, providing an ideal context for establishing a conditioned association with nicotine [5]. Presumably as a consequence of this association, smoking stimuli acquire properties that contribute to smoking. Indeed, de-nicotinized and nicotine-containing cigarettes result in similar levels of smoking during brief laboratory sessions [6], similar decreases in subsequent preferred brand smoking [7], similar responding on a progressive ratio schedule of reinforcement [8,9] and similar decreases in withdrawal and craving [6]. It is also clear that nicotine adds to smoking effects; nicotine-containing cigarettes are preferred over de-nicotinized cigarettes when they are concurrently available [10], produce greater positive subjective effects [7,11–13] and alleviate a wider range of withdrawal symptoms [14].

Together, this research indicates that sensorimotor smoking stimuli may be important for understanding smoking behavior and consequences. However, most

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human research to date has focused on the acute effects of smoking stimuli and their role in short bouts of smoking. Little is known about whether or to what extent these effects are maintained over prolonged exposure. If sensorimotor smoking stimuli acquire their properties through Pavlovian conditioning, their continued presentation in the absence of nicotine should lead to a loss of their effects (i.e. extinction). Recently, Rose & Behm [15] found that exposure to de-nicotinized cigarettes for 2 weeks reduced the subjective reward associated with usual-brand smoking, suggesting that the positive subjective effects of smoking stimuli can be extinguished with repeated exposure and that this process generalizes to preferred brand cigarettes. In contrast, Buchhalter and colleagues [14] found little evidence that the ability of de-nicotinized cigarettes to suppress withdrawal and craving dissipated over 5 days. While smoking behavior was not measured directly, participants turned in fewer cigarette butts during the de-nicotinized condition and had lower expired carbon monoxide (CO) levels than when they had access to nicotine-containing cigarettes [14]. These differences were stable over time, suggesting reduced reinforcement in the de-nicotinized condition, but little evidence of further extinction over the 5-day period.

The purpose of the present study was to examine further whether the effects of de-nicotinized cigarettes dissipate with repeated exposure. We utilized an in-patient design to maximize the extinction process by maintaining a stable and novel context in which new learning could occur [16] and ensure compliance with the smoking conditions [14,15]. A broad range of constructs (e.g. reinforcement, motivation to smoke, subjective effects, mood, withdrawal/craving and puff topography) were assessed repeatedly, allowing us to characterize change thoroughly over time.

METHODS

Participants

Thirty adult, out-patient, volunteers were recruited through advertising, consented to participate and completed the study. Five additional participants did not complete the study. Three of the five left prior to randomization; two left after being randomized to abstinence. The inclusion criteria were as follows: 18–65 years of age, self-reported smoking of 10 or more cigarettes per day for the last year, inhaling while smoking, no intention to quit in the next 3 months and a drug-free urine at screening (opiates, cocaine and benzodiazepines). Exclusion criteria were as follows: significant medical illness, evidence of a major psychiatric illness within past 6 months, females who are pregnant or lactating, current drug abuse treatment, drug dependence (excluding nicotine and caffeine) and diagnosed sleep disorder. The sample was 53% female and predominantly African American (72%). Most participants (93%) completed high school or obtained a general equivalency diploma. Participants, on average, were 37.8 years of age (SD: 11.4), smoked 21.4 (SD: 11.8) cigarettes per day and scored 5.3 (SD: 1.9) on the Fagerström Test for Nicotine Dependence (FTND) (17). Most (77%) reported that their preferred brand was mentholated.

Design

This study employed a between-subjects, double-blind design. We utilized a between-subjects design to limit unblinding resulting from direct comparison of the two experimental cigarettes. Volunteers first participated in a telephone and an in-person screening. If interested and they qualified, participants were admitted to a residential research unit at the Johns Hopkins Bayview Medical Center for 13 nights. Participants were paid for their time and inconvenience.

Procedures

For the first 2 days, all volunteers were allowed to smoke their preferred brand of cigarettes without restriction. On day 1, participants were familiarized with the subjective and behavioral measures. Day 2 served as a baseline assessment of preferred brand smoking; the assessments conducted on day 2 were identical to those taken on days 4–7 and 9–12. On these days (i.e. days 2, 4–7, 9–12), volunteers were allowed to smoke without restriction between 6 a.m. and 10 p.m.; smoking was not allowed between 10 p.m. and 6 a.m. On day 3, 10 participants were assigned randomly to each of three smoking conditions [no smoking, de-nicotinized cigarettes (Denic) and nicotine-containing cigarettes (Nic)] and remained in those conditions throughout the rest of the study. Days 3, 8 and 13 tested the effects of smoking the assigned cigarette (day 3) and change in these effects (days 8 and 13) under controlled conditions. On these days, volunteers were allowed to smoke during only two laboratory sessions (controlled puffing, progressive ratio); unrestricted smoking did not resume until the following day.

Unrestricted smoking assessments

All participants were allowed to smoke their preferred brand of cigarette freely on day 2, and participants in the Denic and Nic conditions were allowed to smoke their assigned cigarettes freely on days 4–7 and 9–12. The first three cigarettes of each day were dispensed from the nurse's station to aid in the collection of subjective and topography data. The subjective effects of smoking,

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craving, CO and cardiovascular measures were assessed before and/or after the first and third cigarette. On days 2, 4, 6, 9 and 11, participants also smoked their first and third cigarettes through the portable topography device (CReSSmicro, Plowshare Technologies, Baltimore, MD, USA). Following the third cigarette, volunteers were given an ample supply of cigarettes for the remainder of the day. Volunteers were responsible for tracking their own cigarette consumption, including recording the time each cigarette was smoked and retaining the unsmoked portion of each cigarette. Cigarette logs were checked against the number of cigarette butts collected daily.

Controlled puffing procedure

On days 3, 8 and 13 (9.00–10.00 a.m.), participants in the Denic and Nic groups inhaled eight 30 cc puffs at an interpuff interval of 1 minute, holding each puff for 3 seconds. Puffing was controlled using real time auditory and visual feedback from desktop topography equipment (CReSS, Plowshare Technologies). Actual volume closely approximated the target volume of 30 cc and did not differ between smoking conditions. Subjective effects, CO and cardiovascular measures were assessed before and/or after smoking. Participants in the no smoking condition read quietly during the time reserved for smoking in the other two groups. Heart rate and blood pressure were taken throughout the session. Participants were instructed that the same cigarettes, inhalation volume and inhalation duration would be available for them later that afternoon in the progressive ratio session.

Progressive ratio procedure

On days 3, 8 and 13 (2.00–5.00 p.m.), participants could earn 30 cc puffs from their assigned cigarettes by pressing the button on the mouse. The number of responses required for each puff increased with each puff earned as follows: 50, 250, 500, 1000, 1500, 2000, 2500, 3000, 3500 and 4000. A minimum inter-response interval of 0.5 seconds was enforced; responding at a faster rate did not count towards completion of the schedule requirements. Volunteers had 3 hours to earn as many puffs as they desired. Puffs were taken immediately after completing each response requirement. Responding was allowed to resume 1 minute after completing the preceding response requirement. Participants in the no smoking condition were allowed to respond, but there was no consequence for completing the ratio requirement. All participants could participate in a neutral activity (e.g. reading) during this time to minimize the influence of boredom on responding.

Additional measures

An extended battery of withdrawal, craving, mood and sleep assessments was administered at 2 p.m. on days 2, 4–7 and 9–12. On day 13, participants were administered an end-of-study questionnaire.

Study cigarettes

Quest brand cigarettes (Vector Tobacco Inc., Research Park Triangle, NC, USA) were used as the study cigarettes. Cigarettes were freely available and all brand labeling was hidden from the staff and volunteers. Nicotine-containing cigarettes (Quest 1) contained 0.6 mg of nicotine and 10 mg of tar and de-nicotinized cigarettes (Quest 3) contained 0.05 mg of nicotine and 10 mg of tar, as measured by the Federal Trade Commission method. It is important to note that nicotine delivery was not directly measured. Menthol versions of both types of cigarettes became available after the study was initiated. Three menthol-preferring participants in both the Nic and Denic groups were assigned to non-mentholated study cigarettes prior to the availability of Quest menthols. Menthol smokers were not excluded because of high rates of menthol smoking in the Baltimore area. Inspection of the data revealed that the pattern of smoking observed was similar in menthol-matched and menthol-mismatched participants. Given the small number of participants, the similarity in observed effects on smoking and the fact that the number of participants who were menthol mismatched was equal in the smoking groups, all individuals were included in the analyses.

Measures

Smoking behavior

Data collected on the time of each cigarette smoked was compiled into three distinct variables: the total number of cigarettes smoked per day, the latency to the first cigarette of the day (i.e. from time of awakening) and the pattern of daily smoking (i.e. cigarettes smoked per 4-hour period of availability). In addition, the average weight of the unsmoked cigarettes was calculated for each day.

Puff topography

Characteristics of puffing behavior were assessed by determining the puff volume, puff duration, peak flow rate, average flow rate, interpuff interval and number of puffs per cigarette. Although volume, duration and flow are measured with each puff, the data reported below represent the mean across puffs for each cigarette.

Cigarettes effects questionnaire

Visual analog scales (0–100; anchored by 'not at all' and 'extremely') assessed the effects of smoking on: satisfying, pleasant, unpleasant, like taste, dislike taste, smoke versus air (anchored with 'mostly smoke' to 'mostly air'), harsh, strength, high in nicotine, like drug effect, dislike

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drug effect, like cigarette, dislike cigarette, calming, relaxing, comforting, less irritable, sense of wellbeing, more awake, easier to concentrate, exhilarating, pleasurable excitement, dizziness, lightheaded, nauseating and nervous [18].

Withdrawal and craving questionnaires

Withdrawal and craving were assessed using standard, validated scales. The Shiffman–Jarvik Withdrawal Scale (SJWS) [19] assesses symptoms of nicotine withdrawal with five subscales: craving, psychological discomfort, physical discomfort, stimulation/sedation and appetite. The Questionnaire on Smoking Urges (QSU) [20] assessing craving using two factors (factor 1: intention to smoke; factor 2: anticipation of relief from withdrawal). The Schuh–Stitzer VAS Craving Scale [21], a five-item visual analog scale, was used as an alternative to the QSU to assess changes in craving after the first and third cigarette on unrestricted smoking days because of its brevity and the desire to reduce task demands.

Mood questionnaires

The Profile of Mood States (POMS) [22] and the Positive and Negative Affect Scale (PANAS) [23] were completed on days 2, 4–7 and 9–12 during the afternoon battery to assess mood.

Sleep questionnaire

The St Mary's Sleep Questionnaire [24] was administered daily and includes measures related to the duration of sleep, time to fall asleep, number of awakenings and subjective assessments of depth of sleep, sleep quality, sleep satisfaction, clear-headedness after awakening, difficulty getting to sleep and difficulty staying asleep.

Physiological measures

Heart rate and blood pressure were measured (non-invasive patient monitor model 506; Criticare Systems, Waukesha, WI, USA) throughout the controlled puffing (9–10 a.m.) session. Expired air CO was taken at 10 a.m., 4 p.m. and 10 p.m. on days 1, 2, 4–7 and 9–12. CO was also assessed hourly from 8 a.m. to 10 p.m. on days 3, 8 and 13 to ensure compliance with smoking restrictions. All CO assessments were taken a minimum of 2 minutes after the last cigarette. Saliva samples were collected each evening at 10 p.m. These samples were not analyzed, but instead used as a bogus pipeline to further deter smoking of non-study cigarettes.

End of study questionnaire

As part of their study evaluation, participants in the Denic and Nic groups were asked the following question: 'In your estimation how much nicotine was in the cigarettes you were asked to smoke?' on a five-point scale. Response categories were (1) nicotine-free: 0 mg; (2) ultra lights: 0.4 mg (e.g. True, Merit ultra lights); (3) lights: 0.8 mg (e.g. Marlboro Light, Vantage); (4) full flavor: 1.1 mg (e.g. Marlboro, Camel); and (4) very strong: 1.7 mg (e.g. Pall Mall).

Data analysis

Data were analyzed for baseline group differences (day 2 only). Data from days 3–13 were analyzed both as raw scores (not including baseline) and after adjusting for baseline (i.e. difference from baseline scores). The results of the difference score analyses are presented only if there were significant group differences at baseline or if the effects of group differed in important ways from the raw data. All data were analyzed using Proc Mixed (SAS) with one or more of the following factors: group, day, cigarette, time of day and pre/post smoking. The repeated-measures variable day was incorporated into the model as a linear and quadratic polynomial contrast for measures taken during unrestricted smoking (i.e. when the number of assessments was large enough to estimate the nature of the change over time). Additional analyses of individual smoking groups were conducted to directly compare change in these groups and to further describe change within each group. Planned comparisons of the different groups within a given day and time were made using *t*-tests. Illustrative examples of effect size are reported as Cohen's *d*. Data reported below focus on main and interaction group effects; other effects are not reported for brevity. Differences with a probability of $P < 0.05$ were considered statistically significant.

RESULTS

Unrestricted smoking behavior

Prior to randomization (i.e. day 2), participants in the three conditions smoked their preferred cigarettes similarly (Fig. 1). Although participants subsequently assigned to the no smoking condition tended to smoke more cigarettes at baseline than did other groups, this trend was driven by a single individual who smoked 37 cigarettes on day 2. Analysis of puff topography taken after the first and third cigarettes of the day revealed a main effect of subsequent group assignment on puff volume ($P = 0.011$) and puff duration ($P = 0.049$). Participants subsequently assigned to the no smoking condition had lower average puff volume (Ps < 0.05) and puff duration (Ps < 0.05) than each of the other groups. Importantly, there were no significant differences between the two subsequent smoking conditions at baseline.

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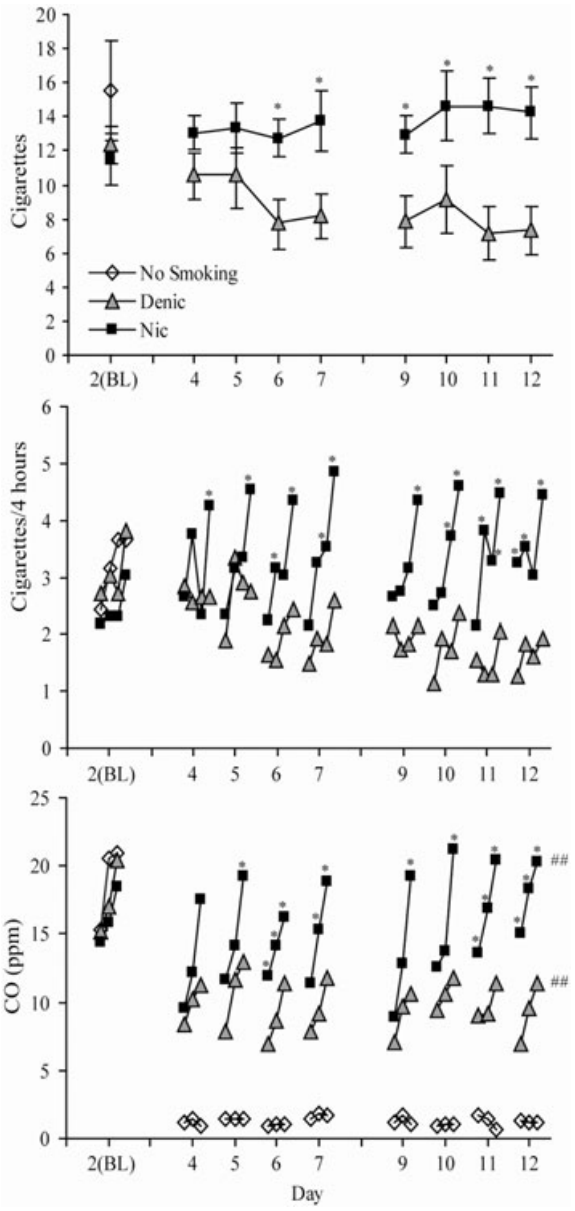


Figure 1 Upper panel: mean (±SEM) total number of cigarettes smoked during each unrestricted smoking day. Middle panel: mean number of cigarettes smoked per 4-hour period for each unrestricted smoking day. Adjacent points within each day represent 6 a.m.–10 a.m., 10 a.m.–2 p.m., 2 p.m.–6 p.m. and 6 p.m.–10 p.m., respectively. Lower panel: mean expired CO at three times each day; adjacent points represent 10 a.m., 4 p.m and 10 p.m., respectively. In all panels, day 2 represents baseline, preferred brand smoking. Participants were randomized on day 3. SEMs are not shown for the middle and lower panel to increase clarity of the figure. *Significant difference compared to Denic. ##Significant differences at all post-baseline time-points compared to no smoking

After randomization, participants in the Nic group smoked more cigarettes and this difference grew over days (Fig. 1). Analysis of the number of cigarettes smoked per day revealed a main effect of group ($P = 0.013$) that was more robust after controlling for baseline ($P < 0.001$). Participants assigned to the Nic group tended to increase the number of cigarettes smoked per day relative to their preferred brand by 2.1 cigarettes (i.e. collapsed across days; $d = 0.46$; $P = 0.13$), while participants in the Denic condition significantly decreased their cigarette consumption by 3.8 cigarettes ($d = 0.80$; $P = 0.041$). A linear group × day interaction ($F_{1,126} = 9.19$, $P < 0.005$) was seen with a significant linear decrease in smoking over days in the Denic group ($P < 0.005$), but no significant change over time in the Nic condition. There were no main or interaction effects of group on puff volume, puff duration, interpuff interval, peak flow or average flow. Puff volume increased relative to baseline, preferred brand smoking, when participants were assigned to either the Denic (+4.8 cc; $d = 0.39$; $P = 0.024$) or Nic condition (+6.8 cc; $d = 0.25$; $P = 0.038$). However, individuals smoking nicotine-containing cigarettes took more puffs per cigarette (11.5 versus 8.8; $d = 0.65$; $P = 0.010$) and tended to leave less of their cigarettes unsmoked (0.45 g versus 0.54 g; $d = 0.47$; $P = 0.11$) than individual smoking de-nicotinized cigarettes. Differences in cigarette butt weight reached significance after controlling for baseline.

Latency to smoke the first cigarette of the day tended to be longer for de-nicotinized than nicotine-containing cigarettes. The change in latency to smoke was abrupt (i.e. seen on day 4) and did not change significantly over repeated days in either cigarette condition. Latencies increased by a mean of 55.8 minutes compared to baseline in the Denic condition while decreasing by a mean of 25.5 minutes in the Nic condition. This difference between groups in the latency to smoke failed to reach significance in the analysis of the raw data ($P = 0.09$), but did reach significance after adjusting for baseline ($d = 0.76$; $P = 0.013$).

The two smoking conditions also differed in the pattern of smoking within the day (Fig. 1). Analysis showed a significant effect of group ($P = 0.008$), a linear day × group interaction ($P = 0.001$) and a linear time of day × group interaction ($P < 0.001$). The within-day increase in smoking rate was much more pronounced for the Nic than for the Denic group; these group differences in within-day smoking patterns did not change over days of exposure.

Biochemical analysis verified the self-report smoking data. CO differed across groups ($P < 0.001$) with both linear day by group ($P = 0.035$) and linear time of day × group interactions ($P < 0.001$). Contrasts focused on the two smoking groups indicated that the Nic group had higher CO levels than the Denic group overall ($P = 0.026$) and that the differences between the smoking groups increased during the day ($P < 0.001$; Fig. 1).

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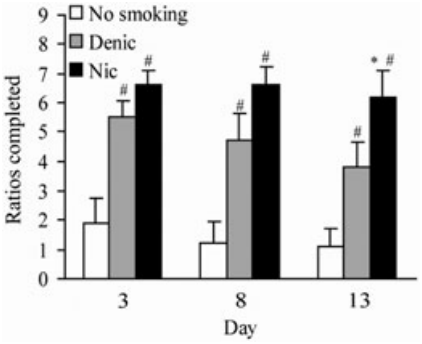


Figure 2 Mean (±SEM) total number of puffs earned during the progressive ratio procedure on days 3, 8 and 13. *Significant difference compared to Denic. #Significant differences compared to no smoking

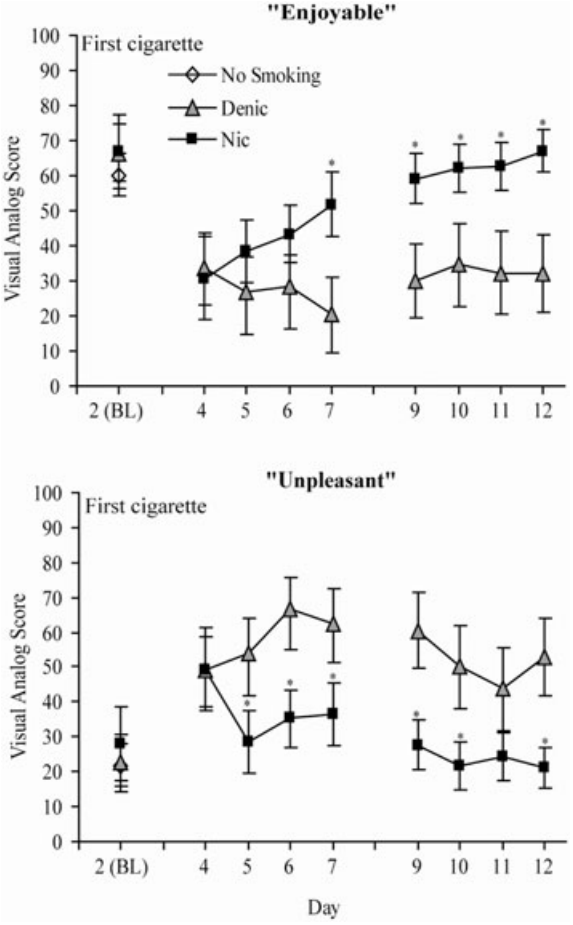


Figure 3 Mean (±SEM) visual analog ratings of how 'enjoyable' (upper panel) and 'unpleasant' (lower panel) smoking the first cigarette was on each of the unrestricted smoking days. Day 2 represents baseline, preferred brand smoking. Participants were randomized on day 3. *Significant difference compared to Denic

Progressive ratio

The number of ratios completed during the progressive ratio procedure (Fig. 2) differed by group ($P < 0.001$). Both smoking conditions completed significantly more ratios across days than the no smoking condition ($Ps < 0.005$) and there was a trend for the two smoking conditions to differ from each other ($P = 0.058$). Pairwise comparisons revealed differences between each of the smoking conditions and no smoking at each day and a significant decline (i.e. day 3 versus day 13) in the Denic group ($d = 0.73$; $P = 0.025$) that was not observed in either of the other groups. The latter observation contributed to a significant difference between the Nic and Denic condition on day 13 ($d = 0.87$; $P = 0.027$).

Cigarette characteristic ratings

There were no main effects of group on the cigarette effects questionnaire at baseline. There was a significant group × cigarette type interaction for ratings of the item dislike drug effect ($P = 0.004$). Pairwise comparisons revealed that ratings of dislike drug effect were higher in the Nic than both the no smoking and Denic groups after the third cigarette ($Ps < 0.05$).

After randomization, analysis of the raw data from cigarettes 1 and 3 on unrestricted smoking days indicated that participants assigned to the Nic group reported significantly higher ratings of pleasant ($P = 0.004$), satisfying ($P = 0.009$), like cigarette ($P = 0.012$) and enjoyable (Fig. 3; $P = 0.007$), and lower ratings of unpleasant (Fig. 3; $P = 0.012$), harsh ($P = 0.034$) and dislike cigarette ($P = 0.007$). Furthermore, some differences emerged or increased over days. These included significant group differences in the linear effect of day on pleasant ($P = 0.043$), calming ($P = 0.043$), less irritable ($P = 0.048$), more awake ($P = 0.033$) and enjoyable ($P = 0.030$) and trends for dislike drug effect, satisfying, like cigarette and comforting ($Ps < 0.10$). There was also a significant quadratic day × group interaction on like cigarette ($P = 0.034$) and dislike drug effect ($P = 0.033$) and trends for dislike cigarette and enjoyable ($Ps < 0.10$). For all group × day interactions except dislike drug effect and dislike cigarette, ratings were higher in the Nic group and these differences increased over days. Analysis of the linear and quadratic effects of day within the Denic condition generally confirmed that there was little change over days; there was a linear decrease in lightheadedness ($P < 0.05$) and an increase in dislike drug effect that slowed over time (quadratic effect; $P < 0.05$). In contrast, analysis of the Nic group revealed significant linear increases in pleasant, like taste, satisfying, calming, like cigarette, relaxing, comforting and enjoyable and linear decreases for unpleasant, dislike taste and dislike cigarette over days ($Ps < 0.05$).

Several items also revealed a group × cigarette interaction. Group differences (Nic > Denic) were greater

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Table 1 Mean (SEM) ratings of cigarette characteristics during the controlled puffing procedure.

	Denic			*Nic*		
	Day 3	*Day 8*	*Day 13*	*Day 3*	*Day 8*	*Day 13*
Like cigarette	25.9 (7.7)	36.4 (10.5)	31.8 (9.1)	34.3 (7.1)	50.5 (9.3)	64.9 (9.4)
Like taste	30.3 (7.5)	39.6 (9.1)	28.4 (7.2)	33.6 (6.4)	38.0 (6.2)	56.5 (10.0)
Pleasant	34.1 (8.0)	34.4 (6.4)	39.0 (7.3)	46.1 (8.0)	52.7 (10.9)	64.5 (10.2)
Pleasurable excitement	26.5 (6.3)	27.8 (9.0)	24.6 (6.9)	39.4 (8.0)	39.1 (9.4)	50.2 (10.2)
Enjoyable	30.6 (7.7	30.9 (9.3)	24.9 (7.6)	35.4 (6.8)	44.5 (9.9)	61.8 (10.7)
Sense of wellbeing	35.1 (8.0)	39.4 (8.5)	32.0 (8.4)	45.6 (10.2)	27.8 (7.6)	41.6 (10.6)
Exhilarating	24.7 (5.7)	28.2 (8.6)	22.3 (6.2)	29.4 (8.5)	24.7 (6.8)	40.7 (8.7)
Calming	36.6 (8.5)	34.4 (6.9)	31.8 (7.6)	45.3 (7.2)	38.2 (8.1)	50.0 (8.8)
Easier to concentrate	35.1 (8.0)	32.5 (6.8)	31.1 (7.0)	31.9 (7.5)	39.8 (8.6)	48.0 (9.4)
Relaxing	31.9 (7.3)	38.3 (8.8)	33.3 (6.9)	41.6 (7.7)	37.7 (7.8)	51.5 (7.6)
Comforting	34.4 (8.2)	38.3 (9.4)	33.5 (7.1)	38.1 (9.2)	40.0 (8.5)	53.5 (7.7)
High in nicotine	26.3 (5.1)	37.5 (8.1)	37.7 (7.7)	40.3 (8.7)	38.2 (8.9)	31.5 (9.4)
Dislike cigarette	69.6 (8.8)	58.5 (10.7)	64.8 (9.4)	48.9 (9.7)	44.4 (10.8)	33.1 (9.5)
Dislike taste	52.7 (10.9)	57.0 (10.5)	67.4 (9.0)	50.2 (11.0)	53.5 (8.8)	40.6 (11.2)
Harsh	37.0 (7.7)	45.6 (9.2)	46.6 (8.3)	39.1 (11.5)	26.3 (9.6)	21.8 (8.0)
Nauseating	18.9 (6.1)	18.1 (7.1)	28.8 (9.1)	6.0 (2.1)	11.7 (7.4)	13.4 (7.0)
Like drug effect	28.6 (5.9)	28.8 (7.8)	28.0 (7.1)	21.3 (6.3)	32.5 (9.5)	45.1 (8.5)

after the third compared to the first cigarettes of the day for relaxing ($P = 0.007$). Ratings of sense of wellbeing, enjoyable and calming showed a similar pattern; however, the third cigarette resulted in greater group differences only during the first few days of the study, after which group differences were similar across cigarettes (group × cigarette × linear day interaction: Ps < 0.05). There was also a group × cigarette interaction for dizziness ($P = 0.027$) and light-headedness ($P = 0.011$). In these cases, ratings were similar after the first cigarette but decreased significantly between the first and third cigarettes in the Nic (Ps < 0.01) but not the Denic group. Adjusting for baseline generally confirmed the results presented above.

Group differences in ratings of cigarette effects after the controlled puffing procedure were similar to those observed during unrestricted smoking (Table 1). Smoking nicotine cigarettes tended to produce fewer negative effects and more positive effects than de-nicotinized cigarettes; some of these differences emerged or became exaggerated over days. There were non-significant trends (Ps < 0.10) for higher ratings of pleasant, like cigarette, pleasurable excitement and enjoyable in the Nic condition, a significant interaction between group and day for like taste ($F_{2,36} = 3.31$, $P < 0.05$) and non-significant trends for group × day interactions for sense of wellbeing, exhilarating and enjoyable (Ps < 0.10). Conversely, ratings of dislike cigarette were significantly lower overall in the Nic group ($F_{1,18} = 4.62$, $P < 0.05$). Pairwise comparisons of responding on each of the test days indicated significantly greater ratings of pleasant, like taste, calming, easier to concentrate, like cigarette ($d = 1.14$), relaxing, comforting, exhilarating, pleasurable excitement and enjoyable ($d = 1.26$) in the Nic than in the Denic group on day 13 ($P < 0.05$). In contrast, the Denic group exhibited significantly greater ratings of dislike taste, harsh, dislike cigarette ($d = 1.06$) and nauseating compared to the Nic condition on day 13 ($P < 0.05$). Significant pairwise comparisons were restricted entirely to day 13. For most of the 'positive' effects (e.g. like taste, like drug effect, like cigarette, easier to concentrate, enjoyable), ratings changed little in the Denic group over time, but increased significantly from day 3 to day 13 in the Nic group. Notably, there were no significant group differences in ratings of high in nicotine.

Craving and withdrawal

Preliminary analyses of background craving and withdrawal taken at 2 p.m. on unrestricted smoking days did not reveal any baseline (day 2) differences on the QSU or the SJWS. After randomization, factor 2 scores in the no smoking group tended to be higher than the Denic condition ($P = 0.084$) and were significantly higher than the Nic condition ($P = 0.028$), but did not differ between the smoking groups. No significant overall main effects of group were found for factor 1 of the QSU or any of the subscales of the SJWS. Pairwise comparison revealed a trend for greater SJWS craving in the no smoking group compared to both the Denic ($P = 0.069$) and Nic ($P = 0.092$) conditions, but no differences between the

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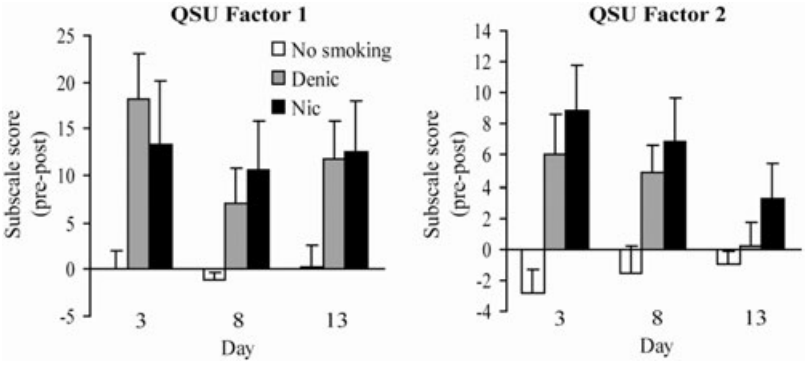


Figure 4 Mean (±SEM) change (pre/post smoking) in factors 1 and 2 subscale scores of the QSU during the controlled puffing procedure on days 3, 8 and 13. Data are presented as change scores for clarity. Analyses were conducted on the raw data and are reported in detail in the text. Briefly, analyses revealed significant differences in the change from pre- to post-smoking for both the Denic and Nic conditions compared to no smoking ($P < 0.05$). No differences were found between the two smoking conditions

two smoking conditions. Analysis of the data after adjusting for baseline revealed a similar pattern of effects.

Craving was also assessed prior to and after the first and third cigarettes each day as measured by the Schuh–Stitzer VAS. These data were not related significantly to group at baseline. After randomization, all differences between the smoking groups were modulated by day. Specifically, there was a linear day × group interaction for 'How pleasant would a cigarette be right now?', 'How much do you want to smoke right now?' and 'How much do you crave a cigarette right now?' (Ps < 0.05) and a quadratic day × group interaction for 'How much do you want to smoke right now?' and 'How much do you crave a cigarette right now?' (Ps < 0.05). Each of these measures indicated that craving decreased slightly more over days in the Denic than the Nic group; however, this magnitude of these differences was small. More importantly, the groups did not differ in the degree to which smoking acutely suppressed craving (i.e. pre/post). There was a significant decrease in all five items of the Schuh–Stitzer after smoking, but this effect was not modulated by group and did not change significantly over days.

The QSU data also revealed suppression of craving after controlled puffing of both types of cigarettes, but little difference in the magnitude of craving suppression. Both nicotine and de-nicotinized cigarettes reduced factor 1 and factor 2 scores compared to not smoking (Ps < 0.01) and the magnitude of craving suppression did not differ between the smoking groups (Fig. 4).

The SJWS administered during the controlled puffing sessions revealed significant effects of group on craving, physical symptoms and appetite. As with the QSU, smoking in the Nic or Denic groups decreased ratings of craving on the SJWS to a similar degree, both producing a significant decrease relative to not smoking (Ps < 0.01). Disruptions in appetite were smaller in the no smoking and Denic conditions compared to the Nic group (Ps < 0.05). However, close inspection of the data revealed that the smoking groups differed prior to smoking on day 3, suggesting that group differences were not a result of the cigarette assignment, but pre-existing differences. Finally, there was a significant decline in physical symptoms after smoking nicotine ($P = 0.048$) but not de-nicotinized cigarettes compared to not smoking. Direct comparison of the two smoking groups, however, failed to reveal any significant differences in physical symptoms.

Estimates of nicotine content

Estimates of nicotine content immediately prior to discharge did not differ significantly between the two smoking conditions, although there was a trend for ratings of nicotine content to be higher in the Nic condition ($P = 0.099$).

Mood measures

There were no reliable differences between groups on the POMS or PANAS. Close inspection of the no smoking and Denic conditions indicated a trend for an increase in POMS-tension, POMS-depression and POMS-total mood disturbance compared to both baseline and the Nic condition, but these differences were small and failed to reach statistical significance. There were no reliable differences in sleep quantity or quality.

Physiological measures

Smoking nicotine-containing cigarettes resulted in a greater increase in heart rate than either smoking de-nicotinized cigarettes ($P = 0.006$) or not smoking ($P < 0.001$). Heart rate also increased across days more in the Nic than the no smoking group ($P = 0.019$). Interestingly, comparison of the no smoking and Denic conditions revealed a group × pre/post interaction such that de-nicotinized cigarettes produced a small but significant increase in heart rate compared to not smoking ($P = 0.014$). There were no reliable group differences in blood pressure.

DISCUSSION

The exclusive, repeated availability of research cigarettes either containing or not containing nicotine differentially

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influenced smoking behavior, the motivation to smoke and the subjective effects of smoking. Throughout the study, participants assigned to the Nic group smoked at levels that were similar to or higher than baseline rates of preferred brand smoking, increased their puff volume and showed no change in the motivation to smoke over repeated progressive ratio tests. Puff volume also increased in participants in the Denic group; however, both their rate of smoking and their motivation to smoke declined over time. Switching from the preferred brand to either novel research cigarette produced an immediate decrease in positive and increase in negative subjective effects regardless of nicotine content. Subsequent ratings changed little over repeated exposure in the Denic group; on parallel measures (e.g. like/dislike cigarette), negative ratings exceeded positive ratings of the assigned cigarettes throughout the study. In contrast, positive ratings of the nicotine-containing cigarettes increased and negative ratings declined with repeated exposure. From the second day of use onwards, participants in the Nic condition reported an overall liking for their assigned cigarettes (i.e. liking > disliking). Notably, the nicotine content of cigarettes had little effect on the suppression of craving by smoking. De-nicotinized cigarettes continued to suppress craving acutely even after 8 days of access.

It is important to note that partial, not complete, extinction of the reinforcing effects of smoking was observed in the present study. While the number of cigarettes smoked during unrestricted access and puffs earned during the progressive ratio session decreased over days in participants smoking de-nicotinized cigarettes, most participants (80%) continued to smoke. Consistent with the present findings, Buchhalter *et al.* [14] also observed a small (non-significant) reduction in smoking over a 5-day out-patient period as assessed by cigarette butts returned. Whether longer periods of exposure would lead to more complete extinction is unknown.

In contrast to the behavioral data, we found no evidence of extinction of the subjective effects of smoking. These data diverge from those reported by Rose & Behm [15]. In that study, the subjective rewarding effects of usual brand smoking were reduced after 2 weeks of exposure to de-nicotinized cigarettes. However, numerous differences make comparison of these studies difficult. Participants in the Rose & Behm study did not smoke de-nicotinized cigarettes exclusively during the 2-week exposure period and change in the subjective effects of the de-nicotinized cigarettes themselves was not assessed. Relatedly, our failure to observe a similar decrease in the positive subjective effects of smoking could be due to a floor effect. Positive subjective ratings were relatively low throughout the present study in participants smoking de-nicotinized cigarettes; in contrast, ratings of usual brand cigarettes in the Rose & Behm study were substantially higher, leaving adequate room to observe a decrease.

The efficacy of de-nicotinized cigarettes to suppress craving was indistinguishable from nicotine-containing cigarettes throughout the study. This observation suggests that craving-suppression may be a particularly robust effect of de-nicotinized cigarettes that is less sensitive to extinction procedures. Consequently, relief from craving may be an important mechanism underlying continued smoking of de-nicotinized cigarettes (i.e. negative reinforcement). Whether even longer periods of exposure would lead to extinction of the craving-suppressing effects of smoking may have implications for the use of smoking stimuli in therapeutic settings.

Withdrawal symptoms other than craving tended to be elevated in both the no smoking and Denic conditions compared to baseline and the Nic condition, but these differences failed to reach significance; therefore, it was difficult to determine whether de-nicotinized cigarettes suppressed withdrawal [14] and whether withdrawal-suppression changed over days. The failure to observe significant withdrawal may have resulted from limited statistical power ($n = 10$ per group) and/or our use of the SJWS. Previous residential studies with larger sample sizes, within-subject designs and utilizing different scales have demonstrated reliable nicotine withdrawal [25,26], suggesting that future studies can effectively address this issue.

There is little experimental evidence supporting the widely held assumption that smoking stimuli acquire their effects through classical conditioning. The present study lends support to this hypothesis by demonstrating that smoking stimuli partly lose their reinforcing effects in the absence of nicotine (i.e. extinction) and that new stimuli acquire similar effects after being paired with nicotine. However, at least two additional hypotheses must also be considered. First, nicotine may contribute to smoking through its sensory effects [27]. Contrary to this hypothesis, the two smoking conditions reported similar initial ratings of taste which diverged only after repeated exposure; this finding is more consistent with learning a new association between taste and nicotine delivery in the Nic condition than stimulus degradation. Another possibility is that nicotine has other actions on the central nervous system that contribute to smoking behavior. Recent animal research has suggested that nicotine can non-associatively enhance reinforcement obtained from both conditioned [28] and unconditioned [29–32] reinforcers. Therefore, nicotine-containing cigarettes may maintain a higher level of smoking not because of nicotine's primary reinforcing effects but because nicotine directly enhances the conditioned reinforcing effects of sensorimotor smoking stimuli or other reinforcers in the

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environment. Additional studies are needed to address this hypothesis directly.

Several limitations are worth noting. First, the use of an in-patient design limits generalizability because the determinants of smoking may differ between in-patient and naturalistic settings. In addition, the population sampled is more likely to be unemployed and/or from a lower socio-economic bracket than many smokers. Secondly, participants had moderate levels of dependence; whether these results would generalize to individuals of lower or higher levels of dependence is a question we intend to address in future studies. Finally, we assume that changes observed over days were a function of exposure to the smoking condition. However, change could also reflect either practice or learning effects, as time is confounded with repeated testing in the present design, or a change in the drive to smoke as time since initial nicotine abstinence elapses and withdrawal begins to subside in the Denic group.

In summary, repeated exposure to de-nicotinized cigarettes resulted in a gradual and incomplete reduction in the number of cigarettes smoked and in the motivation to smoke, but little change in their ability to suppress craving. This profile of effects highlight the important impact of non-nicotine sensorimotor stimuli as a determinant of smoking behavior and suggests that extinction of these effects in the absence of smoking does occur but may be slow. While the present study examined the longest duration of exposure to de-nicotinized cigarette smoking to date, additional research will be needed to follow these effects over even longer periods of time.

Acknowledgements

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In Vitro Model of Platelet-Endothelial Activation Due to Cigarette Smoke Under Cardiovascular Circulation Conditions

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Abstract—Cigarette smoke has been shown to increase platelet activation and endothelial cell (EC) adhesion molecule expression. In the present study, we utilized a hemodynamic shearing device (HSD) to investigate the above effects *in vitro* in a combined system of platelets and cultured HUVECs (Human Umblical Vein ECs) under physiological shear stress. We investigated the alteration of E-selectin expression on ECs upon exposure to: (1) platelets and nicotine-free smoke extract (NFE), (2) platelets alone, (3) NFE alone, under physiological shear stress. We additionally confirmed the protective effect of nicotine on platelet activation. We found that: (i) surface expression of E-selectin on ECs was significantly increased upon simultaneous exposure of ECs and platelets to NFE relative to exposure of ECs to either platelets or NFE alone ($p < 0.05$). (ii) Platelet activation was significantly increased in the presence of NFE ($p < 0.05$). (iii) Nicotine (200 nM) when added to NFE, significantly reduced platelet activation due to NFE ($p < 0.05$), an effect additionally confirmed by conventional cigarette extracts which contain nicotine ($p < 0.05$). We therefore conclude that: (a) NFE and platelets additively increase EC E-selectin surface expression, and (b) nicotine modulates platelet activation regardless of ECs.

Keywords—Cardiovascular diseases, Inflammation, Nicotine, Couette, Cone and plate, Viscometers.

INTRODUCTION

Cigarette smoking has been well established as a risk factor for development of cardiovascular disease.[1,9] A major part of this risk involves increased platelet activation due to cigarette smoke, as has been investigated both *in vivo*[18] and *in vitro*.[23] In two studies involving platelets alone, we have shown that this smoke-induced platelet activation is largely due to the non-nicotine smoke components and their effects can be modulated in the presence of nicotine.[22,23] While the direct desensitizing effect of nicotine on platelet activation is likely associated with reduced cardiovascular disease risk, such investigations need to be extended to a more physiologically relevant scenario to include the presence of the vasculature (endothelial cells (ECs), smooth muscle cells, and extracellular matrix). Additionally, this should be investigated under physiological shear stresses, as those play an important role in the effects of cigarette smoke and nicotine on platelets.

Prior *in vitro* studies have shown that high concentrations of cigarette smoke extract[6,28,29] and nicotine[32–34] increase adhesion molecule expression on ECs. These studies preclude the involvement of physiological shear stresses. Whether inflammatory effects due to smoke extract exposure may be attributed to its nicotine or nicotine-free components also remains unknown. Thrombin-activated platelets when introduced over cultured ECs bind to the cultured cells by several adhesion (receptor–ligand pairs) molecules[4,5,16,31] and may promote increase in EC adhesion molecule expression by downstream signaling. A link has been established between platelet factor-4 and endothelial E-selectin expression,[35] resulting from activation of nuclear factor-kappa B. The same mechanism has been reported for E-selectin expression on ECs due to cigarette smoke.[28] It is likely that if smoke extract and activated platelets both increase E-selectin expression on ECs by similar or distinct mechanisms, the levels of E-selectin surface expression on ECs may be significantly enhanced by their combined activity. Following such hypothesis, ECs simultaneously exposed to smoke extracts and smoke-activated platelets will be expected to increase EC adhesion molecule expression as a result of their combined activity.

The mechanical stress conditions (both fluid shear stress and cyclic strain) largely dictate the development of either pro- or anti-inflammatory phenotype of ECs.[7,12] Investigation of dynamic flow-dependent effects on cultured cells *in vitro* involves the use of devices that impart minimal inertial effects to the fluid

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and avoid re-circulation of fluid. The cone and plate viscometer and the annular Couette viscometer have been extensively used to study spatio-temporal effects of fluid shear stresses on cultured and circulating cells, respectively. Of note is the unique ability of the cone-plate viscometer to study both physiological and pathophysiological flow regimes within the same device,[8] while controlling any secondary flow effects.[27] If the two devices can be combined, both cultured cells and cell suspensions can be subjected to the same shear stress and the effects of interaction between them can be investigated by periodic sampling and subsequently analyzed.

In the present study, we utilize a hemodynamic shear device (HSD)[19] that combines features of the cone-plate with those of the annular Couette viscometer (cross-sectional view, Fig. 1a). The basic advantage of this device is that uniform and identical shear stress levels are maintained in both the cone-plate and Couette regions, and it facilitates convenient periodic cell sampling from the Couette region. We have recently used this device to study fluid shear-induced platelet activation under programmed shear stress.[19] In the present study, we test the hypothesis that platelets and the nicotine-free extract would confer an additive or enhanced inflammatory effect on ECs, by investigating the effects of cigarette smoke extract and platelets together on ECs under physiological shear stresses within the HSD. We additionally hypothesize that in contrast to conventional cigarette extracts, nicotine-free smoke extract would increase platelet activation more significantly, and that this effect may be independent of the presence of ECs.

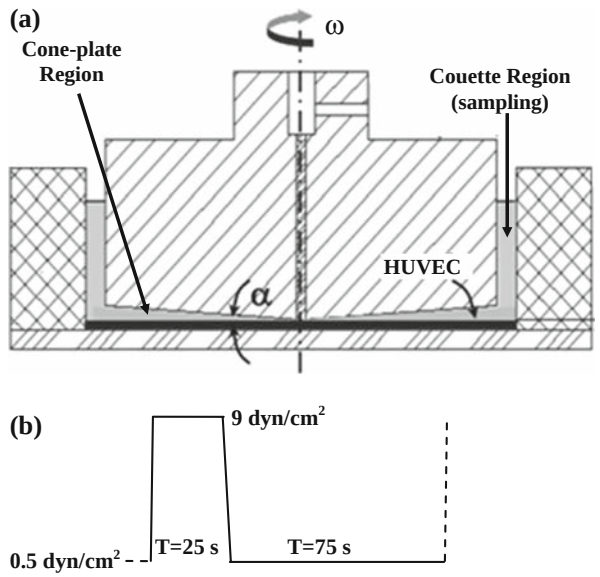


FIGURE 1. (a) (Adapted and modified from Nobili et al (2008) [19]) Cross-sectional view of the hemodynamic shear device (HSD). Platelets ± ECs are exposed to identical shear stress in the HSD. ECs are cultured on disks on top of the base-plate, platelets are sampled from the Couette region for PAS. The cone angle is represented by α, and rotational speed of the cone by ω. (b) Shear-stress waveform applied repeatedly for 33 cycles (100 s each) to the ECs and platelets in HSD.

METHODS

Reagents

Thrombin Receptor Activator Peptide-6 (TRAP-6), nicotine hydrogen tartrate salt, human fibronectin, EDTA, bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4), 10% paraformaldehyde (PFA, EM grade), and Trypsin-EDTA from Sigma (St. Louis, MO); FITC-conjugated mouse anti-human-E-selectin antibody from BD Biosciences (Franklin Lanes, NJ); Human Umbilical Vein Endothelial Cells (HUVECs) from Lonza (Walkersville, MD); Cigarettes—Quest 1 and 3 (Vector Tobacco, Mebane, NC) and Marlboro Red (Philip Morris, Richmond, VA) from a local pharmacy, glycol-modified polyethylene terepthalate (PETG) sheets from McMaster Carr (Robbinsville, NJ), Tumor Necrosis Factor-α (TNF-α; R&D Systems, Minneapolis, MN).

Smoke Extracts

Mainstream smoke extracts from two cigarettes of the same type were prepared in 100 mL of Hepes Buffered Saline (HBS, pH 7.4) as described previously,[23] with a small modification—the smoke was being bubbled through HBS in a vertical column (100 mL measuring cylinder) instead of a beaker used earlier, to facilitate a more efficient transport by increasing smoke-HBS contact time. The following three types of cigarettes corresponding to zero, medium, or high nicotine were used to prepare the three extract solutions: Quest-3 (nicotine-free extract or NFE, 10 mg tar, 0.05 mg nicotine), Quest-1 (medium-nicotine extract, 10 mg tar, 0.6 mg nicotine), Marlboro Red (high-nicotine extract, 16 mg tar, 1.2 mg nicotine). A final concentration of 10% extract was used in all studies.

Platelets

Thirty mL of citrated whole blood was obtained from adult human volunteers after informed consent. Platelet-rich plasma (PRP) was obtained after centrifugation at $300 \times g$ for 6 min, and loaded onto a Sepharose 2B-CL column to separate platelets from plasma proteins. Platelets at a concentration of 2×10^8/mL were eluted in citrated buffer as described previously[23] and used within 3 h of purification. The

order of experiments involving platelets from the same batch on a given day was randomly chosen.

Endothelial Cell Culture

HUVECs (passages 5–10) were cultured on human fibronectin (5 μg/mL)-coated PETG disks (diameter: 3 mm) until complete confluence was obtained as indicated by cobblestone morphology and absence of intracellular gaps. Functionality of HUVECs was confirmed by release of von-Willebrand factor (vWf) in culture and under shear stress. For this purpose, matched antibody-pair (primary and secondary, anti-human vWf) was purchased from Affinity Biologicals (Ontario, Canada) for analysis in an ELISA assay. HUVECs did not detach upon exposure to a constant shear stress of 9 dynes/cm^2 and remained confluent throughout the duration of experiments.

Hemodynamic Shear Device (HSD)

A combined annular Couette and cone-plate viscometer setup (Fig. 1a) was utilized to study the interaction between cultured HUVECs and circulating platelets under shear stress. HUVECs, cultured on PETG disks, occupy the base of the cone and the base of the annular Couette regions. The construction and characterization of this device has been described in our recent study.[19] The platelet suspension occupies the space (cone and annular Couette regions) above the HUVECs. The annular gap (740 μm) was determined to maintain identical shear stress levels under the cone and in the Couette regions. Sampling of platelets was performed from this annular Couette region. Capabilities of the cone-plate viscometer as a compact *in vitro* device to investigate a wide range of dynamic flow regimes experienced *in vivo* have been extensively discussed.[8,10] A repeated shear stress cycle of 100 s as indicated in Fig. 1b was used in the study, for a total of about 33 cycles or 54 min per experiment. All experiments were conducted within the HSD at the same shear stress. The objectives were to determine: (1) Expression of E-selectin on ECs upon exposure to platelets and nicotine-free smoke extract, and (2) Change in platelet activation upon addition of nicotine-free smoke extract and modulation of this effect by nicotine. TRAP-6 was used as a positive control in place of nicotine-free smoke extract, for objective 1. The parameters measured are further explained below, and summarized in Tables 1 and 2.

Platelet Activation State (PAS)

In experiments involving determination of platelet activation (Table 2), sampling was performed from the Couette region every 6 min and platelet activity (PAS or platelet activation state) was determined over the duration of the experiment (54 min), by a modified prothrombinase assay as described elsewhere.[13] This measure of platelet activation reflects primarily the release of factor Va from the α granules to the surface, plus the exposure of anionic phospholipid upon activation.[25] This method has been demonstrated to correlate well with platelet Annexin-5 expression,[14] as determined by flow cytometry. The results were normalized to maximum platelet activity (PAS determined after sonication of the platelet suspension for 10 W at 10 s) for the particular batch of platelets. Additionally, another parameter (Platelet Activation Rate or PAR) was determined to investigate whether PAS changes

TABLE 1. Summary of experiments for investigation of E-selectin expression on: (A) ECs with platelets and nicotine-free smoke extract and (B) ECs with platelets and TRAP-6.

Objective: Investigation of E-selectin expression on ECs by flow cytometry

Cells in HSD	Nicotine-free extract (10%)	Parameter
(A)		
EC	+[a]	E-selectin
EC	–[a]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin
Cells in HSD	**TRAP-6 (100 μM)**	**Parameter**
(B)		
EC	+[b]	E-selectin
EC	–[b]	E-selectin
EC + platelets	+	E-selectin
EC + platelets	–	E-selectin

[a]The signs (+/–) indicate the presence or absence of extract in the experiment.
[b]The signs (+/–) indicate the presence or absence of TRAP in the experiment.

TABLE 2. Determination of platelet activation state (PAS) for platelets exposed to the nicotine-free extract, two commercial cigarette extracts (Quest-1 and Marlboro), control and pure nicotine added to the nicotine-free extract.

Objective: Effect of nicotine on platelet activation state (PAS)

Cells in HSD	Type of smoke extract (10%)	Parameter
Platelets ± EC	NFE (Nicotine-free/Quest-3)	PAS, PAR
Platelets ± EC	NFE + 200 nM nicotine	PAS
Platelets ± EC	Medium-nicotine (Quest-1)	PAS
Platelets ± EC	High-nicotine (Marlboro)	PAS
Platelets ± EC	–	PAS, PAR

Platelet activity rate (PAR) was determined for selected conditions to exclusively determine the effect due to nicotine-free extract on platelets. Note that identical studies were performed for platelets alone and platelets + ECs, as indicated by ± sign.

more rapidly with time upon addition of nicotine-free extract.

E-Selectin Expression on ECs

At the end of studies outlined in Table 1, HUVECs were gently washed with pre-warmed (37 °C) PBS to remove any bound platelets, trypsinized, washed with FACS buffer (5 mM EDTA, 1% BSA, 4 °C, pH 7.4) and fixed with 4% PFA on ice. Prior to flow cytometry, the HUVEC samples were washed and resuspended (1×10^5/mL, FACS buffer) and incubated with saturating concentration (20 μg/mL) of mouse-anti-human-CD62E-FITC mAb for 45 min on ice. A total of 10,000 gated events were used for analysis on a BD FACScan flow cytometer. An IgG1-FITC isotype control was performed as a control to ensure binding specificity. Change in E-selectin expression for each of the four conditions in study (a) above was determined by fraction of total gated cells positive for E-selectin, relative to control. A total of eight experiments were conducted for each set of conditions (Table 1).

Statistical Analyses

All statistical analyses for multiple comparisons of PAS, PAR, and E-selectin expression on ECs, were performed with two-way or one-way ANOVA (SPSS Inc., Chicago, IL). Post-hoc tests to determine individual statistical differences within groups were conducted after Bonferroni's correction. A significance level of $p = 0.05$ was used for all analyses. Specifically, the following parameters across experimental groups were compared:

Platelet Activation State (PAS): The PAS values at times $t = 0$ min and $t = 54$ min obtained for all experimental conditions (Table 2) were averaged over total number of experiments ($n = 5$). Separate comparisons were made for PAS differences at the start ($t = 0$ min) and the end of the experiment ($t = 54$ min) for each set of conditions. We conducted a two-way ANOVA to investigate whether PAS was affected by (a) smoke extracts or nicotine, and (b) presence of ECs.

Platelet Activation Rate (PAR): To examine the effect of nicotine-free smoke extract on platelet activation with time, the PAS values obtained during the 54-min experiment for four conditions (Table 2) were fit to a two-degree polynomial curve. We additionally compared goodness of fit of this data between a 2-degree polynomial and a linear model.

E-Selectin Expression on ECs: E-selectin positive ECs were determined from cytometry for each condition in Table 1. The data obtained from cytometry corresponding to % positive gated cells for each condition fit better to a log-normal distribution (Shapiro-Wilk and Kolmogorov-Smirnov test for normality). The data was therefore log-transformed before multiple comparisons were made with one-way ANOVA separately for the two groups (Table 1A and 1B).

RESULTS

Platelet Activation State (PAS) Increases with Nicotine-Free Smoke Extract

We investigated platelet activation (PAS) in the presence of 10% nicotine-free smoke extract (NFE) and shear stress, both for platelets alone (Fig. 2a) and platelets with ECs (Fig. 2b). The data for each plot represents an average of five experiments. The corresponding results for the average PAS have been reported in Table 3. We found that platelet activation (PAS) was significantly increased upon addition of the nicotine-free extract ($p = 0.001$).

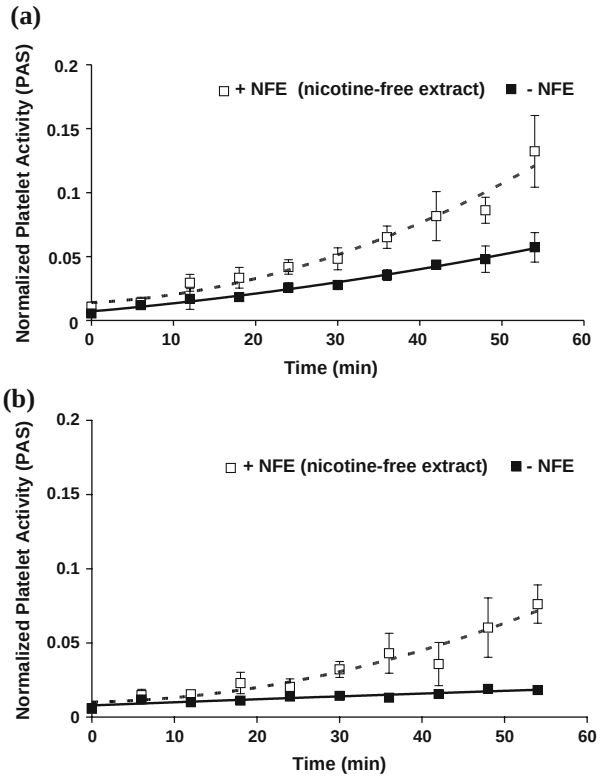


FIGURE 2. Platelet activation state (PAS) over 54 min in the HSD for (a) platelets (±smoke) and (b) platelets + ECs (± smoke). Note 10% nicotine-free smoke extract (NFE) was used. The data are fit to a 2-degree polynomial curve and the average platelet activation rates (PAR) are illustrated in Table 3.

TABLE 3. Summary of platelet activation (PAS and PAR, mean ± s.e.m.) upon exposure to nicotine-free smoke extract.

Condition	PAS at $T = 54$ min	PAR (1×10^{-5} min^{-1})
Platelets	0.06 ± 0.01	1.00 ± 0.60
Platelets + NFE	0.13 ± 0.03	3.02 ± 1.00
Platelets, ECs	0.02 ± 0.00	0.02 ± 0.15
Platelets, ECs + NFE	0.08 ± 0.01	3.00 ± 1.00

All experiments were done under the same shear stress in the HSD.

Platelet Activation Rate (PAR) Increases with Nicotine-Free Smoke Extract

We found that the 2-degree polynomial model was a better overall fit for our PAS data in 3 out of 4 cases shown in Fig. 2 and reported above: $p = 0.01$, 0.02 (platelets ± NFE) and $p = 0.87$, 0.04 (platelets + ECs ± NFE). To maintain consistency, we determined the PAR corresponding to the curvature or quadratic coefficient for the fit for each condition, prior to statistical test with two-way ANOVA. We found that PAR increased significantly due to the presence of nicotine-free smoke extract ($p = 0.006$). The average PAR values have been reported in Table 3.

Control Experiments for Initial Platelet Activation State (PAS at $t = 0$ h)

To ensure that purified platelets did not activate significantly over the 3 h time course of use each day, we performed control experiments and measured the platelet activity overtime with the modified prothrombinase assay. We found that during this time course platelets activated less than 1% when left on the shaker. The mean (±s.e.m.) PAS for this control experiment (repeated 3 times with different platelet batches) at times $T = 0$, 1, 2, 3 h was found to be 0.0051 ± 0.0016, 0.0051 ± 0.0007, 0.0099 ± 0.0021, and 0.0071 ± 0.0009, respectively. Additionally, we compared the PAS data at the beginning of all our experiments as outlined in Table 2 (i.e., PAS at $t = 0$ min) and found that the initial level of platelet activation did not differ between different experimental groups and conditions ($p > 0.05$) and was less than 1% as found in control experiments above. The mean (±s.e.m.) starting PAS ($t = 0$ min) was calculated as 0.0082 ± 0.0008.

E-Selectin Expression on ECs

Three representative cytometry histograms for expression of E-selectin on ECs, when exposed to (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line), are shown in Fig 3a. In all cases, the shift in fluorescence intensity histogram is distinct upon simultaneous exposure of ECs to platelets and smoke (condition iv, thick solid line). The average increase in E-selectin expression for each of these conditions is illustrated in Fig. 3c. The data is an average of eight individual experiments, and mean ± s.e.m values for % positive gated cells are shown. This was statistically confirmed by ANOVA over the log-normalized data for the four conditions mentioned above. The pair-wise differences were then determined after post-hoc Bonferroni's correction after significance was established with ANOVA ($p = 0.001$), and the results are illustrated in Table 4. E-selectin expression obtained upon concurrent exposure of ECs to the nicotine-free extract and platelets, was significantly different from all other conditions and proves the hypothesis of current study.

To confirm this additive effect, another agonist (TRAP-6) which is known to maximally activate P-selectin expression on platelets,[11] was used in place of NFE and the above study was repeated. In contrast with smoke-activated platelets that had a final PAS of 6%, TRAP-6 activated platelets had a PAS of over 90% for all experiments. It should be noted that despite the high PAS, in the absence of fibrinogen there was no platelet aggregation in these studies (confirmed by flow cytometry to P-selectin). An identically statistically significant effect was observed with TRAP-6 ($p < 0.05$, statistical data not shown) as reported above for NFE. As above, three representative cytometry histograms for the expression of E-selectin on ECs when NFE is replaced with TRAP-6 are shown in Fig. 3b. The shift in fluorescence intensity when ECs are concurrently exposed to platelets and TRAP-6 is distinctly identified from individual exposure conditions (Fig. 3b). The average results for % E-selectin positive gated ECs (mean ± s.e.m.) from eight experiments are illustrated in Fig. 3c. Similar statistical results confirming the additive effect were found as in the case of nicotine-free extracts. This further supports the above conclusion that expression of E-selectin on ECs upon concurrent exposure of platelets in combination with the agonist that activates them was significantly increased relative to E-selectin expression obtained upon individual exposure of ECs to either platelets or agonists.

Conventional Cigarette Extracts and PAS

Extracts were prepared from two commercially available cigarettes (that contain nicotine): (1) Marlboro Red (1.2 mg nicotine); and (2) Quest-1 (0.6 mg nicotine) as outlined in the methods. Addition of these

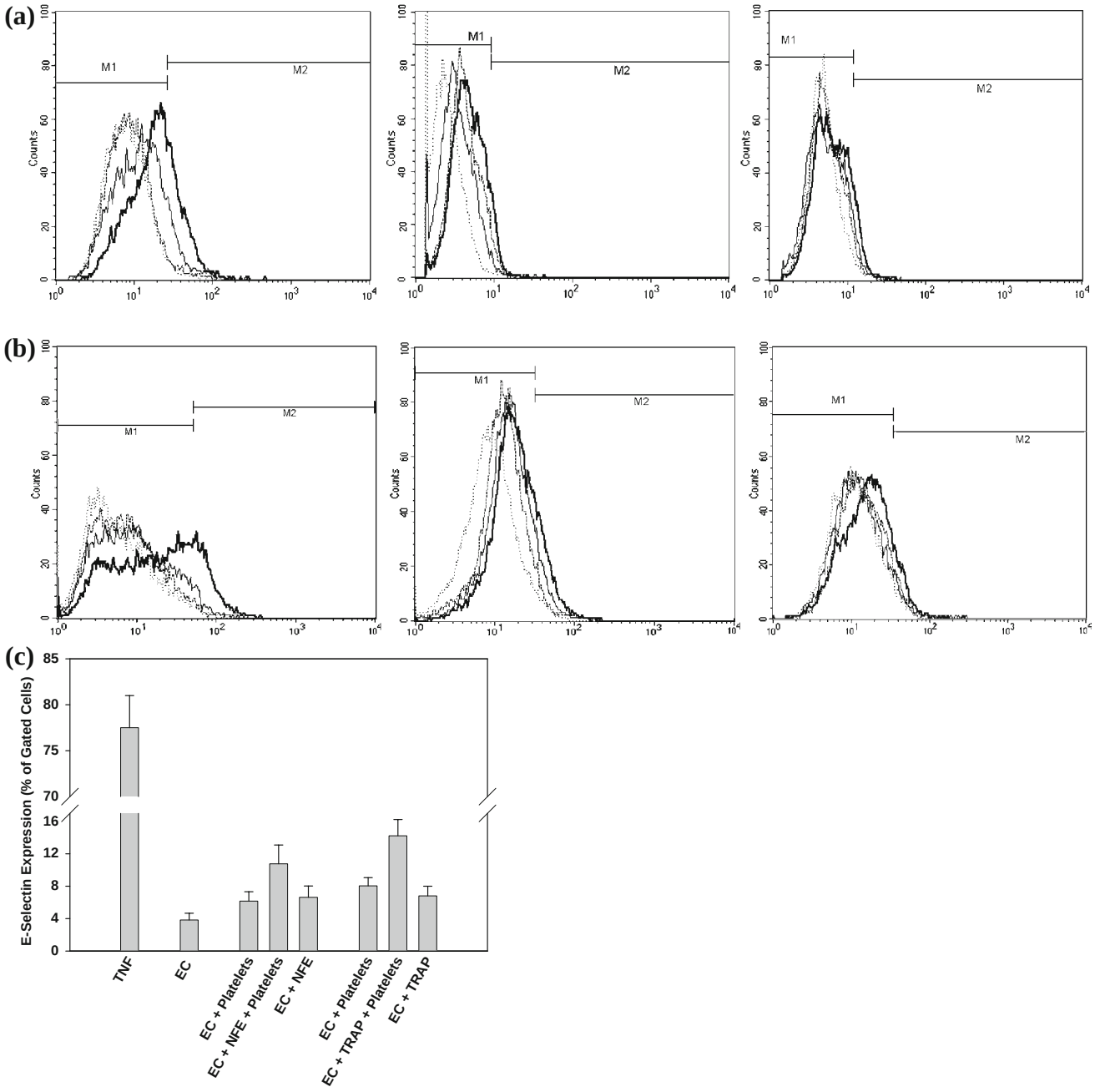


FIGURE 3. (a) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1A: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and NFE (dashed-dotted line), and (iv) shear stress and NFE and platelets (thick solid line). (b) Three typical cytometry histograms for E-selectin expression on ECs subject to conditions outlined in Table 1B: (i) shear stress alone (dotted line), (ii) shear stress and platelets (solid line), (iii) shear stress and TRAP-6 (dashed-dotted line), and (iv) shear stress and TRAP-6 and platelets (thick solid line). (c) Summary of % E-selectin positive gated ECs (mean ± s.e.m.) determined by flow cytometry. All experiments (n = 8) were done under shear stress in HSD as per conditions on horizontal axis, except TNF-α control. Smoke (10% NFE) and TRAP-6 (100 μM final) were used where stated.

extracts (10%) in place of the NFE, reduced platelet activation (PAS) relative to the latter ($p = 0.02$, Fig. 4). PAS was different in the presence of ECs (Fig. 4), but this difference was not influenced by the smoke extracts or nicotine ($p = 0.8$). The pair-wise differences between PAS for each condition (Table 2) were ascertained by post-hoc tests and the results are illustrated in Table 5. Despite the higher tar content of Marlboro cigarettes (16 mg) relative to the Quest cigarettes (10 mg), platelet activation was significantly

TABLE 4. Statistical summary for the expression of E-selectin on ECs.

Conditions	EC	EC + NFE	EC + platelets	EC + platelets + NFE
EC	–	1.00	1.00	0.01*
EC + NFE	1.00	–	1.00	0.03*
EC + platelets	1.00	1.00	–	0.01*
EC + platelets + NFE	0.01*	0.03*	0.01*	–

p-values from post-hoc pair-wise statistical tests for E-selectin expression in the presence of nicotine-free smoke extract, corresponding to conditions in Table 1A. Note that (*) indicates statistical difference.

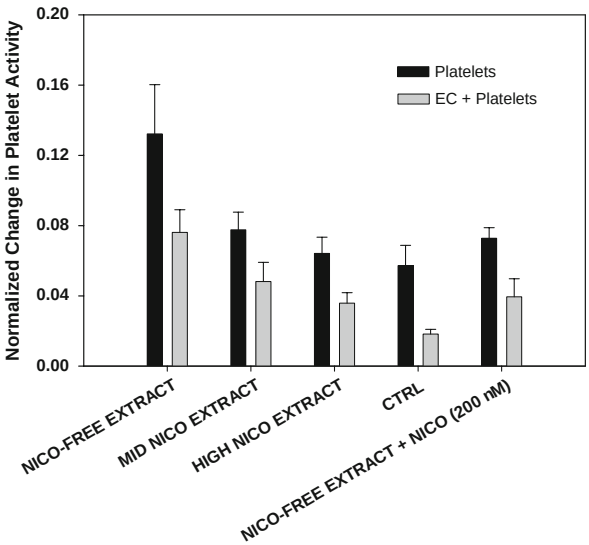


FIGURE 4. PAS for platelets ± ECs, in the HSD for (*left to right*): (1) nicotine-free (10% NFE), (2) medium-nicotine (10%), (3) high-nicotine (10%) extracts, (4) control (no smoke, no nicotine), and (5) condition (1) with pure nicotine (200 nM) added. Data (mean ± s.e.m.) in each bar is representative of five experiments.

reduced by the 2-fold higher nicotine content in the former ($p = 0.001$). Specifically, the PAS measured with Marlboro extract was less than that measured with the NFE by 48% (platelets only) and 47% (platelets + ECs). The PAS measured with the Quest-1 extract was also significantly lower compared to the NFE extract ($p = 0.03$). These findings clearly support the role of nicotine as a key modulator of platelet activation in smokers, at least within the short experimental durations as in the current study.

Further, when 200 nM nicotine was added in addition to 10% NFE, the resulting PAS decreased by 45% (platelets only) and 49% (platelets + ECs) relative to activation in the presence of NFE as reported above ($p = 0.004$). This confirms our earlier findings that nicotine is a key modulator of smoke-induced platelet activation.

DISCUSSION

Hemodynamic Shear Device

Platelet activation and aggregation at elevated shear stresses has been successfully investigated previously in the cone-plate viscometer.[15,30] The interaction between platelets and ECs has also been investigated in parallel-plate flow chambers[17] and also the cone-plate viscometer.[20] Equally well described is the annular Couette viscometer with a narrow annular gap and inner rotating cylinder, which has been used to investigate shear-induced blood damage and hemolysis of red blood cells.[21] The HSD design incorporates features of these two viscometers to maintain the same shear stress and facilitate sampling, as discussed under methods and in our recently published study.[19] In the present study, we used an intermittent shear stress of 9 dynes/cm^2 which is representative of flow in a normal coronary artery. Under these conditions, the flow is laminar ($\tilde{Re} < 0.5$) and secondary flow effects in the cone region therefore may not impose significant

TABLE 5. Statistical summary for the effect of nicotine on platelet activation (PAS at *T* = 54 min).

Conditions	Nicotine-free	Medium-nicotine	High-nicotine	Control	NFE + 200 nM nicotine
Nicotine-free	–	0.03*	0.00*	0.00*	0.00*
Medium-nicotine	0.03*	–	1.00	0.36	1.00
High-nicotine	0.00*	1.00	–	1.00	1.00
Control	0.00*	0.36	1.00	–	1.00
NFE + 200 nM nicotine	0.00*	1.00	1.00	1.00	–

The experimental conditions are described in Table 2. The table shows *p* values, determined from post-hoc pair-wise tests after significant difference for effect of experimental condition on PAS was established with two-way ANOVA ($p = 0.02$). Note that (*) indicates statistical difference.

localized forces and edge effects in the present system.[27] The present system can therefore be utilized to investigate EC-platelet or EC-leukocyte interactions under physiological or pathophysiological shear stresses.

E-Selectin Expression on ECs

We show that nicotine-free smoke extract or TRAP-6, when introduced together with platelets over ECs, result in a relatively greater increase in E-selectin expression on ECs. Although the mechanisms are unknown, it is likely that both smoke extract (or TRAP-6) and platelets contribute to the enhanced expression: (1) Downstream signaling resulting from engagement of receptor–ligand pairs on apposite cells may increase E-selectin expression on ECs. For instance, the presence of endothelial vWf in the cell suspension may act as bridging molecule between platelets and ECs, and this could be supplemented with integrin-like interactions between adhesion receptors on both cell types.[4] (2) ECs have been previously shown to increase E-selectin expression in response to smoke extracts[6] and also by TRAP-6 binding receptors on the surface.[26] We did not get significantly different E-selectin expression with platelets or nicotine-free extract introduced alone over ECs relative to ECs exposed to shear stress (control). However upon simultaneous introduction of platelets and nicotine-free extract on ECs, the E-selectin expression obtained is significantly different from the other three conditions (Table 4). We obtained similar results with TRAP-6 (in place of NFE), thus confirming the above mechanism of additive E-selectin expression.

Peak E-selectin expression on ECs in the presence of pro-inflammatory cytokines such as TNF-α and IL-1-β typically occurs at 4–6 h. The duration of our experiments was 54 min, after which ECs were immediately fixed. This may explain the lower E-selectin expression rates after 54 min in our experiments, but is comparable to levels found after TNF-α treatment beyond 2 h.[3] Such levels may be sufficient to induce leukocyte adhesion and progression of inflammation. The time limitation in our study arises from maintaining identical experimental conditions with the same passage of ECs and batch of platelets for one set of results (Table 1, four conditions) involving four experiments of 54 min each, done on the same day. Nevertheless, a relatively smaller but significant increase in E-selectin is observed and confirms our hypothesis. We may therefore conclude that such expression of E-selectin observed when activated platelets and agonists are introduced together over ECs, may represent the initial inflammatory response similar to the one induced by cytokines.

Modulation of PAS by Nicotine

The addition of pure nicotine to nicotine-free smoke extract has been previously shown to reduce platelet activation due to smoke extracts and shear stress.[22,23] We confirmed this protective effect of nicotine in our present study, albeit in the presence of ECs (Fig. 4). ECs have been known to express a subset (alpha-7) of cholinergic receptors on the surface, which can bind nicotine.[24] A recent *in vivo* study has demonstrated that nicotine may confer immunosuppressive effects on ECs by this mechanism.[24] Possible modulatory effects of nicotine on ECs (as mentioned above) may influence the differences observed with respect to reduction in PAS relative to nicotine-free extract (Fig. 4), however longer experiments are needed to investigate such effects. Platelet activation (PAS) was not statistically significant in the presence of medium- and high-nicotine extract relative to control ($p = 0.36$ and $p = 1.00$, respectively, Table 5). This shows that the presence of nicotine in the cigarette at concentrations above 200 nM may compensate for the harmful effects of non-nicotine cigarette components on platelet activation. Additionally, our conclusions regarding the protective effects of nicotine on platelet activation are well supported by our recent study with adult smokers whereby smoking zero-nicotine cigarettes caused a significantly higher (~3-fold) increase in platelet activation, than smoking medium-nicotine cigarettes.[11]

It should, however, be noted that the conclusion holds only for the short experimental duration of the study. Longer experiments with purified platelets are not feasible and the effects of nicotine and cigarette smoke extract over extended periods can only be investigated in animal models (*in vivo*). An alternative approach that involves pre-treatment of ECs with nicotine and cigarette smoke extract prior to exposure with platelets, under physiological conditions, will be investigated in the future.

PAS in Presence of ECs

Endothelial cells protect platelets from shear-induced activation by a variety of mechanisms.[2] Such mechanisms may be initiated by mechanotransduction under shear stresses, and may be influenced by the topography and the net-forward magnitude of the stresses.[7] Our shear stress condition is laminar and atheroprone effects associated with disturbed flow conferring an inflammatory phenotype to ECs may therefore not be expected.[7,12] Given the short duration and lower shear stress conditions in our study, antithrombotic effects due to release of NO and prostacyclins may be significant only during the acceleration and peak shear stress phase (~25% experimental time or 14 min). We believe that the difference in PAS arise

due to a combination of the above factor and the fact that ECs may form a passivating layer for the platelets, which may be less thrombogenic than the material surfaces of our device. As observed in Fig. 4, PAS was different in the presence of ECs, however this difference was not influenced by nicotine or smoke extracts. This is indicated by the lack of significance of the effect of interaction between group (platelets, ECs and platelets) and experimental conditions (i.e., nicotine and smoke) in two-way ANOVA ($p > 0.05$).

Our primary focus here is to demonstrate the pro-inflammatory effects of nicotine-free smoke on platelets in the presence of ECs, thereby bringing the system close to physiology. We clearly show that PAS increases significantly in the presence of the nicotine-free extract, relative to control (Fig. 2). Additionally, we observe that the rate of increase of PAS in the presence of such extracts (i.e., PAR) is non-linear. This fact corroborates our earlier findings of non-linear increases in PAS due to platelet-platelet co-operativity at physiological (2×10^8/mL) concentrations. We attributed such increase to the enhanced Factor Va release by activated platelets. In the present study, we observed that the PAR was statistically more non-linear (Figs. 2a and 2b) when smoke extract was added to the platelets (irrespective of the presence of ECs). Such non-linearity may be enhanced by external agonists such as cigarette smoke which may induce much higher Factor Va release by platelets.

In summary, we show that the HSD is a compact *in vitro* device to study the interaction between circulating and cultured cells under physiological shear stresses. More importantly, we also show that E-selectin expression on ECs is enhanced significantly only upon simultaneous exposure to platelets and nicotine-free cigarette smoke extract. We additionally confirm that nicotine decreases platelet activation due to cigarette smoke, in the presence of ECs. In this *in vitro* study this effect of nicotine was studied within a combined system of ECs and platelets, which brings this study closer to a physiologically relevant scenario. Such a system may therefore be used to develop precursor models to study inflammation *in vitro*. More studies that predispose the ECs to an inflammatory phenotype, before platelets or agonists are added are clearly needed.

In conclusion, our study supports the hypothesis that cigarette smoke is likely associated with increased cardiovascular disease risk, and this may be effectively modulated by nicotine.

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The Influence of Instructions and Nicotine Dose on the Subjective and Reinforcing Effects of Smoking

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Subjective and reinforcing effects of smoking a cigarette were examined within a 2 × 2 modified balanced-placebo design, which manipulated instructions about nicotine content (i.e., told regular nicotine vs. told low nicotine) and actual nicotine dose (given a regular nicotine brand vs. a denicotinized brand). Most ratings of the nicotine content and reward value of cigarettes were higher for those told regular nicotine versus told low nicotine, and for those given regular nicotine versus given low nicotine. Nicotine and instructions did not affect craving, withdrawal, or smoke-reinforced responding, but instructions affected the number of puffs earned for those given low nicotine (i.e., placebo effect). Thus, verbal information (instructions) can influence some responses to smoking consistent with the presence of placebo and antiplacebo effects.

Aside from direct pharmacological actions, consumption of drugs can exert nonpharmacological effects on subjective, behavioral, and physiological responses. One source of these effects is the individual's expectancies about the drug content of the substance (i.e., stimulus expectancy) and about the likely results of consuming the substance (i.e., response expectancies; Kirsch, 1997; see also Perkins, Sayette, Conklin, & Caggiula, 2003). Laboratory researchers have commonly manipulated stimulus expectancy by means of instructional set or substance cue presentation (i.e., drug-paired stimuli), along with manipulation of exposure to the drug itself (see Brandon, Juliano, & Copeland, 1999; Fillmore & Vogel-Sprott, 1992; Juliano & Brandon, 2002).

The most typical method of studying the specific influences of stimulus expectancy and active drug, separately and together, is through use of the balanced-placebo design (BPD; Marlatt & Rohsenow, 1980). With the BPD, participants are randomly assigned to one of four conditions, corresponding to each combination of instructions about the drug content of the substance they are about to receive (told drug vs. told no drug) and actual drug content of the substance (given drug vs. given no drug) for a 2 × 2 between-subjects manipulation. Thus, half of the participants are given a drug and half are given a substance with no drug, and within each group half are told they are receiving a drug and half are told they are receiving no drug. (Note that the term *no drug* is used to more clearly convey what participants are told or given in regard to the drug content of the control substance they receive. Also, *placebo* more precisely refers to the observed effect of instructions rather than to the instructional manipulation itself; see Martin & Sayette, 1993; Perkins et al., 2003.) Among those given no drug, greater responses in those told they are receiving a drug versus those told they are receiving no drug indicate placebo effects, or effects of instructions in enhancing response to the substance. Among those given a drug, smaller responses in those told they are receiving no drug versus those told they are receiving a drug indicate antiplacebo effects, or effects of instructions in attenuating response to the (drug) substance (Carpenter, 1968; see also Martin & Sayette, 1993), the opposite of the direction of the placebo effect. In other words, the antiplacebo effect captures the effect of expecting no drug in the presence of pharmacological actions of the drug. In theory, the BPD procedure tests the independent and interactive effects of a drug and instructions on responses to drug administration (Martin & Sayette, 1993).

Alcohol researchers have made frequent use of the BPD (Hull & Bond, 1986; Marlatt & Rohsenow, 1980). In contrast, the BPD has rarely been used in smoking research. However, Juliano and Brandon (2002) recently used the BPD to examine the influence of nicotine dose and instructions about the nicotine content of cigarettes on craving and anxiety reduction in a laboratory study. Briefly abstinent smokers (3 hr) were given a stressor (preparing a potentially embarrassing speech) while receiving a nicotine or denicotinized cigarette to smoke ad libitum, along with instructions about its nicotine content (i.e., told nicotine or told no nicotine). Results showed an interaction of instructions and nicotine on craving, as only those told nicotine reported a decrease in smoking urge among those given a denicotinized cigarette (i.e., placebo effect), but both groups given a

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nicotine cigarette reported a decrease in urge regardless of what they were told about its nicotine content (i.e., no antiplacebo effect). However, only nicotine, and not instructions, influenced self-reported anxiety or overall withdrawal.

These findings suggest that the manipulation of instructional set regarding nicotine dose may influence craving, at least under some conditions. However, the degree to which the manipulation was successful across the different conditions of the BPD is not clear. Juliano and Brandon (2002) found that one third of participants in the placebo condition (i.e., told nicotine but given no nicotine) and nearly 40% of those in the antiplacebo condition (told no nicotine but given nicotine) did not believe their respective instructions. (It is interesting that although only 3% of those told and given nicotine did not believe their instructions, 27% of those told and given no nicotine did not believe their instructions.)

The difficulty Juliano and Brandon (2002) observed in attempting to manipulate instructional set in the placebo and, especially, the antiplacebo conditions matches findings from the placebo alcohol literature (Martin & Sayette, 1993). Investigators have found that many participants in the antiplacebo condition do not believe the instructions of no alcohol when given a large dose of alcohol. Similarly, participants in the placebo condition (i.e., given no alcohol) often do not believe their instructions if told the dose of alcohol they are receiving is large. In response to this difficulty, a modified BPD has been proposed, whereby comparisons are made between two amounts of alcohol (e.g., low vs. high), rather than between no alcohol and a high alcohol dose (Ross & Pihl, 1989). To maximize the distinction between instructional sets, instructions also include information about the likely effects to expect. With this manipulation, Ross and Pihl (1989) found that belief in instructions persisted for a longer period of time and that intoxication ratings were more likely to differ because of instructions, relative to the standard BPD (see also Martin & Sayette, 1993).

In the present study, we sought to test the usefulness of a similar modified BPD on responses to smoking to determine whether we might be more successful in manipulating instructional set. Toward this end, we used smaller amounts of smoking exposure than a full cigarette smoked ad libitum, as in Juliano and Brandon (2002), and used instructions to convey that the denicotinized cigarette contained low rather than no amount of nicotine. To increase the difference in instructional set between groups, however, we also included instructions about likeability of the cigarettes. In piloting of smoking exposure amounts less than a full cigarette, we found that most participants in the antiplacebo condition did believe their instructions when given just two puffs but not when given four. Two puffs also appeared to be sufficient exposure for participants to be able to rate the cigarette on various characteristics (i.e., none complained that the exposure was inadequate to complete the ratings). These observations may be consistent with the notion that instructional sets for a small drug dose can be manipulated experimentally more easily than instructional sets for a large drug dose (Kirsch, 1997; Martin & Sayette, 1993). We were also mostly interested in the influence of instructions on cigarette ratings related to reward under conditions typical of smoking in the natural environment, that is, smoking in the absence of deprivation or environmental challenges (e.g., the stressor in Juliano & Brandon, 2002). We also wanted to examine whether instructions would influence actual smoking behavior (i.e., reinforcement), which to our knowledge has not been examined in placebo research with cigarette smoking.

Finally, a secondary interest in this study was to examine sex differences in the influence of instructional set and nicotine. Past research in this laboratory and elsewhere suggests that women are more responsive than are men to environmental cues associated with nicotine (Perkins et al., 2001) and are less responsive than are men to manipulations of nicotine dose (Perkins, Jacobs, Sanders, & Caggiula, 2002; for a review, see Perkins, Donny, & Caggiula, 1999). Other researchers suggest similar sex differences in responses to cocaine cues (Robbins, Ehrman, Childress, & O'Brien, 1999) and cocaine dose manipulations (Lukas, Sholar, Fortin, Wines, & Mendelson, 1996). Because drug cues can influence placebo effects (Perkins et al., 2003), greater responses of women to drug cues could suggest that women also may respond to a greater extent to manipulation of instructional set.

In sum, in the current study we used a modified BPD in minimally abstinent male and female smokers to examine the influence of nicotine and instructional set on subjective and reinforcing effects of cigarettes, as well as on craving and withdrawal. We hypothesized main effects of both instructions and nicotine. As indicated in Figure 1A, a main effect of nicotine alone would be shown by greater responses in those given regular nicotine versus those given low nicotine, regardless of instructions. A main effect of instructions alone would be shown by greater responses in those told regular nicotine versus those told low nicotine, regardless of cigarette nicotine dose (see Figure 1B). Effects of both nicotine and instructions would be shown by a combination of these (see Figure 1C). Secondary hypotheses were that the effects of instructions and nicotine would interact with sex, such that effects of nicotine would be smaller in women and effects of instructions would be greater in women, relative to their effects in men. Thus, we expected responses in women to look more like those in Figure 1B and less like those in Figure 1A, compared with men.

Method

Participants

Participants were 96 healthy young smokers (56 men, 40 women) who smoked at least 10 cigarettes per day and had been smoking for at least 2 years. Their preferred brand was required to be one with a nicotine yield of at least 0.7 mg, so that the denicotinized brand used here would be distinctly different from their usual brand (see below). Those with any history of smoking ultralight cigarette brands (defined as nicotine yield < 0.3 mg) for more than 2 weeks were excluded. Participants were recruited

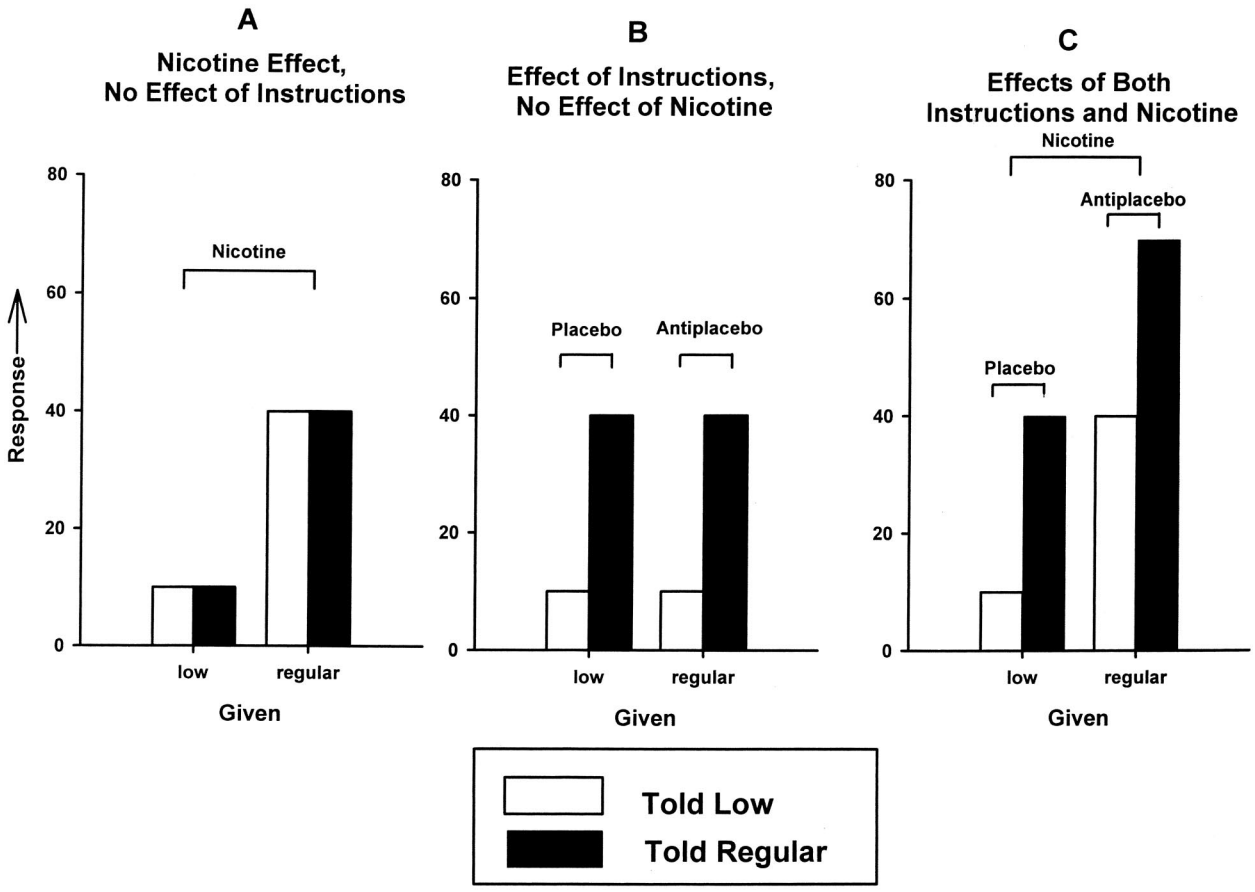


Figure 1. Idealized results illustrating a main effect of nicotine alone (i.e., given low nicotine vs. given regular nicotine) (A), a main effect of instructions alone (i.e., told low nicotine vs. told regular nicotine) (B), and main effects of both (C). Brackets indicate the comparisons between conditions that correspond to the significant main effects.

from flyers and ads placed in the surrounding university community and were paid for their participation. Sample characteristics were as follows: age ($M \pm SE = 27.4 \pm 1.00$ years), nicotine yield of preferred brand (0.96 ± 0.02 mg), daily smoking rate (17.4 ± 0.60 cigarettes/day), number of years smoking (11.0 ± 0.90 years), and Fagerstrom Test of Nicotine Dependence (FTND; Heatherton, Kozlowski, Frecker, & Fagerstrom, 1991) score (4.70 ± 0.20). Men and women were similar on these characteristics.

Cigarettes

Two cigarette brands were used to manipulate cigarette nicotine dose: the regular nicotine cigarette used was Meridien brand (yield of 0.90 mg nicotine, 9 mg tar), and the low-nicotine cigarette used was a prototype of Quest denicotinized brand (yield < 0.05 mg nicotine, 9 mg tar) provided by Vector Group, Ltd. (Miami, FL). Identifiable markings on the cigarettes were hidden. All smoking was done according to computerized puffing instructions used in numerous other studies to standardize smoke intake (e.g., Perkins et al., 2002).

Subjective Measures

Cigarette ratings. The reward value of smoking was assessed using two items from the Rose Sensory Questionnaire (see Westman, Behm, & Rose, 1996), the ratings of "liking" and "satisfying," along with a third measure asking "How much would you pay to smoke another of the same type of cigarette right now?" (in U.S. dollars). Another Rose Sensory Questionnaire item, "how much nicotine," was used to check the manipulation of instructions to participants about the perceived nicotine content of the cigarette (see instructional manipulation below). The Rose Sensory Questionnaire also has an item asking how similar the cigarette is to one's own brand. Each item in the Rose Sensory Questionnaire was rated on a visual analog scale (VAS) ranging from 0 (*not at all*) to 100 (*extremely*). Participants also used a VAS ranging from 0 to 100 to rate the cigarette on a measure asking "How strong was this cigarette?" These measures have been used in past similar studies from this laboratory (Perkins et al., 2001, 2002).

Craving and withdrawal. Craving was assessed with two measures: a single-item VAS ranging from 0 to 100 of "desire for a cigarette" and the four-item version of the Questionnaire on Smoking Urges (QSU; Tiffany & Drobes 1991), a widely used clinical and laboratory measure of urges to smoke. The four items in this version, which is described by Carter and Tiffany (2001), are "Nothing would be better than smoking a cigarette right now," "I have an urge for a cigarette," "All I want now is a cigarette," and "I crave a cigarette right now." Responses to the items were scored from 1 (*strongly disagree*) to 7 (*strongly agree*) and then were averaged. Nicotine withdrawal was assessed with the scale devel-

oped by Hughes and colleagues (Hughes & Hatsukami, 1986), with each item rated on a VAS ranging from 0 to 100. Items were averaged across symptoms (other than "desire for a cigarette") to get a total withdrawal score.

Progressive Ratio (PR) Measure of Smoking Reinforcement

Smoking reinforcement was assessed using a computer task involving an increasing response requirement for each reinforcer, one puff on the designated cigarette. This task has been described in detail elsewhere (Perkins et al., 2002). Participants engaged in the task under PR conditions, with response requirements presented in an ascending order typical of the PR. Response requirements for each puff (i.e., finding an apple) were 12, 28, 65, 152, 354, 620, and 886. The task was paused as the puff was immediately consumed, followed by a return to the task. The task ended when the participant failed to respond for 5 min. Smoking reinforcement was determined by the total number of responses for smoke puffs, the breakpoint (largest response requirement completed), and the number of puffs earned. (Because participants could quit at any time, even in the middle of a response requirement, number of responses and number of puffs earned were not necessarily redundant measures.) This task is similar to other PR procedures in human drug research (e.g., Comer, Collins, & Fischman, 1997) and has been found to be sensitive to cigarette nicotine dose in within-subjects designs (Perkins et al., 2002).

Procedure

Participants participated in one session, preceded by ad-lib smoking (i.e., no abstinence). They provided informed consent to participate in a study to evaluate the characteristics of different types of cigarettes and how much smokers like them. Participants were randomly assigned to one of the four conditions in the balanced-placebo design: told regular nicotine/given regular nicotine, told regular nicotine/given low nicotine, told low nicotine/given regular nicotine, told low nicotine/given low nicotine. Groups were stratified by sex so that each group had 14 men and 10 women.

Participants smoked one of their preferred brand cigarettes ad libitum upon arrival and then rested quietly for 1 hr (to standardize the duration since the last cigarette). During the rest period, they completed the Balanced Inventory of Desirable Responding (BIDR; Paulhus, 1991). This measure was obtained to examine any relationship between socially desirable responding and responses to the instructional manipulation. At the end of the 1-hr rest period, they completed baseline measures of craving and withdrawal. They then were introduced to the designated cigarette (regular nicotine vs. low nicotine) and were given the following instructions, depending on their group assignment:

> Told regular nicotine: We are interested in why smokers like certain brands of cigarettes. Regular Full-Flavor brands, those higher in nicotine, often are liked. So, nicotine levels may have something to do with brand liking. Today, you will be smoking a regular higher nicotine cigarette that is well-liked.
>
> Told low nicotine: We are interested in why smokers do not like certain brands of cigarettes. Low nicotine brands often are not liked, so nicotine levels may have something to do with brand liking. Today, you will be smoking a low nicotine cigarette that is not well-liked.

Regardless of their actual nicotine content, the cigarettes in each condition were labeled by a letter code, depending on what instructions the participants were given ("R" for regular and "L" for low). Participants then took two puffs to sample the cigarette (exposure period), using the standardized puff procedure noted previously. After the two puffs, they rated the cigarette on the Rose Sensory Questionnaire and related items, including the "how much nicotine" item, which was used as an initial check of the instructional manipulation. They also completed craving and withdrawal measures. Then participants performed the PR task for additional puffs, as described previously. They were told that they would remain in the laboratory until the scheduled completion time for the session and that stopping the PR task early would not result in their being able to leave the laboratory early (in order to be able to smoke their own brand ad libitum). Participants were given 1 hr to earn smoke puffs, and those who stopped responding before the hour was up rested quietly while reading until the end of that hour.

After completing the PR task and consuming the earned puffs, participants completed a final measure asking them to circle what brand cigarette they believed they received from among a list of low- and moderate-nicotine brands, each with their nicotine yield presented. This measure was designed to determine whether participants, by the end of the session, believed the instructions about the nicotine content of the cigarette. Thus, to verify belief in the instructions, those in the told regular nicotine groups were expected to circle any one of the moderate-nicotine brands, and those in the told low nicotine groups were expected to circle any one of the low-nicotine brands. When participants circled one of the brands on the list different from their instructions (e.g., one of the low-nicotine brands for participants in the told regular nicotine groups), this response was interpreted as indicating disbelief in the instructions about the nicotine content of the cigarette they received. This procedure was adopted because of concern about the reliability of the typical approach taken in most studies to ascertain belief in instructions, which is to ask participants a direct question as to whether or not they believed the instructions, which can lead to response bias (Hull & Bond, 1986; Martin & Sayette, 1993).

Upon completion of the session, participants were debriefed as to the true purpose of the study, which was to examine responses to smoking as a function of what smokers are told about the cigarettes. They were told that some smokers did and others did not receive a brand consistent with the instructions about nicotine content and what they actually received. This study was approved by the University of Pittsburgh Institutional Review Board.

Data Analysis

We analyzed the rating of "how much nicotine" first, as a check of the manipulation of instructions. We analyzed this rating using analysis of variance (ANOVA), with Instructions (told regular nicotine vs. told low nicotine), Nicotine (regular- vs. low-nicotine cigarette), and Sex as between-subjects factors. We analyzed the ratings of "overall strength" and "similar to own brand" using similar ANOVAs. Because the other three cigarette ratings (Rose Sensory Questionnaire items of "liking" and "satisfying," and the amount pay item) were designed to capture smoking reward, we first examined responses to these items by means of multivariate analysis of variance (MANOVA), with Instructions, Nicotine, and Sex as factors. When appropriate, we followed up the MANOVA with ANOVAs of these effects on the individual items. We analyzed amount of responding on the PR task for smoke puffs and the number of puffs earned (reinforcement) using ANOVA, again with Instructions, Nicotine, and Sex as factors. Because of the nonnormal distribution of PR responses, these values were log-transformed prior to analysis. We also analyzed the breakpoints (highest completed response requirement for one puff) by means of the Kruskal–Wallis nonparametric chi-square test. We analyzed crav-

ing and withdrawal measures using similar ANOVAs, with the added within-subjects factor of Time (baseline, postexposure). We conducted follow-up tests for comparisons between groups using Fisher's least significant difference *t* test. As recommended by Martin and Sayette (1993), we also made specific comparisons between the told low nicotine and told regular nicotine subgroups within each nicotine dose condition (i.e., given low nicotine, given regular nicotine) to determine the significance of instructions for each dose condition.

Successful manipulation of expectancies about drugs or their effects is believed to be a critical requirement of studies using the BPD (Hull & Bond, 1986; Martin & Sayette, 1993). Thus, we repeated analyses after excluding those participants who apparently did not believe the instructions they received regarding the nicotine content of the cigarette to which they were assigned, using the end-of-session form described previously. With this analytic approach, also conducted by Juliano and Brandon (2002), we addressed the more specific question as to whether instructions (and nicotine) influence responses among only those who believe the instructions. We also examined the relationship between socially desirable responding and responses to the instructional manipulation by means of hierarchical regression. Nicotine, instructions, and the Impression Management scale of the BIDR were entered in Step 1, and the interaction of Instructions × Impression Management was added in Step 2, to determine the influence of impression management after controlling for the effects of nicotine and instructional set.

Results

To simplify presentation of the ANOVA results for cigarette ratings, the *F* ratios for significant main and interaction effects are presented in Table 1, collapsed across sex on the top and for interactions involving sex on the bottom. Because a number of the interactions involving sex were significant for the cigarette ratings, means for all of these ratings are presented in Figure 2, for men and women separately.

Belief in Instructions: Postexposure

We analyzed the Rose Sensory Questionnaire item, "how much nicotine," first to evaluate the influence of instructions on participants' expectations of the nicotine in the designated cigarette. As expected, the main effect of instructions was highly significant for responses to this item, suggesting successful manipulation of dose expectancy by means of instructions, as shown in Table 1 and Figure 2. This effect of instructions was significant in comparisons within each of the nicotine dose (given) conditions for both men and women (*t*s for each comparison are not presented to save space). Thus, higher responses due to told regular nicotine versus told low nicotine among those given low nicotine indicates a placebo effect, whereas lower responses due to told low nicotine versus told regular nicotine among those given regular nicotine indicates an antiplacebo effect (cf. Figure 1).

Responses on the "how much nicotine" item were also influenced by the main effect of nicotine (see Table 1), but the interaction of Instructions × Nicotine was not significant for this or any other measure, except for number of puffs earned (see below). These ratings differed between the given low nicotine and given regular nicotine groups among women but not among men (i.e., Nicotine × Sex interaction; see bottom portion of Table 1), suggesting that women were more sensitive to dose than men were. The interaction of Instructions × Nicotine × Sex also was significant, such that the sex difference due to nicotine was seen among those in the told regular nicotine group but not among those in the told low nicotine group.

Table 1
Significant F Ratios (df = 1, 88) for Analyses of Variance of the Effects of Instructions and Nicotine on Cigarette Ratings and Interactions Involving Sex

Measure	Nicotine	Instructions	Instructions × Nicotine
	Effects on cigarette ratings		
How much nicotine[a]	16.80**	104.38**	
Overall strength	15.45**	55.81**	
Similar to own brand	10.79**	12.34**	
Liking	8.86**	23.08**	
Satisfying	16.59**	15.27**	
Amount pay for cigarette			

Measure	Nicotine × Sex	Instructions × Sex	Instructions × Nicotine × Sex
	Effects on interactions involving sex		
How much nicotine	10.23**		4.80*
Overall strength	6.17*		
Similar to own brand			
Liking			2.84†
Satisfying			4.52*
Amount pay for cigarette			4.51*

[a] Manipulation check.
† $p < .10$. * $p < .05$. ** $p < .01$.

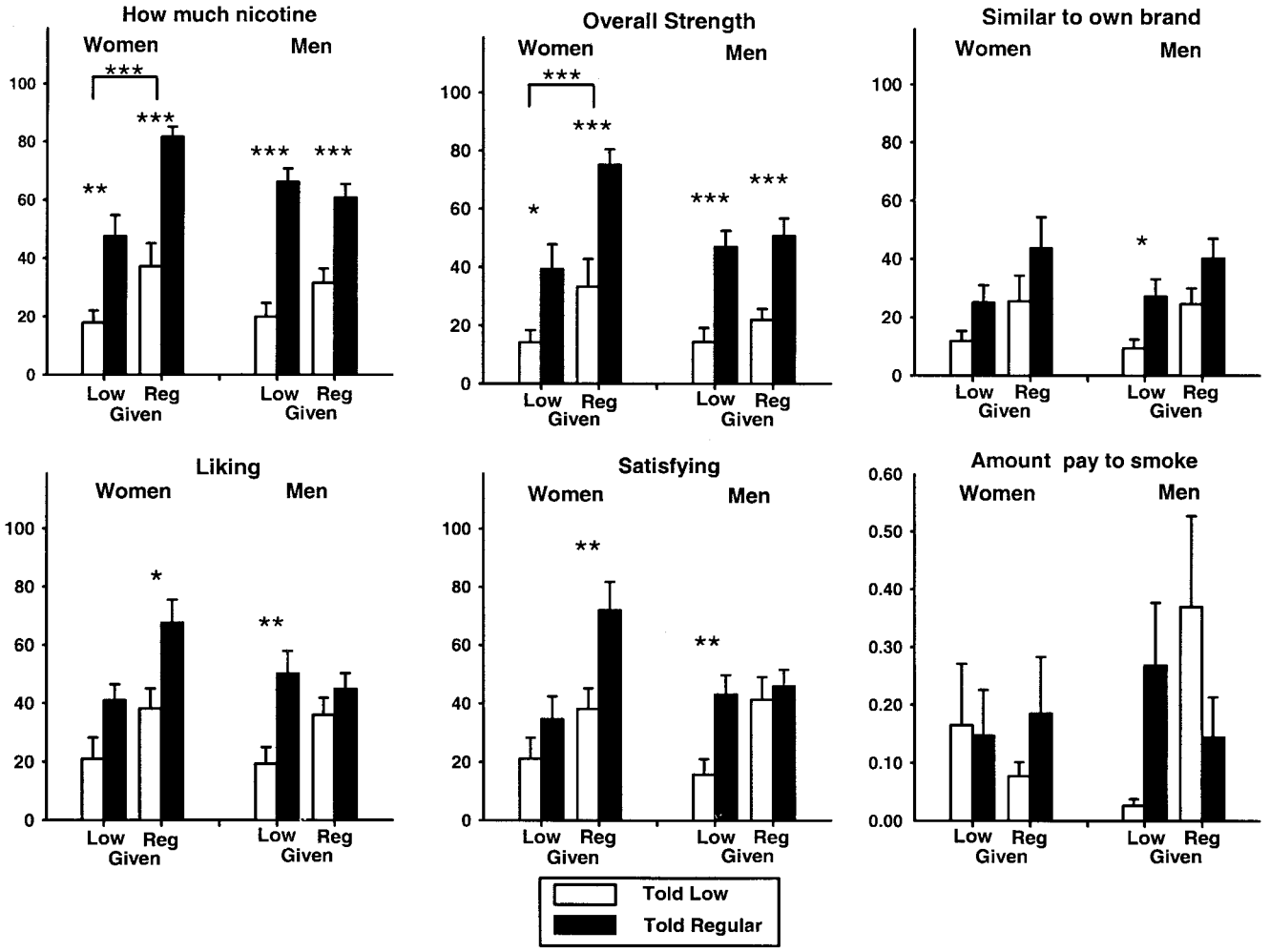


Figure 2. Cigarette ratings on visual analog scales (range = 0–100) following two puffs on the designated cigarette during the exposure period. Also shown is amount of money (in U.S. dollars) participants would pay for another cigarette. Values are presented for men and women given either the denicotinized (Low) cigarette or the regular (Reg) nicotine cigarette as a function of instructions (told low nicotine, told regular nicotine). The main effects of nicotine and instructions were significant for all measures, other than the amount pay item, and are not explicitly shown in the figure. Also significant were the interaction of Nicotine × Sex for "how much nicotine" and "overall strength" and the interaction of Instructions × Nicotine × Sex for "how much nicotine," "satisfying," and the amount pay item (see Table 1). Brackets indicate a significant effect of nicotine collapsed across instructions for just one sex (within-women). Other comparisons are for the effects of instructions within each nicotine dose condition separately by sex. *$p < .05$. **$p < .01$. ***$p < .001$.

Results for "overall strength" largely mirrored those for "how much nicotine," with main effects of instructions and nicotine and a Nicotine × Sex interaction (see Table 1 and Figure 2). In comparisons of the effect of instructions within each dose condition, instructions were significant for both the given low nicotine and given regular nicotine conditions, $t(47) = 4.94$ and 5.65, respectively, both ps $< .01$. The rating of "similar to own brand" also was affected by instructions and nicotine, but there were no interactions. In comparisons, the effect of instructions was significant for both the given low nicotine and the given regular nicotine conditions, $t(47) = 2.46$ and 2.58, respectively, both ps $< .05$.

Cigarette Reward Ratings

Results for the MANOVA of the three reward ratings revealed significant main effects of instructions, $F(3, 86) = 8.44$, $p < .001$, and of nicotine, $F(3, 86) = 5.65$, $p < .01$. The interaction of Instructions × Nicotine ×

Sex was marginally significant, $F(3, 86) = 2.22$, $p < .10$. Because of our a priori interest in sex differences, we included this interaction in examination of the ANOVA results for the individual ratings. No other effects were significant.

For "liking," the main effects of instructions and of nicotine were significant (see Table 1 and Figure 2), as in the prior measures. "Liking" was lower for those in the told low nicotine versus the told regular nicotine subgroups, both for the given low nicotine, $t(47) = 4.02$, $p < .01$, and the given regular nicotine conditions, $t(47) = 2.70$, $p < .01$. The effects of instructions here are consistent with placebo and antiplacebo effects. The interaction of Instructions × Nicotine × Sex was marginally significant (see the bottom of Table 1). In comparisons by sex, instructions were significant only for the given regular nicotine condition among women and for the given low nicotine condition among men, as shown in Figure 2.

Ratings of "satisfying" also were influenced by main effects of instructions and nicotine. Ratings were lower for those in the told low nicotine condition versus those in the told regular nicotine condition among both the given low nicotine, $t(47) = 3.04$, $p < .01$, and the given regular nicotine conditions, $t(47) = 2.37$, $p < .05$. The interaction of Instructions × Nicotine × Sex also was significant, as instructions affected ratings only for the given regular nicotine condition among women and for the given low nicotine condition among men (see Figure 2). There were no differences in the amount pay item, although again the interaction of Instructions × Nicotine × Sex was significant. Unlike the pattern of results for this interaction for "liking" and "satisfying," the amount willing to pay tended to be higher for those in the told regular nicotine condition versus those in the told low nicotine condition among men given low nicotine, but the opposite was true among men given regular nicotine, although both comparisons were only marginally significant. No effects were seen in women.

Craving and Withdrawal

Baseline levels of the two craving measures of QSU and "desire for a cigarette" were at the midpoint of the range of each measure (3.7 ± 0.2 on a scale from 1 to 7 for QSU, 49.2 ± 2.6 on a scale from 0 to 100 for the "desire for a cigarette" item), suggesting moderate craving despite only 1 hr of abstinence. Baseline total withdrawal was mild (16.3 ± 1.4 on a scale from 0 to 100), as expected. Instructions and nicotine had no significant main effects on the craving measures or withdrawal. The interaction of Instructions × Sex × Time was significant for the QSU, $F(1, 88) = 3.95$, $p < .05$, as the change from baseline to the exposure period decreased more for the told regular nicotine group versus the told low nicotine group among women but not among men (data not shown). For total withdrawal, the main effect of time was significant, $F(1, 85) = 17.80$, $p < .01$, as withdrawal decreased from baseline to the two-puff smoking exposure.

Smoking Reinforcement

Total responses for smoke puffs in the PR task are presented on the left side of Figure 3 (without log-transformation for clarity), and number of puffs earned is presented on the right side. In contrast to the reward ratings, smoke-reinforced responding was not influenced by either instructions or nicotine, nor by sex or any interactions involving these factors. Similarly, there was no significant difference in breakpoint across factors (data not shown). However, the number of puffs earned was significantly greater in those given the regular-nicotine cigarette than in those given the low-nicotine cigarette (i.e., main effect of nicotine), $F(1, 88) = 4.80$, $p < .05$. The interaction of Instructions × Nicotine also was significant for puffs earned, $F(1, 88) = 5.43$, $p < .05$. The number of puffs earned was greater in the told regular nicotine group versus the told low nicotine group among those given the low-nicotine cigarette, $t(47) = 2.35$, $p < .05$, consistent with placebo effects, but instructions had no effect among those given the regular-nicotine cigarette (see Figure 3).

Belief in Instructions: End of Session

The percentages of each group who, at the end of the session, identified a cigarette brand consistent with the nicotine level in the instructions given to that group were as follows: told regular/given regular (88%), told low/given low (88%), told regular/given low (79%), and told low/given regular (62%). Thus, according to this measure, 21% of those in the placebo condition did not believe that the denicotinized cigarette they were given was regular nicotine, as instructed. More than one third (38%) of those in the antiplacebo condition did not believe that the regular nicotine cigarette they were given was low nicotine, as instructed.

When we reanalyzed the data from this study with only those participants who believed the instructions about nicotine content, the results were largely unchanged from the analysis results presented above, in which all participants were used. All main effects of instructions that were significant in analyses of all participants were still significant in analyses of only those believing the instructions, but the main effect of nicotine was no longer significant for "liking," "satisfying" (became marginal), and puffs earned.

In an exploratory analysis, we conducted an ANOVA of ratings on the manipulation check item, "how much nicotine," obtained after the two-puff exposure period, as a function of whether participants indicated that they did or did not believe the instructions on the basis of this end-of-session form. The main effect of instructions was significant, as expected, but the effect of nicotine was not. Moreover, the interaction of Instructions × Belief (did/did not believe) was significant, $F(1, 81) = 31.64$, $p < .01$. Ratings of "how much nicotine" were higher for the told low nicotine condition among those who did not believe those instructions, and ratings were lower for the told regular nicotine condition among those who did not believe those instructions. This observation supports the validity of the

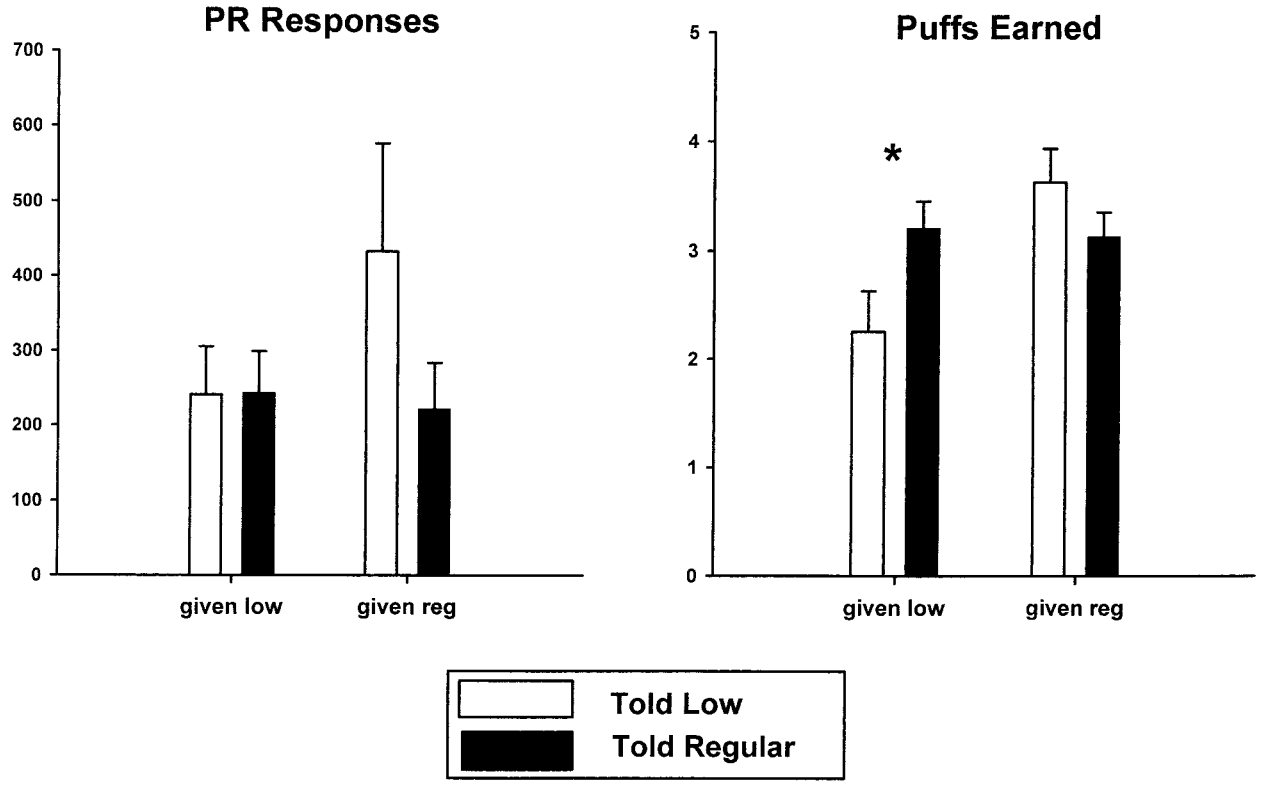


Figure 3. Measures of smoking reinforcement (total progressive ratio [PR] responses for smoke puffs, smoke puffs earned) across the four groups (as identified in the Figure 2 caption). The main effect of nicotine and the interaction of Instructions × Nicotine were significant for puffs earned. No effects were significant for total PR responses. *$p < .05$.

end-of-session form as a measure of belief in the instructions. It also suggests that those who did not believe the instructions formed this belief after the two puffs of the exposure period, rather than gradually over time while consuming the earned puffs. In other exploratory analyses to determine characteristics that distinguished believers from nonbelievers, only cigarettes per day differed significantly between these subgroups. Those who did not believe their instructions smoked more than those who did believe their instructions, 20.2 ± 1.6 vs. 16.7 ± 0.6 cigarettes per day, respectively; $t(94) = 2.37$, $p < .05$.

Finally, we determined whether responses on the "how much nicotine" and "liking" scales—those most directly relevant to the instructions—may have been influenced by participants' desire to create favorable responses to the experimenter instructions. For each rating, we performed a hierarchical multiple regression, with nicotine, instructions, and the Impression Management scale score from the BIDR entered in Step 1, followed by addition of the interaction of Instructions × Impression Management in Step 2. Step 1 determined whether impression management was generally related to these responses to smoking, independent of the influence of nicotine or instructions, whereas Step 2 demonstrated whether impression management may have moderated the influence of instructions on responses to smoking. For both ratings, neither impression management alone nor the interaction of impression management with instructions was related to responses (βs = .014 and .119 for main effect and interaction on "how much nicotine," respectively; βs = −.052 and −.051 for main effect and interaction on "liking," respectively; $t < 1$ for all effects).

Discussion

In this study, instructions about the nicotine content and expected likeability of a cigarette influenced ratings of the cigarette on items relevant to the perceived nicotine content and strength of the cigarette, as well as on some measures of smoking reward ("liking" and "satisfying" but not the amount pay item). As shown in Figure 2, the greater ratings for the denicotinized cigarette among those told regular nicotine versus those told low nicotine support a significant placebo effect. Similarly, the lower ratings for the regular nicotine cigarette among those told low nicotine versus those told regular nicotine support a significant antiplacebo effect (Perkins et al., 2003). It is notable that there was no significant interaction of Instructions × Nicotine on any of these ratings, suggesting that instructions had comparable effects regardless of the nicotine dose of the cigarette. Thus, within the limits of a modified BPD, verbal information, and

presumably the expectancies that information creates (Brandon et al., 1999; Kirsch, 1997), can have significant effects on responses to brief smoking exposure.

As noted, these results demonstrate the potential influence of placebo and antiplacebo effects of smoking on subjective ratings of cigarettes under neutral laboratory conditions. Nevertheless, these differences in ratings due to instructions (and nicotine) were not accompanied by similar differences in craving or withdrawal. This is perhaps not surprising here, given the brief deprivation before the session (1 hr) and the modest exposure to smoking prior to ratings (two puffs). These procedures were incorporated in this study to examine placebo effects on cigarette ratings under nondeprived conditions and to improve the credibility of the antiplacebo manipulation, as noted previously.

However, these results contrast somewhat with those of Juliano and Brandon (2002), who found greater craving reduction, but not withdrawal, due to told nicotine instructions among participants given a denicotinized cigarette (i.e., an interaction of Instructions × Nicotine). In their study, Juliano and Brandon provided a full cigarette, smoked ad libitum, to participants who had been abstinent for 3 hr. Baseline craving in their study averaged almost 70% of the maximum on their measure (about 14 on a 21-point scale), compared with our baseline craving level of nearly 50% of the maximum. The lower level of baseline craving in our study could have limited our ability to find reduced craving after smoking. Moreover, Juliano and Brandon examined the effects of instructions on responses to smoking within the context of a stressor (preparation of a speech), whereas in the current study we examined these effects under standard laboratory conditions. The influence of instructions (and nicotine) may be greater in some contexts than in others (e.g., Wigmore & Hinson, 1991; Willner & Jones, 1996). Whether the greater smoking exposure, the longer presession abstinence, or the inclusion of a stressor in the Juliano and Brandon study was critical to their obtaining effects of instructions on craving remains to be determined.

By and large, women did not show stronger responses to instructions compared with men, in contrast with our secondary hypotheses. However, the triple interaction of Instructions × Nicotine × Sex was marginal or significant for the three reward items. Although the pattern of this interaction was not completely consistent across items, two of the three items, "liking" and "satisfying," were significantly influenced by instructions for women given a regular- but not a low-nicotine cigarette, whereas the opposite was the case for men (see Figure 2). This pattern suggests the presence of antiplacebo effects alone in women but the presence of placebo effects alone in men (cf. Figure 1). Thus, rather than differing in overall responsiveness to instructions as hypothesized, men and women may differ in the robustness of placebo versus antiplacebo effects, although much more research is needed to gauge the reliability of this finding.

On the other hand, the significant interaction of Nicotine × Sex on "how much nicotine" and "overall strength," items that may reflect perception of nicotine intake from smoking, indicated greater effects due to nicotine in women compared with men (see Figure 2). This observation is contrary to our expectation that nicotine effects would be smaller for women than for men. A smaller effect of cigarette nicotine dose in women than in men was found in a recent within-subjects study in which the same subjective and reinforcement measures were used but no instructions about dose were provided (Perkins et al., 2002). Other studies not involving instructional manipulations have shown that women may be less sensitive than men to nicotine's reinforcing and discriminative stimulus effects (see Perkins et al., 1999, for review). Although speculative, it is conceivable that the introduction of specific dose instructions, as in this study, may eliminate or even reverse this sex difference in the influence of nicotine dose on the subjective and reinforcing effects of smoking.

Findings for smoking reinforcement were mixed, as no differences were seen in responses on the PR task or on breakpoint, but nicotine and the interaction of Instructions × Nicotine influenced the number of puffs earned. As expected, puffs earned were greater among those told regular nicotine than they were among those told low nicotine in participants given the denicotinized cigarette, consistent with the notion of a placebo effect on smoking reinforcement. However, puffs earned did not differ because of instructions among participants given the regular-nicotine cigarette. These results for puffs earned mirror the craving results reported by Juliano and Brandon (2002), in which instructions influenced craving among those given a denicotinized cigarette but not among those given a regular-nicotine cigarette. These results also may help explain past research showing no reduction in reinforcement from denicotinized versus nicotine cigarettes when either cigarette was the only one available to smokers given no instructions about the cigarette (Shahan, Bickel, Madden, & Badger, 1999). In the absence of instructions, the conditioned stimuli (cues) from a denicotinized cigarette may be sufficient to maintain reinforcement (i.e., conditioned reinforcement; see Caggiula et al., 2001). However, accurate instructions that a very low nicotine cigarette contains little nicotine may override the influence of these conditioned stimuli and eliminate this conditioned reinforcement, resulting in an overall decrease in the reinforcing effects of such a cigarette.

Our results showing the effects of instructions on puffs earned for those given low- but not regular-nicotine cigarettes are only partly consistent with nicotine gum research by Hughes and colleagues (Hughes, Pickens, Spring, & Keenan, 1985). They found in one study (Study 2) that instructions suggesting that nicotine gum was placebo and that placebo gum was nicotine significantly decreased self-administration of nicotine gum and increased self-administration of placebo gum, suggesting equal influence of instructions on reinforcement for nicotine and placebo gum. Aside from the different form of nicotine used, reasons for the difference in results may be the concurrent access given to both gums, longer duration of access to gums, and inclusion of smokers wanting to quit permanently in the Hughes et al. (1985) study.

The instructions used in this study were reasonably successful in manipulating expectations for nicotine from the given cigarettes, as quantitative ratings of "how much nicotine" after the two-puff exposure clearly differentiated the told regular nicotine and told low nicotine groups. Similarly, at the end of the session, following PR responding for smoke puffs, the vast majority of participants identified a cigarette brand consistent in nicotine content with the instructions they were given at the start of the session (e.g., those told regular nicotine identified a regular nicotine brand). Nevertheless, more than one fifth of participants in the placebo condition—told regular/given low—did not believe their instructions according to this measure. This rate is somewhat less than the one third in the Juliano and Brandon (2002) study who did not believe the instructions for the placebo condition. Moreover, in the current study, 38% of participants in the antiplacebo condition—told low/given regular—did not believe their instructions, similar to the 40% in the Juliano and Brandon study who did not believe the instructions for the antiplacebo condition. The rate of disbelief in the antiplacebo condition of the current study was found despite extensive piloting to devise instructions that would be more credible to participants in this condition, specifically the use of low-nicotine rather than no-nicotine instructions. Yet, results did not differ between analyses that did or did not exclude the participants that did not believe their instructions, as also found by Juliano and Brandon. In any case, our findings provide only limited support for the use of a modified BPD on smoking responses. Clearly, further research is required to develop stronger procedures for effectively manipulating instructional sets for nicotine in smoking studies.

This study has several limitations that may affect the generalizability or interpretation of results. First, as noted, we selected a brief period of abstinence and a modest amount of smoking exposure. Results certainly may differ with use of longer abstinence or greater smoking exposure, as suggested by Juliano and Brandon (2002). Second, the participants in this study tended to be young smokers, and the effects of instructions (and nicotine) may be greater among those who are older and more dependent. In a review of studies with smokers given no explicit instructions about nicotine content, Brauer and colleagues (Brauer et al., 2001) found that the "liking" of denicotinized cigarettes was greater among high- versus low-dependent smokers. On the other hand, those in the current study who disbelieved their instructions were heavier smokers compared with those who believed their instructions, suggesting that research manipulating instructions among more dependent smokers might be more difficult to do. Third, the PR task may have been an insensitive measure of reinforcement under the conditions of this study. Other research shows that the PR task and similar procedures are insensitive in nonabstinent smokers, as in the current study, but are sensitive in abstinent smokers (Madden & Bickel, 1999; Rusted, Mackee, Williams, & Willner, 1998). Perhaps consistent with this notion, we recently found the PR task to be sensitive to a within-subjects manipulation of cigarette nicotine dose in abstinent smokers (Perkins et al., 2002), despite finding no differences in the current study with nonabstinent smokers.

Finally, the instructions used here included a statement regarding the expected likeability of the cigarette, as well as the nicotine content of the brand. This element of the instructions was included to provide greater differentiation between the told regular nicotine and the told low nicotine instructions, as done in prior modified BPD procedures (Ross & Pihl, 1989). However, the combining of instructions about likeability and nicotine content complicates the identification of which element produced the effects of instructions on cigarette ratings and other responses. It also increases experimenter demand for responses from participants that are consistent with these instructions (e.g., "liking" ratings in Figure 2; see also Martin & Sayette, 1993). Yet, we found that ratings for "how much nicotine" and "liking," those most relevant to the instructions, were unrelated to scores on one measure of socially desirable responding, the Impression Management scale of the BIDR. Future research could separately manipulate instructions about expected likeability of a cigarette to determine whether such instructions alone, without mention of nicotine content, could be sufficient to produce these effects. In any case, the current study demonstrates that verbal instructions per se about a cigarette are sufficient to influence most ratings of that cigarette and that, among those given the denicotinized cigarette, they affect the number of puffs earned.

In sum, instructions about nicotine dose and likeability can influence cigarette ratings in ways consistent with the presence of placebo and antiplacebo effects. Instructions can also influence at least some measures of the reinforcing effects of denicotinized cigarettes. However, instructions may not affect craving or withdrawal under the conditions in this study of minimal deprivation and brief smoking exposure. Men and women appear relatively similar in their overall responses to instructions, but women may be more sensitive to effects of nicotine on ratings regarding perception of nicotine, in contrast to some past research. Future research should examine the influence of instructions on these and other responses to smoking under other conditions, such as varying abstinence duration, smoking population, and environmental context.

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The airway sensory impact of nicotine contributes to the conditioned reinforcing effects of individual puffs from cigarettes⋆

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Abstract

Puffs from cigarettes are the fundamental unit of smoking reward. Here, we examined the extent to which reward from puffs can be derived from the airway sensory effect of nicotine, in the absence of a direct central nervous system effect of nicotine. We did this by assessing the self-reported reward obtained from individual puffs from nicotinized, denicotinized and unlit cigarettes within 7 s of inhalation, which is before nicotine had an opportunity to reach the brain. We also assessed the self-reported strength of airway sensations elicited by the puffs. We found that nicotinized puffs were rated as both stronger and more rewarding than denicotinized and unlit puffs. We also found that the extent to which nicotine elicited reward was directly correlated with the extent to which nicotine elicited airway sensations. This indicates that the airway sensory effects of nicotine contribute to the reward from puffs, above and beyond the reward derived from the airway sensory effects of non-nicotine constituents. These findings have implications for the interpretation of studies that use puffs as experimental units to examine nicotine reward. They also have implications for the use of denicotinized and low nicotine cigarettes as aids to smoking cessation.

Keywords

Cigarette smoking; Nicotine; Denicotinized cigarettes; Reward; Hedonic impact; Conditioned reinforcement; Airway sensation

1. Introduction

Cigarette smoking is a dependence behavior, characterized by compulsion to smoke despite awareness of its negative consequences (American Psychiatric Association, 2001). Much of the research on cigarette smoking dependence is predicated on the idea that smoking is subjectively pleasurable and behaviorally reinforcing (i.e. it is rewarding). The fundamental unit of reward from smoking is the puff, an act repeated more than a hundred times a day by a typical pack-per-day smoker. Individual puffs from cigarettes are subjectively experienced as pleasurable and desirable (Rose, 1984; Herskovic et al., 1986; Baldinger et al., 1995) and smokers will perform work to obtain them (Willner et al., 1995; Shahan et al., 1999; Perkins

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et al., 2002), indicating that individual puffs reproduce some of the rewarding characteristics of smoking whole cigarettes. For this reason, an increasing number of studies are using individual puffs as a way to address the psychological and neural processes that underlie smoking reward (e.g. Rose et al., 1985a,b; Herskovic et al., 1986; DeGrandpre et al., 1993; Bickel et al., 1997; Madden and Bickel, 1999; e.g. Shahan et al., 1999; Tidey et al., 1999; Perkins et al., 2002). Despite their popularity as experimental tools, the relative contributions of pharmacologic processes (i.e. direct central nervous system effects of nicotine) and sensory-motor processes (i.e. conditioned reinforcement) to the reward from individual puffs are not known.

In addition to delivering nicotine to the central nervous system, each puff from a cigarette stimulates an array of sensations within the airway that are transmitted to the central nervous system through peripheral nervous pathways. These sensations may be more important than the direct central nervous system effects of nicotine for smoking reward. For example, it has been shown that puffing on denicotinized cigarettes, which stimulates a component of the sensory impact of puffing without delivering an appreciable quantity of nicotine to the plasma, elicits feelings of exhilaration, euphoria and satisfaction, as well as reductions in urge, that are greater than those elicited by intravenous nicotine infusion (Westman et al., 1996; Rose et al., 2000). Conversely, blockade of airway sensations by anesthetizing the airway reduces smoking desirability and also the reduction in urge obtained from smoking (Rose et al., 1984 a,b, 1985a,Rose et al., b). Thus, airway sensations are both necessary and sufficient to elicit smoking reward.

The reward derived from airway sensory stimulation (‘‘sensory reward’’) is distinct from the reward derived from nicotine’s direct central effects (‘‘pharmacologic reward’’) in a number of ways. Firstly, sensory reward is elicited immediately following each puff from a cigarette, in the time it takes for airway sensation to be signaled in the brain. This makes individual puffs natural behavioral units for sensory reward, in a manner analogous to the way in which a bite of food is the natural unit of taste reward. In contrast, pharmacologic reward requires nicotine to be absorbed in the airway and transported to the central nervous system via the circulation, a process that has been estimated to take anywhere from 7 to 19 s (Russell and Feyerabend, 1978; Benowitz, 1990; Henningfield and Keenan, 1993). Furthermore, whereas pharmacologic reward is unlearned, or ‘‘primary’’ reward, sensory reward is learned. This learning has been proposed to be a form of conditioning, wherein airway sensations acquire reward value through repeated association with the pharmacologic reward obtained from nicotine (Rose and Levin, 1991). The role of learning in sensory reward is evidenced by the finding that the airway sensory effects of smoking are aversive to naïve smokers (Lee et al., 1993). Also, it has been shown that more heavily dependent smokers derive more reward from airway sensory stimulation than less heavily dependent smokers (Brauer et al., 2001), implying that this learning develops along with smoking dependence. Together, this suggests that sensory reward and pharmacologic reward, though both ultimately arising in the brain, may be mediated by psychological and neural processes that are at least partially distinct.

Experiments with denicotinized cigarettes demonstrate that airway stimulation by non-nicotine constituents is sufficient to elicit rewarding effects. However, a greater component of the airway sensory effects of a puff may actually be due to nicotine. Ginzel (1975) was the first to describe the sensory effects of nicotine within the pulmonary system and distinguish these from nicotine’s pharmacologic (direct central nervous system and ganglionic) effects. More recently, Lee et al. (1993) have shown that inhalation of the nicotinic receptor antagonist hexamethonium reduces the airway irritation elicited by smoking. It has also been shown that the airway visceral afferent discharge elicited by a single breath of nicotinized cigarette smoke is more than 3 times greater than the discharge elicited by a breath of denicotinized smoke

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(Lee et al., 1989; Kou and Lee, 1990). This latter observation implies that nicotine is the primary agent in tobacco smoke responsible for airway sensory stimulation.

The airway sensations elicited by nicotine are known to be a source of reward. For example, Pritchard et al. (1996) found that the increase in satisfaction obtained by increasing cigarette nicotine content is related as much to the perceived sensory impact of the cigarettes as it is to the plasma nicotine level. In addition, Rose et al. (1999b) found that systemic blockade of peripheral nicotinic acetylcholine receptors using the ganglionic blocker trimethaphan reduces the satisfaction obtained from smoking. This manipulation also reduced the intensity of airway sensations, implying that nicotine's airway sensory effects contribute to reward from smoking.

While previous studies have established a role for the sensory effects of both nicotine and non-nicotine tobacco constituents in smoking reward, the relative contribution of airway sensory stimulation by nicotine and non-nicotine constituents to smoking reward is not known. This is necessary to know in order to understand exactly how denicotinized cigarettes are different from nicotinized cigarettes, which has implications for the use of denicotinized cigarettes both as aids to smoking cessation and as control stimuli in experimental studies of nicotine's pharmacologic effects. Also, no study has addressed nicotine sensory reward at the level of individual puffs. This is important because individual puffs are the natural behavioral units of airway sensory stimulation by smoking. This is also necessary for interpreting the results of studies that use individual puffs to address the psychological and neural processes underlying nicotine reward.

In this study, we sought to quantify the extent to which the airway sensory effects of the nicotine delivered by individual puffs give rise to reward, above and beyond the reward derived from the airway sensory effects of non-nicotine tobacco constituents. To isolate the airway sensory effects of nicotine from the direct central nervous system effects of nicotine, we assessed self-reported reward from individual puffs within 7 s of inhalation. This interval allowed airway sensations to be elicited by the nicotine in a puff, but did not allow the nicotine in a puff to reach the brain. We compared the reward from puffs from nicotinized cigarettes to the reward from puffs from denicotinized and unlit cigarettes. We also assessed the self-reported strength of airway sensations stimulated by puffs from each type of cigarette, which allowed us to correlate the reward elicited by nicotine with the airway sensations elicited by nicotine. All of the subjects in this study smoked less than one hour before testing. This was done in order to diminish the pharmacologic effects of nicotine. This also approximated the usual conditions of smoking for a typical one pack-per-day smoker.

2. Method

2.1. Subjects

All procedures were approved by the University of Iowa Institutional Review Board for Human Subjects Research. Twenty cigarette smokers were recruited through advertisements in the university and local community. All reported smoking more than 20 cigarettes (1 pack) per day for at least 1 year. Subjects were screened by self-report to exclude any current medical, neurological or psychiatric disorders, including a history of dependence upon substances other than tobacco. Subject characteristics are described in Table 1.

2.2. Pre- and post-procedure assessments

Subjects were instructed to smoke ad libitum in the hours before the experiment and to smoke one of their own cigarettes just before they entered the facility, a university hospital. The experiment took place in a stainless steel chamber equipped with a high-performance ventilation system. Upon arriving at the facility, subjects gave informed consent, completed

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the Fagerstrom Test for Nicotine Dependence (FTND) (Heatherton et al., 1991) and reported the time of smoking the last cigarette and their usual brand of cigarette.

Heart rate, withdrawal symptoms and self-reported urge were measured both before and after the controlled smoking procedure in order to determine subjects' response to the pharmacological effects of the nicotine delivered during the procedure. Heart rate was measured over a two-minute interval at rest, with eyes closed. Subjects were administered the Minnesota Withdrawal Form (MWF) (Hughes, 1992) and the Brief Questionnaire of Smoking Urges (QSU-B) (Cox et al., 2001). Exhaled carbon dioxide (CO) was also measured before and after the procedure using a Microsmokerlyzer (Bedfont, Kent, UK).

2.3. Stimuli

All cigarettes were Quest cigarettes (Vector Tobacco, Inc., New York, NY). Quest cigarettes are made from tobacco in which a key enzyme for nicotine synthesis (quinolinate phosphoribosyl transferase) has been inactivated through gene duplication. Varying nicotine levels are achieved by blending of modified and wild-type strains. This method of denicotinization leads to a nearly complete removal of nicotine and is presumed to spare more of the sensory properties of the non-nicotine constituents of tobacco smoke than chemical denicotinization. Nicotinized cigarettes were Quest 1: Low Nicotine (0.6 mg nicotine; 10 mg tar; FTC method). Denicotinized cigarettes were Quest 3: Nicotine Free (less than 0.06 mg nicotine; 10 mg tar; FTC method). Unlit cigarettes were either Quest 1 or Quest 3. All identifying marks were covered so that the cigarettes appeared identical. Cigarettes were handed to the subject by the experimenter, who sat directly across from the subject, separated by a small table. A screen obscured the view of the lit cigarettes, ashtrays and lighter.

2.4. Controlled smoking procedure

Eighteen individual puffs were presented in six blocks. Each block consisted of three trials, with each puff type (nicotinized, denicotinized and unlit) being presented once per block in pseudo-random order. The subject was not aware of the sequence. On each trial, the subject was handed a cigarette and instructed to hold the cigarette close to his or her face, without touching it to the lips. After 5 s, the subject was instructed to take a single puff from the cigarette, inhaling and exhaling as if smoking one of his or her own cigarettes. Four seconds after the instruction to puff, the subject was prompted to rate the puff as follows: ''Rate pleasure'' [rating], ''Rate strength'' [rating], ''Rate desire for more'' [rating]. A 4-s interval was chosen to allow the subjects time to inhale and exhale fully before rating the puffs. The onset of inhalation usually followed this instruction by 2–3 s, during which the subject brought the cigarette to the lips and drew smoke into his or her cheeks. Pleasantness ratings were nearly always completed within 7 s of the instruction to puff. This means that most pleasantness ratings actually occurred within 4–5 s following the onset of inhalation. Ten seconds after the desirability rating, the next cigarette was handed to the subject. Subjects took two practice puffs from unlit cigarettes before the beginning of data collection, in order to gain familiarity with the procedure. New cigarettes were lit after every 6 trials, such that no more than two puffs were taken from any one cigarette and the char lines never reached more than 2/3 of the way to the filter. During lighting of the new cigarettes, subjects were allowed to sip water and to communicate with the experimenter.

2.5. Self-report ratings of puffs

Ratings were made verbally using 7-point (1–7), Likert-type scales. Before the procedure, subjects were instructed on the use of the rating scales, which were displayed before the subject throughout the procedure. The first item was ''How pleasant was the puff?'' Subjects were instructed that this referred to how much they liked the puff, or found it pleasurable. The second

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item was ‘‘How strong was the puff?’’ Subjects were instructed that this item referred to the strength of the sensations in the throat and chest caused by the puff. The third item was ‘‘How much do you desire another puff from this cigarette?’’ Subjects were instructed that this referred specifically to the desire for another puff from the cigarette from which a puff had just been taken, and not a desire for puffs from other cigarettes or and overall urge to smoke. Subjects were instructed that they would be prompted to rate the 3 items after each puff and were told to refer to the scales as necessary throughout the procedure.

2.6. Heart rate

EKG was measured using the standard configuration for lead II. Signals were acquired at 1 KHz. The transducers, amplifiers, analog to digital converter (MP100) and data acquisition and analysis software were all from Biopac, Inc. (Santa Barbara, CA). The mean heart rate was measured pre- and post-procedure over the two-minute interval during which subjects rested with eyes closed. Heart rate for each beat was measured using a peak detection algorithm implemented within the Biopac software. The mean heart rate was the average of the series of individual heart beat measurements.

2.7. Data analysis

Responses from the first block were excluded in order to reduce novelty effects. Only trials on which pleasantness ratings were completed within 7 s of the instruction to puff were included. The remaining puff-related responses were averaged within each subject for each puff type. The dependent measures for each puff type were thus: mean pleasantness rating (pleasantness), mean desirability rating (desirability) and mean strength rating (strength).

Repeated measures ANOVA was used to examine the overall effect of puff type on each of the dependent measures. The degrees of freedom were Hyunh–Feldt corrected as deemed necessary by Mauchly’s test of sphericity. Post-hoc *t*-tests were used to compare puff types pair-wise. The critical *t*-values (2-tailed) for significant effects were adjusted for multiple comparisons using Tukey’s correction. This led to a critical $t(19) = 3.59$ for $p < 0.05$ and $t(19) = 4.67$ for $p < 0.01$, which applied to all comparisons made between puff types. In addition, an effect size estimate (*d*) was calculated for each comparison, taking into account the correlation between dependent variables due to a repeated measures design (Dunlap et al., 1996).

For each subject, the difference in the mean rating for nicotinized puffs and the mean rating for denicotinized puffs was calculated for the pleasantness, desirability and strength reports. This provided, for each subject, measures of the degree to which nicotinized puffs were reported as stronger, more pleasant and more desirable than denicotinized puffs, respectively. The difference scores for pleasantness and desirability were respectively correlated with the difference score for strength using Pearson product moment correlation.

3. Results

Fig. 1 shows the mean self-report responses for each puff type. The ANOVA revealed a main effect of puff type on ratings of pleasantness [$F(2,38) = 57.42$, $p < 0.0001$], desirability [$F(2,39) = 43.19$, $p < 0.0001$] and strength [$F(1.5,29.6) = 61.83$, $p < 0.0001$, Hyunh–Feldt corrected]. Post-hoc *t*-tests revealed that, compared to denicotinized puffs, nicotinized puffs were rated as more pleasant ($t = 5.06$, $p < 0.01$, Tukey-corrected), more desirable ($t = 5.24$, $p < 0.01$, Tukey-corrected) and stronger ($t = 5.70$, $p < 0.01$, Tukey-corrected). Pleasantness, desirability and strength were all greater for both denicotinized and nicotinized puffs than they were for unlit puffs (all *t*’s >5.0, all *p*’s <0.01, Tukey-corrected). Table 2 shows the effect size calculations for each of these comparisons.

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As shown in Fig. 2, the degree to which smokers reported nicotinized puffs as stronger than denicotinized puffs was significantly positively correlated with the degree to which smokers found nicotinized puffs more pleasurable than denicotinized puffs ($r = 0.70$, $p = 0.001$) and the degree to which they found nicotinized puffs more desirable than denicotinized puffs ($r = 0.71$, $p = 0.001$).

There were no pre- to post-procedure changes in heart rate ($t = 0.79$, $p = 0.44$), MWF score ($t = 0.5$, $p = 0.31$) or QSU-B score ($t = 0.79$, $p = 0.44$), indicating that, as a group, subjects' responsiveness to nicotine's pharmacological effects was diminished. Exhaled CO increased significantly from pre- to post procedure ($t = 2.61$, $p = 0.02$). These data are summarized in Table 3.

4. Discussion

In this study we show that individual puffs from nicotinized cigarettes are subjectively appreciated as being more pleasurable and desirable and as eliciting stronger airway sensations than puffs from denicotinized cigarettes when these are rated immediately after inhalation. We find this in the context of a diminished pharmacological effect of nicotine, as shown by a failure of the procedure as a whole to increase heart rate and decrease withdrawal symptoms or smoking urges. Furthermore, we show that the extent to which nicotinized puffs are rated as more pleasant and more desirable than denicotinized puffs are respectively correlated with the extent to which nicotinized puffs are rated as eliciting stronger airway sensations than denicotinized puffs.

Previous studies (Herskovic et al., 1986; Baldinger et al., 1995; Perkins et al., 1996, 2002; Shahan et al., 1999) have shown that increasing the nicotine content of individual puffs increases their reward value, but these studies did not make an effort to distinguish between the reward derived from nicotine's airway sensory effects and the reward derived from nicotine's direct central and ganglionic effects. The present results provide direct evidence that nicotine delivered by individual puffs can give rise to reward through airway sensory effects alone. These results are consistent with previous findings showing that nicotine exerts sensory effects upon the airway (Ginzel, 1975; Lee et al., 1993) and with findings that have provided correlative evidence that nicotine's airway sensory effects contribute to smoking reward (Pritchard et al., 1996; Rose et al., 1999a,b). This study provides new information by completely isolating nicotine sensory reward from nicotine pharmacologic reward. This allowed for a quantification of the relative contributions of nicotine and non-nicotine sensory stimulation to smoking reward at the level of individual puffs.

Subjects were asked to rate the pleasantness of puffs beginning 4 s after the instruction to puff, and always completed this rating within 7 s of inhalation. Seven seconds is the minimum estimate of the time required for nicotine to reach the brain following inhalation (Benowitz, 1990). This estimate is based upon the lung–brain transit time of inhalation anesthetics (Mapleson, 1973). Unlike inhalation anesthetics, which are lipophilic, nicotine is positively charged at the pH of cigarette smoke, and is therefore likely to be exchanged more slowly across the alveolar surface. Indeed, the absorption kinetics of nicotine have been found to be significantly slower than previously assumed (Rose et al., 1999a,b). Studies in rodents (Brewer et al., 2004) indicate that the delivery of nicotine to the brain may be slowed significantly by binding within the lung. This means that 7 s may actually be an underestimation of the minimum inhalation–brain delay for nicotine. This makes it extremely likely that, in the present study, pleasantness ratings were always completed before nicotine had a chance to bind to receptors within the brain, which would mean that differences in pleasantness ratings between

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nicotinized and denicotinized puffs cannot be attributed to nicotine's direct central nervous system effects.

Because ratings of desirability were usually made after 7 s following inhalation, it is possible that these ratings were influenced by nicotine's direct central effects. However, if this were the case, then the effect size for the difference between nicotinized and denicotinized puffs should have been greater for ratings of desirability than for ratings of pleasantness, since this delay would have allowed time for nicotine pharmacologic reward to add onto nicotine sensory reward. However, these effect sizes differed by only a very small amount. One possibility is that ratings of desirability, like ratings of pleasantness, also occurred before nicotine reached the brain. The maximum estimate of the inhalation–brain transit time for nicotine has been reported to be 19 s (Henningfield and Keenan, 1993). Though the interval between inhalation and desirability rating was not measured in the present study, this interval is likely to have been less than 19 s for a large proportion of the trials.

Another possibility is that the dose of nicotine delivered to the brain by each puff was too low to exert an effect on any of the self-report measures, regardless of the inhalation-rating interval. It has been shown that intravenous nicotine delivered in doses obtained from smoking whole cigarettes does not lead to a subjectively appreciable acute reward above that obtained from smoking denicotinized cigarettes (Rose et al., 2000). Also, a recent meta-analysis of studies examining nicotine self-administration in humans (Dar and Frenk, 2004) has shown that dependent smokers do not self-administer nicotine in the absence of the sensory-motor process of smoking. Together, these findings suggest that the pharmacologic effects of nicotine, even in doses delivered by whole cigarettes, is not a source of acute reward from smoking. Though controversial (see Perkins, 2004), these findings would imply that the nicotine delivered to the brain by individual puffs from nicotinized cigarettes would not be enough to elicit a subjectively rewarding effect above the reward obtained from the airway sensory effects of non-nicotine constituents. This would suggest that differences in reward value between nicotinized and denicotinized puffs are due primarily to the airway sensory impact of nicotine. However, this interpretation requires some caution, since the precise contribution of nicotine's pharmacologic effects to the reward from individual puffs is not known.

In the present study, the ability of nicotine's pharmacologic effects to contribute to the reward from individual puffs was further diminished by acute tolerance to the pharmacologic effects of nicotine. Subjects possessed a diminished response to nicotine's pharmacologic effects, as indicated by a lack of effect of the procedure on heart rate, withdrawal symptoms or urge to smoke. If the procedure as a whole, which delivered 6 puffs from nicotinized cigarettes, did not exert a significant pharmacologic effect, then individual puffs from nicotinized cigarettes were unlikely to have exerted a pharmacologic effect, including pharmacologic reward. Acute tolerance to nicotine's pharmacologic effects has been shown to last for approximately 2 h after nicotine exposure (Perkins et al., 1995). This means that for a typical one pack-per-day smoker, who smokes on average every hour, most cigarettes are smoked under conditions of acute tolerance to nicotine's pharmacologic effects. For them, like for the subjects in the present study, the majority of reward obtained from nicotine may actually be due to its airway sensory effects, rather than to its pharmacologic effects.

Despite tolerance to nicotine's pharmacologic effects, subjects were still able to appreciate and derive reward from nicotine's airway sensory effects. This suggests that the airway sensory effects of nicotine are less susceptible to acute tolerance than the pharmacologic effects of nicotine are. One explanation for this may be that, unlike nicotine's pharmacologic effects, nicotine's airway sensory effects are not mediated by nicotinic acetylcholine receptors; rapid desensitization of nicotinic acetylcholine receptors may underlie acute tolerance, so a non-

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receptor-mediated process could potentially circumvent acute tolerance. This is unlikely, however, since the airway expresses an abundance of nicotinic acetylcholine receptors (Wang et al., 2001; Keiger et al., 2003; Proskocil et al., 2004) and it has been shown that blockade of nicotinic acetylcholine receptors significantly reduces the airway sensory effects of smoking (Lee et al., 1993; Rose et al., 1999a,b). An alternative explanation is that the airway sensations stimulated by nicotine may be transduced by an isoform of the nicotinic acetylcholine receptor that is not susceptible to rapid desensitization, or does not undergo as complete a desensitization as the receptors that mediate nicotine's pharmacologic effects.

Though the primary difference between nicotinized and denicotinized cigarettes in the present study was assumed to be the airway sensory impact of nicotine, these may have also differed with respect to some non-nicotine sensory factor. This may have occurred either as a result of the genetic denicotinization process or, more likely, through the manipulation of the denicotinized tobacco by the manufacturer in order to increase its sensory impact. Tobacco industry documents (Bates, 1983, 1995) show that cigarette manufacturers have a long history of manipulating the pH of mainstream tobacco smoke in order to modify its sensory impact. One way that pH may affect sensory impact is by modulating the absorption of nicotine; a higher pH of mainstream smoke has been shown to lead to a greater absorption of nicotine in the airway (Armitage and Turner, 1970). In this case, however, increasing pH would only increase the sensory impact of nicotinized cigarettes. Another possibility is that pH acts upon sensory impact of non-nicotine constituents independently of its effects on nicotine absorption. However, evidence for such effects is lacking. Apart from pH, it is possible that any number of non-nicotine constituents may have been introduced into the tobacco that may have modulated the sensory impact of the denicotinized and nicotinized cigarettes used in the present study. The extent of such manipulations cannot be known without a full chemical analysis of the mainstream smoke of each of the cigarettes, which is beyond the scope of this study. Though the potential effects of such manipulations are important to consider in any study that compares nicotinized and denicotinized cigarettes, they are likely to be small in relation to the airway sensory effects of nicotine.

In this study, the estimates of effect size allowed for a quantitative comparison between the airway sensory impact of nicotine and the airway sensory impact of non-nicotine tobacco constituents. For each of the self-report measures, the effect size of the difference between nicotinized puffs and denicotinized puffs was approximately equal the effect size of the difference between unlit puffs and denicotinized puffs. This indicates that, for cigarettes with a nicotine content of 0.6 mg and a tar content of 10 mg, the airway sensory impact of nicotine is approximately equal to the airway sensory impact of non-nicotine constituents. This also indicates that the reward derived from the airway sensory impact of nicotine is approximately equal to the reward derived from the combined effects of the airway sensory impact of non-nicotine constituents and the motor act of puffing. The nicotinized cigarettes used in this study, which may be considered ''light'', have a relatively low nicotine/tar ratio: 0.06. For full-flavored cigarettes, which have higher nicotine /tar ratios, the proportion of sensory impact that can be explained by nicotine would be even higher.

This means that any study that quantifies the reward obtained from the nicotine in individual puffs will include a significant component of reward derived from airway sensation. This includes studies that use puffs from denicotinized cigarettes as ''control'' stimuli. For instance, Shahan et al. (1999) have shown that subjects will perform more work to obtain puffs from nicotinized cigarettes than they will to obtain puffs from denicotinized cigarettes. If one assumes that denicotinized cigarettes control completely for the sensory-motor process of smoking, then this result can be taken as evidence that reward is derived from the pharmacological effects of nicotine. However, if, as the present results suggests, a significant

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proportion of the reward from individual puffs can be attributable to the sensory impact of nicotine, the difference in reinforcement value between nicotinized and denicotinized puffs may be due to differences in sensory impact. Perkins et al. (2002) used a similar reinforcement paradigm to address sex differences in the reward from nicotine. In this study, it was found that the reinforcement value of puffs is more sensitive to nicotine content in males than in females. This was taken as evidence supporting the hypothesis that males are more sensitive than females to the primary reinforcing effects of nicotine, in contrast to females, who are more sensitive to conditioned reinforcement from non-nicotine stimuli (reviewed in Perkins et al., 1999). The present results indicate that nicotine in puffs is in itself is a source of conditioned reinforcement. This points to the possibility that males may actually be more sensitive to the conditioned reinforcement derived from the airway sensory effects of smoking than females are. A number of studies have also shown that smokers' preference for the nicotine content of individual puffs increases as a result of nicotine deprivation (Rose et al., 1984a,b; Herskovic et al., 1986; Perkins et al., 1996; Madden and Bickel, 1999). The present results suggest that what is modulated by deprivation is the reward derived from the airway sensory effects of nicotine. This is similar to the way in which hunger modulates the reward obtained from the sensory impact of food, a phenomenon known as alliesthesia (Cabanac, 1971). Because they are temporally discrete units of smoking reward, individual puffs may also soon be used in combination with event-related functional imaging techniques, such as functional magnetic resonance imaging (fMRI), to address the neural substrates of nicotine reward. The present results imply that any study that compares the neural response to nicotinized puffs to the neural response to denicotinized puffs will reveal neural correlates of reward derived from nicotine's airway sensory effects. Indeed, the methods presented in the present study are ideally suited to address the neural substrates of sensory reward from smoking.

A clinical implication of these results is that smokers who switch to low nicotine and denicotinized cigarettes as a way to wean themselves off of nicotine will find that they receive less reward from each puff than they are used to, due to a reduced sensory impact. This may reduce the efficacy of this smoking cessation strategy. It has been suggested that switching to reduced nicotine cigarettes may be more acceptable to smokers if their sensory impact could be somehow increased (Rose and Behm, 2004). These manipulations, which may include increasing the pH of the denicotinized tobacco smoke, would increase the efficacy of denicotinized cigarettes for reducing smoking urges and would also increase compliance, both of which would tend to reduce the rate of relapse to smoking the usual brand.

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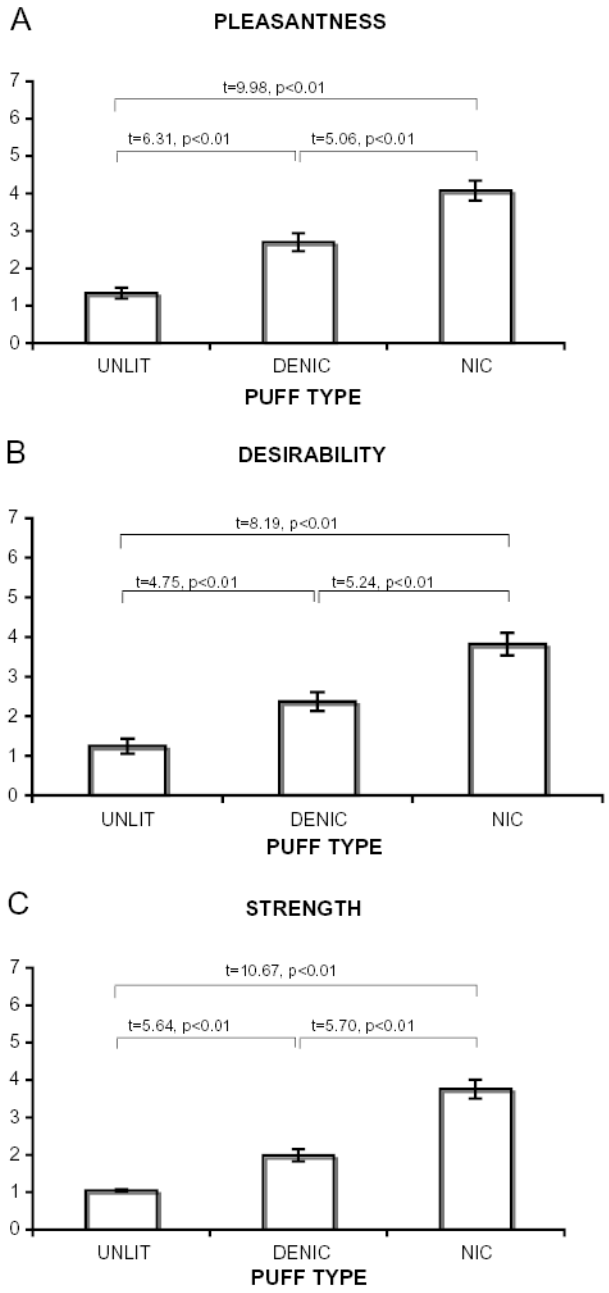


Fig 1.

Self-report pleasantness (A), desirability (B) and strength (C) of unlit, denicotinized and nicotinized puffs. Error bars represent 1 standard error of the mean. All *t*-tests are 2-tailed and all *p*-values are Tukey-corrected for multiple comparisons.

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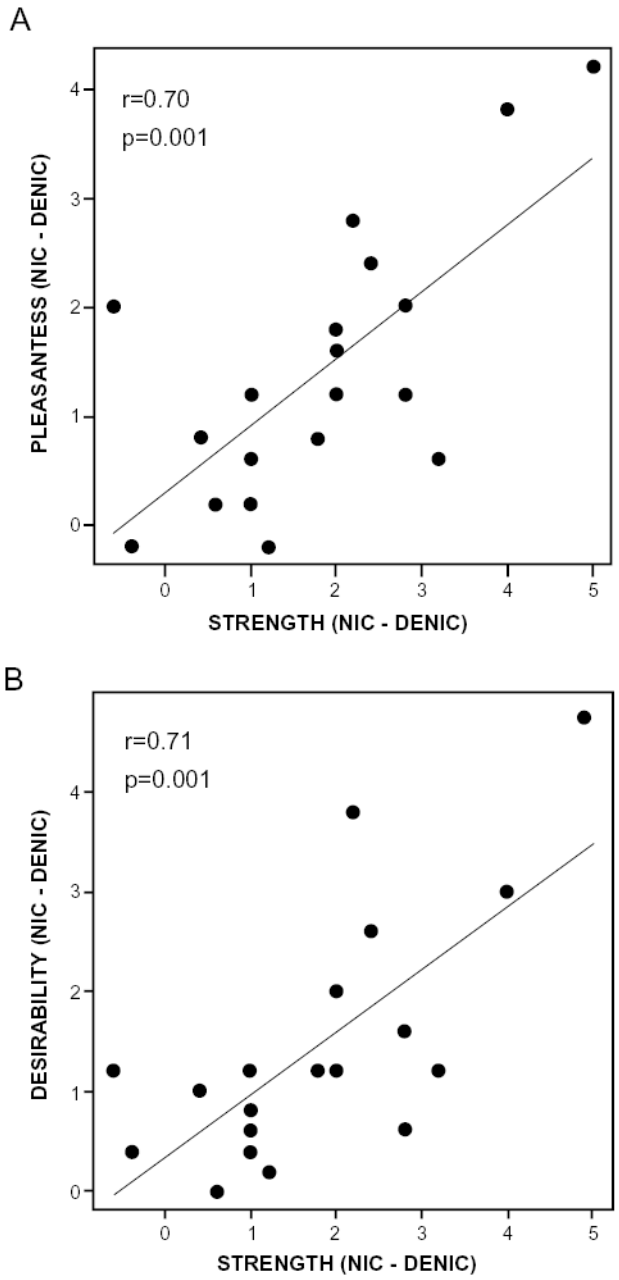


Fig 2.

Correlation of the difference in strength between nicotinized and denicotinized puffs and (A) the difference in pleasantness between nicotinized and denicotinized puffs and (B) the difference in desirability between nicotinized and denicotinized puffs. Points represent difference scores for individual subjects.

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Table 1

Subject characteristics

	N	Mean	Range	S.D.
Females	9			
Males	11			
Menthol smokers	2			
Hand-rolled smokers	1			
Age		29	19–60	12.72
Cigarettes/day		25.5	20–40	7.05
Years smoking this much		16.93	1–38	12.49
Minutes since last cigarette		18.6	8–30	0.16
FTND score		5.45	2–9	2.06
Usual brand nicotine*		0.91	0.1–1.2	0.23
Usual brand tar*		12.24	5–16	3.39

* Hand-rolled cigarettes excluded from calculation of nicotine and tar content.

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Table 2

Effect size (*d*) estimates comparing different puff types

	Unlit vs. Denic	Unlit vs. Nic	Denic vs. Nic
Pleasantness	1.49	2.85	1.24
Desirablity	1.18	2.45	1.27
Strength	1.75	3.23	1.88

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Table 3

Changes from pre- to post-procedure

	Pre-procedure	Post-procedure	SD of change	t	p
Heart rate	81.63	81.16	4.85	0.79	0.44
QSU-B score	12.20	12.90	8.62	0.79	0.44
MWF score	2.90	3.50	2.19	1.05	0.31
Exhaled CO	22.40	28.75	4.00	2.61	0.02

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ORIGINAL INVESTIGATION

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Naltrexone reduces the relative reinforcing value of nicotine in a cigarette smoking choice paradigm

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Abstract *Rationale:* Human behavioral pharmacology studies can examine how medications that target different neurotransmitter systems influence different aspects of smoking. Naltrexone and bupropion have been shown to alter ad lib smoking behavior; however, medication effects on nicotine reward in a cigarette choice paradigm have yet to be investigated. *Objective:* This study explored the effects of an acute dose of naltrexone, bupropion, or placebo on the relative reinforcing value of nicotine from cigarette smoking using new nicotine and de-nicotinized (Quest, 0.6 and 0.05 mg = "denicotinized") cigarettes. *Methods:* In a double-blind, within-subjects design, 26 dependent smokers participated in three experimental cigarette smoking sessions following pretreatment with either naltrexone (50 mg), bupropion (300 mg), or placebo. After medication administration and 2 h of monitored deprivation from cigarettes and food, participants rated their responses to the initial exposure to the cigarettes and then participated in four choice sessions over a 2-h period during which they could take four puffs from either cigarette. *Results:* The relative reinforcing value of nicotine, as measured by the number of nicotine puffs chosen out of 16, was significantly lower following naltrexone compared to placebo. There were no effects of an acute dose of bupropion on nicotine choices. *Conclusions:* These results suggest that naltrexone may reduce the relative reinforcing effects of nicotine *via* cigarette smoking and support ongoing investigation of opioid antagonists as potential smoking cessation pharmacotherapies.

Keywords Nicotine · Smoking · Naltrexone · Human · Opiates

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Introduction

The use of medications to help people quit smoking has shown promise. Although nicotine replacement therapies and bupropion are effective medications for smoking cessation, as many as 80% of smokers relapse (Fiore et al. 1994; Hughes et al. 2003; Hurt et al. 2002; Litten and Allen 1999; Peters and Morgan 2002; West 2003). Several neurotransmitter systems have been shown to play a role in the rewarding effects of nicotine, including dopamine, acetylcholine, and endogenous opioids; however, little is known about how medications influence smoking behaviors (Balfour 2002; George and O'Malley 2004; Litten and Allen 1999). Human behavioral laboratory studies provide a model to examine mechanisms that contribute to smoking behavior and to test how medications that target different neurotransmitter systems influence different aspects of smoking.

Several studies have utilized behavioral pharmacology paradigms to investigate the mechanisms of action and the effects of pharmacotherapies on smoking behavior, with conflicting results. Bupropion, the only non-nicotine medication approved by the FDA for smoking cessation, has been shown to be effective in several placebo-controlled trials (Collins et al. 2004; Ferry and Johnston 2003; Hayford et al. 1999; Holm and Spencer 2000; Hurt et al. 1997, 2002; Jorenby et al. 1999). Bupropion, like amphetamine, elevates synaptic dopamine and norepinephrine levels (Ferris and Beaman 1983; Ferris et al. 1982, 1983; Nomikos et al. 1990; Trulson 1985; Trulson and Crisp 1985). In contrast to the effects when administered chronically, acute administration of a single dose of bu-

propion and amphetamine increased the number of cigarettes smoked *ad libitum* and decreased sensory responses to smoking (Cousins et al. 2001). Blunted "intensity" and "buzzed" sensations while on bupropion may have led smokers to smoke more ad lib to compensate for the reduced sensations, an effect that may be attributable to bupropion's non-competitive antagonist effects on nicotine receptors (Slemmer et al. 2000). Acutely, bupropion could also increase smoking through a priming effect (de Wit 1996).

Pre-clinical research documenting nicotine–opioid interactions (Boyadjieva and Sarkar 1997; Davenport et al. 1990; Wewers et al. 1999) led to studies of the effects of naltrexone, an oral opioid receptor antagonist, on smoking behavior. In animals, nicotine acutely increases plasma levels of beta-endorphins (Conte-Devolx et al. 1981) and endogenous opioid metenkephalin levels in the nucleus accumbens (Houdi et al. 1991; Pierzchala et al. 1987). Opiate antagonism with naloxone precipitates nicotine/opiate withdrawal symptoms in both animals and humans (Krishnan-Sarin et al. 1999; Malin et al. 1993, 1996). As with bupropion and mecamylamine, naltrexone may also be a noncompetitive nicotine antagonist (Almeida et al. 2000).

Naltrexone and naloxone have been investigated for effects on smoking in behavioral pharmacology studies (Epstein and King 2004; Hutchison et al. 1999; King and Meyer 2000) and in clinical settings (Covey et al. 1999). Acute pre-treatment with naloxone reduces cigarette consumption (Gorelick et al. 1989; Karras and Kane 1980) and satisfaction (Palmer and Berens 1983) in some studies, but not in others (Nemeth-Coslett and Griffiths 1986). King and Meyer (2000) found that a single oral dose of 50 mg naltrexone reduced craving, desire to smoke, and sensory effects of smoking, and reduced the total number of cigarettes smoked *ad libitum* relative to placebo. In another recent acute dose study, naltrexone significantly reduced the total number of cigarettes smoked and expired carbon monoxide levels compared to placebo (Epstein and King 2004). Naltrexone administered orally for 1 week increased the aversive effects of the first cigarette smoked each day and attenuated the increased wakefulness after smoking without altering ad lib smoking in the laboratory setting (Brauer et al. 1999). Taken together, these studies suggest that naltrexone may influence some aspects of cigarette smoking and not others.

Participants in the previous studies smoked *ad libitum*, meaning that any differences between the effects of naltrexone and placebo could reflect medication effects on either the rewarding value of nicotine or on other, non-nicotine conditioned rewarding aspects of smoking (Robinson et al. 2000; Rose et al. 2000; Rusted et al. 1996). An alternative approach to evaluating the relative reinforcing effects of nicotine is *via* a choice paradigm (Perkins et al. 1996, 2001, 2002). This paradigm has been adapted from similar procedures with other drugs such as caffeine (Oliveto et al. 1992), alcohol (de Wit and McCracken 1990), and marijuana (Chait and Burke 1994). In this choice procedure, participants are given the opportunity to select puffs from either nicotine or non-nicotine cigarettes at discrete intervals over a 2- to 3-h period in a double-blinded fashion. The number of nicotine choices divided by the total number of opportunities to choose, a behavioral measure of nicotine's relative rewarding effects, is sensitive to differences in baseline smoking deprivation (Perkins et al. 1996). Similarly, choice of nicotine vs non-nicotine nasal spray is sensitive to baseline smoking deprivation (Perkins et al. 1996) and smoking status (Perkins et al. 2001), and predicts duration of smoking abstinence in a non-medication smoking cessation trial (Perkins et al. 2002).

In a majority of the Perkins et al. choice studies, nicotine was presented *via* nasal spray, which lacks the naturalistic elements of smoking. One remaining study used tobacco vs herbal cigarettes, which differed from one another in taste as well as nicotine content (Perkins et al. 1996). The present study extends previous works by using the new Quest 0.6- and 0.05-mg ("denicotinized") cigarettes recently made available commercially. Quest cigarettes utilize a proprietary process that enables the production of nicotine-free tobacco that tastes and smokes like the tobacco in other cigarette products (http://www.vectortobacco.com, http://www.questcigs.com, accessed 02/04/04). These cigarettes have identical levels of tar and other cigarette constituents, differ only in nicotine level, and provide an excellent model for evaluating nicotine self-administration and reinforcement.

Using Quest cigarettes in the nicotine choice paradigm, we examined the effects of acute oral doses of naltrexone and bupropion (vs placebo) on the relative reinforcing value of nicotine from cigarette smoking. Because a 50-mg single oral dose of naltrexone produces sufficient pharmacological blockade of the mu opioid receptor (Lee et al. 1988), we hypothesized that pre-treatment with naltrexone would reduce the number of nicotine cigarette choices (i.e., relative reinforcing value of nicotine). Although prior human laboratory work with bupropion (Cousins et al. 2001) found that a single acute dose increased ad lib smoking, the effects on the relative reinforcing value of nicotine have not been investigated. We hypothesized that bupropion would lead to fewer nicotine cigarette choices.

Materials and methods

Study participants

Smokers responding to local advertisements to participate in a study for non-treatment seeking smokers were screened for eligibility in July–November 2003. Persons were eligible if they were current smokers age ≥18 years who had smoked ≥10 cigarettes per day for the previous 12 months. Exclusion criteria included: allergies to bupropion and/or naltrexone, history of seizures, opiate, cocaine or stimulant dependence, current opiate use for pain; pregnancy, lactation or planning pregnancy; unstable angina, history of heart attack or stroke in the previous 6 months, insulin-dependent diabetes, current diagnosis or history of bipolar

disorder or psychotic disorders, current major depression, and/or current use of bupropion, naltrexone, nicotine replacement therapies or prescription medications. Persons who smoked menthol cigarettes were excluded because Quest cigarettes were available only as non-menthol at the time of the study. All of the 26 eligible and consenting smokers completed the three experimental sessions.

Procedures

Eligibility screening All trial procedures were reviewed and approved by the University of Pennsylvania Institutional Review Boards. Participants were screened at the Tobacco Use Research Center where they provided written informed consent, participated in a brief structured clinical interview and physical examination, and provided a sample for urine drug screen (and urine pregnancy test for females) to determine eligibility. They also completed baseline questionnaires to assess smoking history.

Experimental sessions Study participants took part in three experimental sessions, identical except for the medication administered, in a comfortable sitting room that was designed and ventilated for smoking studies. Sessions were separated by at least 5 days (medication wash-out period). The order of the medications for the three sessions was determined by random assignment and counterbalanced between participants.

Participants were asked to maintain usual cigarette smoking patterns before arriving at the sessions and to refrain from drinking alcohol or using recreational drugs or medications for 24 h prior to and 12 h following each session. Participants scheduled all three sessions for the same time of day (9:00 A.M., 12 P.M. or 4:00–5:00 P.M.). Upon arrival, participants had their expired breath alcohol levels and blood pressure measured, and all participants tested negative for pregnancy (women only) and drug screens. To standardize the time since last cigarette, participants were asked to smoke one of their preferred/own brand of cigarettes after which their CO was measured. Immediately after the baseline cigarette, participants were monitored while they ingested two capsules containing either 50 mg naltrexone (Mallinckrodt/Tyco, St. Louis, MO), 300 mg bupropion (Zyban-SR 150 mg tablets over encapsulated from Glaxo-SmithKline of RTP, NC), or placebo, all provided in identical opaque colored capsules with a small glass of water. The doses were chosen to be consistent with prior laboratory studies of smoking and naltrexone (Brauer et al. 1999; King and Meyer 2000; Sutherland et al. 1995) or bupropion (Cousins et al. 2001).

Initial cigarette exposure To give participants an initial basis for making judgments in the ensuing choice trials, they were exposed to the nicotine and denicotinized test cigarettes. This also provided an opportunity for us to assess the subjective effects of each cigarette. To standardize deprivation from cigarettes and food, the initial exposure occurred 2 h after the baseline cigarette and following a small snack (energy bar). The two test cigarettes were color-coded; for half the participants, the nicotine cigarette had a blue band, and for the others, it had a red band. For any given participant, this color scheme was maintained for all test cigarettes presented at all three sessions.

To standardize puffing (i.e., to reduce the likelihood of increased puffing on the lower nicotine cigarette), the test cigarettes were attached to a Clinical Research Support System (CReSS) smoking topography machine (Plowshare, Baltimore, MD). This device is designed to collect smoking topography variables by placing a cigarette in a sterilized flow meter mouthpiece, which is connected to a pressure transducer. The pressure transducer measures pressure changes that occur during inhalation. The pressure changes are amplified, digitized and sampled at 1,000 Hz. CReSS software converts the signal to airflow (ml/s) in real-time (s), and from this provides number of puffs, puff volume, puff duration, maximum flow, and inter-puff interval (time between puffs). The equipment was calibrated prior to each visit per the instructions of the manufacturer. For half the participants, the blue cigarette was in the left-hand machine; for the others, it was in the right-hand machine. Both the participants and research assistants were blinded to the nicotine content of the cigarettes throughout the study.

During each initial cigarette exposure, participants were instructed to take four puffs. To standardize puff volume, participants were instructed when to initiate and discontinue each puff by instructions presented on a computer monitor. After exposure to four puffs of the first cigarette, the participant completed the Cigarette Evaluation Scale (CES) and Sensory Questionnaire (SQ) (see "Dependent measures," below). Twenty minutes later, participants followed the same cigarette exposure procedure, smoking four puffs from the second colored cigarette and completing the CES and SQ. The 20-min interval was selected to prevent participants from becoming satiated with smoking during the course of the session, and is consistent with other smoking studies with multiple cigarettes in a single session (Pickworth et al. 1999; Zacny and Stitzer 1985; Zacny et al. 1987), as well as nicotine choice studies (Perkins et al. 2001).

Cigarette choice procedure The choice procedure, described elsewhere in detail (Perkins et al. 1996), was initiated following 30 min of monitored abstinence. Participants were presented with two freshly lit cigarettes, one blue and one red, each of which had nicotine levels corresponding to the initial exposure. Both cigarettes were placed in the mouthpieces of CReSS smoking topography units. Participants were permitted to choose any combination of four puffs from the two cigarettes, and were instructed *via* computer monitor to take a puff every 45 s. There were a total of four choice sessions, each 30 min apart. As in the initial exposure phase, the purpose of the 30-min interim intervals was to prevent nicotine saturation. During these intervals, participants were allowed to watch movies or read in the smoking laboratory room.

To verify that participants did not compensate for the lower levels of nicotine by attempting to increase their puff volume or duration for the denicotinized cigarette, we used paired *t*-tests to compare puff volume and duration for nicotine vs denicotinized cigarettes at initial exposure during the placebo session. No significant differences by cigarette type were found for puff volume or duration ($p>0.10$).

Dependent measures

Nicotine choice

Because puffing topography did not differ between cigarette types, as noted above, the numbers of puffs of each type were assessed as the units of reinforcement (i.e., self-administration). The primary outcome measure was the total number of times out of 16 that participants selected the 0.6-mg over the 0.05-mg nicotine cigarette during the four choice trials.

Subjective effects

The Questionnaire of Smoking Urges (QSU) was administered at the start of each treatment session and again 2 h after medication was administered. The QSU consists of 32 Likert-scaled items that measure acute smoking craving and urges (Tiffany and Drobes 1991). Each item on the QSU consists of a smoking urge statement for the participant to score from 1 (strongly disagree) to 7 (strongly agree). The QSU has two factor-derived subscales; factor 1 reflects desire to smoke for stimulation ("desire to smoke and anticipation of pleasure"); factor 2 assesses urge to smoke to relieve nicotine withdrawal ("anticipation of relief from negative affect and withdrawal"). The subscales are created as the means of their component items (15 and 11 items, respectively). We also created a total urge score as the mean of all 32 items. Internal consistency of these scales was high (Cronbach's alpha= 0.95, 0.92, and 0.96 for factor 1, factor 2, and total score, respectively).

The Cigarette Evaluation Scale (CES) was administered twice at the initial exposure to the test cigarettes during each treatment session, once for each type of cigarette. The CES is a self-report instrument, developed by Westman et al. (1992), consisting of 11 items on which participants rate the cigarette they just smoked, using response scales ranging from 0 (not at all) to 7 (extremely). Nine of the 11 items formed three subscales—satisfaction ("Was it satisfying," "Did it taste good"); psychological relief (e.g., "Did it calm you down," "Did it help you concentrate"); and toxicity ("Did it make you dizzy," "Did it make you nauseous"). Subscales were created as the means of the responses to the component items. The toxicity subscale was not used in the analyses due to low variance. The internal consistency of the satisfaction and psychological relief scales, as measured by Cronbach's alpha, was 0.93 and 0.86, respectively, for ratings of the non-nicotine cigarette, and 0.89 and 0.86, respectively, for ratings of the nicotine cigarette.

The Sensory Questionnaire (SQ), which was administered at the same times as the CES, contains nine items on which participants rate the puffs they took from the cigarette they just smoked, using response scales ranging from 0 (not at all) to 7 (extremely) (Rose et al. 2000). The items formed two subscales—"liking" (four items, e.g., "How satisfying were the puffs you just took"), and "strength" (five items, e.g., "Strength of puffs in chest"). Subscales were created as the means of the responses to the component items. Cronbach's alpha coefficients for the liking and strength scales were 0.90 and 0.96, respectively, for ratings of the non-nicotine cigarette, and 0.84 and 0.91, respectively, for ratings of the nicotine cigarette. Both the CES and SQ measures have been used previously in studies assessing subjective effects of low-nicotine cigarettes (Brauer et al. 2001; Robinson et al. 2000; Rose et al. 2000; Shahan et al. 1999).

Adverse effects

The Symptoms Evaluation Checklist (SEC) was administered following the four choice trials. The SEC consists of 17 items representing potential drug side effects (e.g., headache, nausea, fatigue) accompanied by response scales ranging from 0 (none) to 3 (severe). Several summary scales can be generated from this instrument; we used the total symptom count, which is the sum of the number of non-zero responses across all 17 items.

Data analysis

The primary outcome variable of interest in the analysis was cigarette choice. Secondary outcomes were variables representing: (1) changes in urge to smoke—specifically, difference scores (i.e., differences between ratings obtained prior to, and 2 h after, medication administration) on the QSU factor 1, factor 2, and combined scales; (2) side effects; and (3) subjective ratings of the cigarettes—specifically, ratings of the nicotine and non-nicotine cigarettes on the CES "satisfaction" and "psychological relief" scales, and the SQ "liking" and "strength" scales.

Effects of treatment on cigarette choice, urge to smoke, total number of side effects reported, and cigarette ratings were assessed using linear mixed-effects models (implemented *via* the MIXED procedure in SPSS). In addition to treatment (bupropion, naltrexone, placebo), each model also included session (first, second, third) as a factor, to account for possible period effects. As is usual in the analysis of cross-over studies, other predictors considered for inclusion in the models were the interaction of session by treatment; treatment given at previous session (none, bupropion, naltrexone, placebo), to take account of possible carryover effects; nicotine level of the participant's regular cigarette brand; and the interaction of nicotine level of regular brand by treatment. In addition, in light

of previous reports that gender may moderate the effects of naltrexone (Covey et al. 1999), the mixed-effects model of cigarette choice described above was repeated including gender and the interaction of gender by treatment as additional predictors. Similarly, we examined the role of baseline nicotine dependence, measured by the Fagerström Test for Nicotine Dependence (FTND) (Heatherton et al. 1991), as a possible covariate, by repeating the model with the main effect of FTND and the interaction of FTND by treatment as additional predictors. In all analyses, the contribution of these variables was not significant and they were excluded from the models reported here.

Results

Study participants

Fifteen (58%) of the 26 participants were male, 12 (46%) were married, 12 (46%) were college graduates, and 24 (92%) were white. Ages ranged from 18 to 68 (mean=44.0, SD=15.2). Participants reported smoking between ten and 30 cigarettes per day (mean=17.9, SD=6.6). Nicotine levels of participants' regular cigarette brands ranged from 0.2 to 0.5 mg for four (15%) of the 26 participants; from 0.6 to 1.0 mg for 16 participants (62%); and from 1.1 to 1.5 mg for the remaining six (23%). The mean score on nicotine dependence (FTND) at baseline was 4.38 (SD=2.37).

Effects of treatment on cigarette choice

The mean numbers of puffs taken from the nicotine cigarette in each of the three treatment conditions were as follows: bupropion, 10.92 (SD=4.47); naltrexone, 9.81 (SD=5.12); placebo, 11.31 (SD=4.24). A linear mixed-effects model was used to assess the effect of treatment on cigarette choice. The overall effect of treatment approached significance [$F_{(2,48)}$=2.48, p=0.095]. Pair-wise comparisons revealed that the nicotine cigarette was chosen significantly less often in the naltrexone sessions than in the placebo sessions (t=−2.17, p=0.035). There was no significant difference in cigarette choice between the bupropion and placebo sessions (t=−0.66, p=0.515).

Effects of treatment on urge to smoke

Baseline (pre-medication) scores on the QSU factor 1, factor 2, and combined scales were comparable across the three treatments. In general, scores tended to increase from pre-medication to 2 h post-medication, presumably due to the fact that participants were not smoking during this period. The mean change scores on the combined scale were as follows: bupropion, 1.55 (SD=0.93); naltrexone, 1.08 (SD=1.15); placebo, 1.51 (SD=1.06).

The overall effect of treatment on changes in QSU scores approached significance for the QSU combined scale [$F_{(2,48)}$=2.44, p=0.098] but not for QSU factor 1 [$F_{(2,48)}$=1.75, p=0.18] or factor 2 [$F_{(2,48)}$=1.49, p=0.24]. Pair-wise comparisons revealed that scores on the QSU combined scale increased less in the naltrexone sessions than in the placebo sessions (t=−1.85, p=0.07). Non-significant trends in the same direction were found for QSU factor 1 (t=−1.48, p=0.14) and factor 2 (t=−1.56, p=0.13). Differences between bupropion and placebo on these measures did not approach significance (p>0.80).

Side effects by treatment

Overall, the frequency of side effects was low in this study. The mean numbers of side effects reported, by treatment, were as follows: bupropion, 1.12 (SD=1.58; range=0–7); naltrexone, 2.12 (SD=2.39, range=0–7); placebo, 1.77 (SD=1.68, range=0–6). Based on a mixed-effects model, the overall effect of treatment on number of side effects reported was significant [$F_{(2,48)}$=3.24, p=0.048]; however, pair-wise comparisons showed no significant difference between placebo and either naltrexone (t=0.87, p=0.39) or bupropion (t=−1.63, p=0.11) with respect to the number of side effects reported. Bivariate associations of each individual side effect with treatment were examined using Friedman ANOVAs (nonparametric repeated-measures ANOVAs). The results indicated elevated levels of nausea (p=0.04) and dizziness (p=0.007) in the naltrexone session. However, neither nausea nor dizziness, nor an index denoting the sum of the two items, was significantly associated with nicotine choice, either across or within treatments (Spearman correlations=0.07 to −0.13; p>0.50).

Effects of treatment on cigarette ratings

Mean ratings of the nicotine and non-nicotine cigarettes on the CES satisfaction and psychological relief scales, and the SQ liking and strength scales, by treatment, are given in Table 1. We estimated linear mixed-effects models of the ratings on each of these scales. These models included the two cigarette type responses (i.e., evaluations of the nicotine and non-nicotine cigarettes) within each of the three treatment sessions. The effects of greatest interest were interactions between treatment and cigarette type; a significant interaction would indicate a difference across treatments in the extent to which participants evaluated the two types of cigarettes differently. There were no significant interaction effects for any of the four scales (p≥0.35). With the interaction terms removed from the models, significant main effects of cigarette type were found for CES satisfaction (mean difference between nicotine and non-nicotine=0.49, SE=0.15; t=3.35, p=0.001), SQ liking (mean difference=0.54, SE=0.13; t=4.22, p<0.0001), and SQ strength (mean difference=0.47, SE=0.13; t=3.67, p=0.0004). The overall effect of treatment was not significant for any of the four scales (p≥0.14). Pair-wise comparisons showed no significant differences between

Table 1 Mean (SD) scores on cigarette rating scales, by treatment and cigarette type

Variable	Bupropion		Naltrexone		Placebo	
	Nicotine	Non-nicotine	Nicotine	Non-nicotine	Nicotine	Non-nicotine
Cigarette Evaluation Scale (CES) satisfaction	3.44 (1.00)	2.87 (1.49)	2.94 (1.23)	2.65 (1.33)	3.40 (1.36)	2.79 (1.46)
CES relief	1.45 (0.60)	1.52 (0.79)	1.36 (0.61)	1.41 (0.68)	1.48 (0.73)	1.42 (0.81)
Sensory Questionnaire (SQ) liking	3.38 (0.98)	2.75 (1.24)	3.00 (1.26)	2.45 (1.21)	3.25 (1.00)	2.81 (1.19)
SQ strength	3.05 (1.10)	2.36 (1.07)	2.99 (1.20)	2.52 (1.31)	2.98 (0.97)	2.74 (1.25)

placebo and either naltrexone ($p \geq 0.15$) or bupropion ($p \geq 0.36$).

Patterning of cigarette choices across trials

Thus far, the data on cigarette choice have been presented as the number of puffs taken from the nicotine cigarette, collapsed across all four choice trials within a given experimental session. This use of aggregated data might have obscured patterns of choice within each session that might have differed across treatments. In an attempt to address this issue, we fitted non-linear mixed effects models to the data, using trial, treatment, and the trial by treatment interaction as predictors. Non-linear models were required because the response (number of nicotine puffs in a trial) can only take values 0 through 4, and is modeled as following a binomial distribution. Models were fitted separately for each of the three sessions, using treatment as a between-subjects factor. For each session, the models showed no significant trend over time and no significant differences in trend across the three treatments.

Discussion

The primary purpose of this study was to test whether naltrexone, compared to placebo, would alter the choice of nicotine compared to denicotinized cigarettes. We postulated that naltrexone would reduce the number of nicotine cigarette choices as a measure of the relative reinforcing value of nicotine. In support of our hypothesis, naltrexone significantly reduced the choice of nicotine cigarettes following a brief monitored period of abstinence. Our findings are consistent with those of a study in which a single dose of naltrexone significantly reduced the total number of cigarettes smoked ad lib (Epstein and King 2004). In contrast to that study, in which acute naltrexone increased total side effects in smokers after overnight abstinence (Epstein and King 2004), naltrexone was well tolerated in the present study and did not differ from placebo in terms of overall incidence of side effects.

Although research has provided evidence that naltrexone blocks the effects of nicotine (Brauer et al. 1999), previous laboratory studies examining the effects of naltrexone on smoking behavior have been limited by the inability to separate the pharmacological effects of nicotine from the sensory components (sight, smell, sensations of inhaling) that contribute to the reinforcing effects of smoking (Brauer et al. 1999; Epstein and King 2004; Sutherland et al. 1995). In the current study, this problem was surmounted by the use of a choice paradigm involving two commercially available cigarettes identical in all respects except for nicotine content. This is the first study of naltrexone and smoking behavior to show that naltrexone reduces the relative reinforcing value of nicotine per se, controlling for the non-nicotine aspects of smoking.

Participants' ratings of the test cigarettes and of their own smoking urges provided insight into potential mechanisms by which naltrexone might influence cigarette choice. Ratings of the test cigarettes showed neither a significant main effect of treatment nor a significant treatment by cigarette type interaction, suggesting that naltrexone does not affect the hedonic value of smoking in general or nicotine in particular. Compared to placebo, naltrexone did reduce smoking urges. Although the effect was marginal, it is in the direction consistent with other studies in which naltrexone decreased difficulty in abstaining (Covey et al. 1999), urge to smoke (Houtsmiller et al. 1997), post-cigarette craving, and desire to smoke during a subsequent 1-h nonsmoking interval (King and Meyer 2000). Another laboratory study reported that naltrexone did not alter smoking urges or nicotine withdrawal, yet it reduced expired carbon monoxide and number of cigarettes smoked (Epstein and King 2004). While the mechanism remains unclear, this and other studies suggest that naltrexone alters smoking behavior.

Concentrations of naltrexone in plasma following a single oral dose of 50 mg naltrexone, as used in the present study, have been reported to be in the range of 1–10 ng/ml (Kogan et al. 1977; Meyer et al. 1984; Verebey et al. 1976; Wall et al. 1981), and are much lower than the 25-μmol EC_{50} required for acute inhibition of nicotinic receptors in whole-cell recordings from rat hippocampal neurons in culture as reported by Almeida et al. (2000). However, humans, in contrast to rats, metabolize naltrexone to 6 beta-naltrexol, which is an opiate antagonist shown to have the ability to block the reinforcing properties of alcohol in rats independent of the effects of naltrexone (Rukstalis et al. 2000). Thus, in humans, both naltrexone and 6 beta-naltrexol may contribute to the effects found in the current study.

In contrast to most of the bupropion studies in smokers trying to quit (Hurt et al. 1997), a single dose of bupropion did not affect smoking behavior in the non-treatment seeking smokers in the current study. It is possible that bupropion effects on smoking behavior differ in non-treatment compared to treatment seeking smokers. In ad-

dition, the presumed action of bupropion on dopaminergic systems may take several days of medication administration (Ascher et al. 1995; Ferris and Beaman 1983; Ferris et al. 1982, 1983). In smoking cessation treatment, bupropion is typically administered 1 week prior to the quit-smoking date because the pharmacokinetics require this duration for effective levels to be attained. Thus, the single-dose design of the current study limits the interpretability of the results regarding bupropion.

In the current study, no significant differences were found between the effects of bupropion and placebo on smoking satisfaction, in contrast to the results of a study by Cousins et al. (2001). This discrepancy in results may have been due to differences in the smoking procedures in the two studies. In the current study, satisfaction was assessed following four puffs of the experimental cigarettes attached to a topography unit, whereas Cousins et al. assessed the subjective effects of smoking one of the smoker's usual brand, smoked at their normal rate.

In summary, compared to placebo, naltrexone reduced the relative reinforcing value of nicotine in a cigarette choice paradigm. The results warrant replication given the small sample size and small absolute difference between naltrexone and placebo. A limitation of the current study is that smoking was intentionally controlled to standardize puffing *via* smoking topography machine; thus, findings may not generalize to all smokers or to smoking of other nicotine containing products. In addition, although we assessed medication side effects, we did not include a general measure of mood. It is possible that naltrexone effects on nicotine choice were attributable, in part, to increases in negative affect or other non-specific mechanisms. Nonetheless, these findings are consistent with previous studies showing that naltrexone alters different aspects of smoking behavior. These data suggest that the cigarette choice paradigm is sensitive to medication effects and support the ongoing investigation of opioid antagonists as potential medications for smoking cessation.

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ORIGINAL INVESTIGATION

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Sex differences in the influence of nicotine dose instructions on the reinforcing and self-reported rewarding effects of smoking

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Abstract *Rationale:* Compared to men, the smoking behavior of women may be less responsive to nicotine and more responsive to nonpharmacological factors, perhaps including verbal information (e.g., dose instructions). *Objective:* This study compared the influence of the presence vs absence of dose instructions on the subjective and reinforcing effects of nicotine via cigarette smoking in men and women. *Methods:* Subjects (n=120) abstained overnight from smoking and were randomly assigned to one of four groups. Half of the subjects received nicotine cigarettes (Quest 1, yield of 0.6 mg), and the other half received denicotinized cigarettes ("denic"; Quest 3, yield of 0.05 mg). Furthermore, half of each subsample was accurately instructed they were receiving a "normal nicotine" or a "no nicotine" cigarette, while the other half received no instructions. Subjects completed baseline measures of craving and mood (positive and negative affect), took two puffs from the cigarette after receiving dose instructions or no instructions, and then rated the cigarette's "reward" value (liking, satisfying) and other characteristics. They also repeated the craving and mood measures. Subjects then smoked more of that same brand ad libitum over the next 30 min to measure reinforcement (puff number and latency to first puff). *Results:* Overall, nicotine increased reward, other cigarette ratings, and positive affect, but did not affect craving or smoking behavior. However, results varied by sex. Dose instructions enhanced the effects of nicotine on smoking reward and reinforcement in women, while instructions tended to dampen or even reverse these effects of nicotine in men (i.e., interaction of sex×nicotine×instructions). *Conclusions:* In women but not in men, the influence of nicotine on smoking reward and reinforcement is enhanced by accurate verbal information about the cigarette's nicotine dose. These results are consistent with the notion that the smoking behavior of women, relative to men, may be more responsive to nonpharmacological factors.

Keywords Nicotine · Dose instructions · Sex differences · Reinforcement · Smoking reward

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Introduction

Drug responses are influenced by pharmacological and nonpharmacological factors. The most common procedure of testing pharmacological effects of a drug in human research is under "blind" conditions, in which subjects are given no explicit verbal information about the drug (or told the substance may or may not contain active drug). Blind conditions hold constant nonpharmacological factors, such as expectancies about drug (Kirsch 1997), allowing the isolation of the drug's pharmacological effects. Conversely, research on nonpharmacological factors in drug effects usually involves the opposite manipulation, holding constant pharmacological exposure while varying nonpharmacological factors. For example, verbal information provided to subjects regarding the drug content of a substance (e.g., instructions that it contains "drug" vs "no drug") can influence the responses of subjects to consuming the substance (e.g., Fillmore and Vogel-Sprott 1992; Kirsch 1997). Such information can influence responses to cigarette smoking (Juliano and Brandon 2002), sometimes to a greater extent than actual nicotine dose (Perkins et al. 2003, 2004). Because accurate information about substances is usually available to drug users in the natural environment,

testing drug effects in the presence of dose instructions may better capture drug responses as they occur under naturalistic conditions, compared to testing in the absence of instructions (Kirsch and Rodino 1993; Martin and Sayette 1993).

Several studies have directly compared responses to drug as a function of the presence vs absence of dose instructions to determine if providing such instructions enhances responding. A few studies assessing physiological and subjective arousal effects of caffeine failed to show differences due to the presence vs absence of instructions. However, these studies did not manipulate dose, administering only caffeine (Nash et al. 2002) or only a placebo beverage (i.e., no caffeine; Mikalsen et al. 2001, experiment 2; Walach et al. 2002). Yet, Kirsch and Rodino (1993) also manipulated dose and found that self-reported tension was greater for caffeine vs placebo only among those given instructions that the beverage contained caffeine and not among those given no instructions or those told it contained no caffeine. In a study with alcohol, Bradlyn and Young (1983) found greater intoxication ratings due to 0.3 g/kg alcohol in those given accurate dose instructions vs those given no instructions, but instructions had no effect on ratings among subjects given a drink with no alcohol or a higher dose (0.6 g/kg).

In a clinical study involving quitting smokers, Hughes et al. (1989) found that under accurate dose instructions, but not under blind conditions, nicotine vs placebo gum medication influenced the number of cigarettes smoked, the rated helpfulness of the gum, and gum self-administration (trend only). On the other hand, other measures, such as self-reported side effects of the gum and smoking abstinence, tended to show the opposite influence of instructions—smaller effects of drug when subjects were accurately instructed about dose relative to blind conditions—suggesting that dose instructions dampened, rather than enhanced, some drug effects. To our knowledge, no study has compared the influence of the presence vs absence of dose instructions on the acute effects of nicotine in cigarettes (i.e., an abused form of nicotine).

In addition, unexplored are individual differences in the influence of explicit dose instructions on drug responding. There is reason to believe that men and women may differ in responsivity to some instructional manipulations, at least when it comes to smoking or nicotine reinforcement (self-administration) and reward (i.e., hedonic characteristics of the drug, such as self-reported "liking"). We have found that, relative to men, these effects in women may be more sensitive to nonpharmacological factors such as nonverbal information about smoking, or "cues" (e.g., sensory stimuli from smoking; Perkins et al. 2001), and less sensitive to the pharmacological factor of actual nicotine dose (i.e., assessed under blind conditions; Perkins et al. 1996; 2002). Other research suggests that women are more susceptible than men to instructional manipulations when reporting on perceived internal states (Roberts and Pennebaker 1995; Tremblay et al. 2004).

Recently, we examined the influence of the type of verbal information about nicotine dose (accurate vs inaccurate dose instructions), as well as the actual dose, on responses to smoking (Perkins et al. 2004). This study used a modified 2×2 balanced-placebo design (see Martin and Sayette 1993), in which subjects were divided into groups receiving a cigarette containing nicotine or one containing no nicotine (i.e., placebo), and within each group, half of the subjects received accurate instructions and the other half inaccurate instructions about the nicotine content of the cigarette they received. Thus, all subjects got dose instructions of some kind; there was no blind condition (i.e., a "no instruction" condition). We found that under accurate, but not inaccurate, dose instructions, nicotine increased smoking reward more in women than in men. However, there was no sex difference in smoking reinforcement, measured in this study by a computer task with escalating response requirements (i.e., progressive ratio) for earning cigarette puffs.

Thus, it appears that the rewarding and reinforcing effects of smoking in women, compared to men, are less sensitive to cigarette nicotine dose under blind conditions (Perkins et al. 2002), while the rewarding effects may be more sensitive to nicotine under conditions of accurate dose instructions (Perkins et al. 2004). However, confirmation of this notion requires a direct test of the effects due to the presence vs absence of dose instructions within one study, given the variability in procedures between these studies. If confirmed, this finding would extend prior observations regarding sex differences in response to nonverbal information about smoking (i.e., cues; Perkins et al. 2001) to another type of nonpharmacological factor in drug reward and reinforcement, verbal information about smoking (i.e., nicotine dose instructions).

In the present study, we directly compared the influence of accurate dose instructions vs no dose instructions on the subjective and reinforcing effects of nicotine via cigarette smoking in men and women. We hypothesized that the presence of dose instructions would enhance the influence of nicotine on these effects, compared to no instructions (i.e., blind conditions). We also hypothesized that the impact of instructions on the rewarding and reinforcing effects of nicotine (but not other effects) would be greater in women than in men, consistent with the notion that smoking behavior of women is more responsive than that of men to nonpharmacological factors.

Method

Subject eligibility

Eligible subjects were healthy young men and women who smoked more than ten cigarettes per day for at least 1 year. Their preferred brand had to have a nicotine yield of at least 0.6 mg, so that the denicotinized brand used in the study would be distinctly different from their usual brand (see

below). Subjects were excluded if they had ever smoked "ultra-light" brands (defined as nicotine yield <0.3 mg) for more than 2 weeks. Participants were recruited from ads placed in the surrounding community and were paid for participation.

Cigarettes

To manipulate nicotine exposure, the "normal" nicotine brand was Quest 1 (yield of 0.6 mg nicotine, 9 mg tar) and the denicotinized ("denic") brand was Quest 3 (yield <0.05 mg nicotine, 9 mg tar), both sold commercially by Vector Group, Ltd. (Miami, FL). Menthol smokers received menthol Quest; nonmenthol smokers received nonmenthol Quest. All cigarettes had identifiable markings covered.

Subjective measures

Cigarette ratings Smoking reward was assessed with two items from the Rose Sensory Questionnaire (see Westman et al. 1996): the ratings of "liking" and "satisfying." The Rose measure also asks "how high in nicotine" and "how similar to own brand" the cigarette is, which were included to assess other perceptual responses to the cigarettes that might differ as a function of nicotine and dose instructions. Each item in the Rose Questionnaire was rated on a 0 ("not at all") to 100 ("very much") visual analog scale (VAS). This measure has been used in similar studies from this lab (Perkins et al. 2001, 2002).

Mood Self-reported mood was assessed with the Diener and Emmons (1984) Mood Scale, consisting of nine items rated on 0 (not at all) to 100 (very much) VAS that yield a positive affect (PA) and negative affect (NA) score. PA scale items are happy, joyful, pleased, and enjoyment/fun, while NA scale items are depressed/blue, unhappy, frustrated, worried/anxious, and angry/hostile.

Craving Craving was assessed with the 4-item version of the Questionnaire on Smoking Urges (QSU), a widely used clinical and laboratory measure of urges to smoke (Tiffany and Drobes 1991). The four items in this version, which is described by Carter and Tiffany (2001), are "Nothing would be better than smoking a cigarette right now," "I have an urge for a cigarette," "All I want now is a cigarette," and "I crave a cigarette right now."

Smoking behavior

Smoking reinforcement was determined by behavioral observation of the number of puffs and latency to first puff during the 30-min ad lib smoking period (see Procedure, below). These measures were obtained by videotaping the subjects during this period (see Perkins et al. 1997; Conklin and Perkins 2005). Inter-rater reliability of assessing smoking behavior in this manner exceeds 99% (Perkins et al. 1997).

Procedure

Subjects participated in one session, preceded by overnight abstinence from smoking (at least 14 hrs), verified by expired-air CO<13 ppm, as in previous research (e.g., Perkins et al. 2002). They provided written informed consent to participate in a study to "evaluate the characteristics of different types of cigarettes and how much smokers like them." Subjects were randomly assigned to one of four conditions manipulating dose instructions and cigarette nicotine content in a 2×2 between-subjects design: accurate instructions about the cigarette's nicotine dose vs no dose instructions (i.e., kept blind) and given nicotine vs denic cigarettes. Groups were stratified by sex, so that each condition had 15 men and 15 women.

After a brief rest, subjects completed baseline measures of craving and mood. They then were given general instructions about the session, namely, that they would take a few puffs on a cigarette, rate it, and then be able to smoke more of that cigarette if they wanted. Subjects were introduced to the designated cigarette (nicotine vs denic) and, depending on their group assignment, given either no instructions or the following dose instructions:

> Subjects given the nicotine brand (Quest 1) were told: "This cigarette has a normal amount of nicotine."
> Subjects given the denic brand (Quest 3) were told: "This cigarette has no nicotine."

Subjects then took two puffs to sample the cigarette (exposure period), rated it on the Rose Questionnaire, and repeated the craving and mood measures. Then, subjects were given ten more of the same cigarettes and told they were free to smoke as much or as little as they wanted over the subsequent 30 mins. Subjects were given reading material and allowed to listen to the radio.

Upon completion of the session, subjects were debriefed and told that our interest was to examine conditions that affect smokers' preferences for different cigarettes. This study was approved by the University of Pittsburgh Institutional Review Board.

Data analysis

Analyses of variance (ANOVAs) were used to analyze Rose Questionnaire items after the exposure puffs, as well as the change in mood (negative, positive affect) and craving from baseline to after the exposure puffs. Instructions (presence vs absence of dose instructions), nicotine (nicotine vs denic cigarette), and sex were between-subjects factors. Time (baseline to postexposure) was an added within-subjects factor for mood and craving ANOVAs. The number of puffs during the 30-min ad lib smoking period and the latency to first puff were each analyzed by similar ANOVAs. Follow-up tests were conducted by Fisher's

LSD *t* test (see Huitema 1980). We hypothesized an interaction of nicotine×instructions for all measures, indicating that the presence of instructions would enhance nicotine's effects. Based on prior research (see Introduction), we also hypothesized that this influence of instructions would be greater for women vs men in analyses of the smoking reward and reinforcement measures (i.e., significant interaction of sex×nicotine×instructions). Because men and women differed on cigarettes per day (see Subject characteristics, below), reward and reinforcement ANOVAs with significant interactions involving sex were repeated using cigarettes per day as a covariate to determine whether the interaction could be due to the difference in smoking frequency.

Results

Subject characteristics

Characteristics (mean±SE) for the 60 men and 60 women, respectively, were as follows: age, 32.0±1.6 vs 28.6±1.3; nicotine yield of preferred brand, 1.04±0.03 vs 0.99±0.03 mg; daily smoking rate, 19.7±1.0 vs 15.9±0.5; number of years smoking, 14.8±1.5 vs 11.7±1.2; and Fagerstrom Test of Nicotine Dependence (FTND; Heatherton et al. 1991) score, 4.9±0.3 vs 4.5±0.2. Men and women did not differ significantly on these characteristics, except daily smoking rate [$t(118)=3.37$, $p<.001$], as commonly observed in the general population of smokers (compare with means of 20.6 vs 17.0 cigarettes per day for American men and women daily smokers, respectively; Centers for Disease Control 2003).

ANOVA results for all measures

The ANOVA results are summarized for all measures in Table 1.

Cigarette ratings

Reward As expected, nicotine increased the cigarette reward items of liking and satisfying (see Table 1). More importantly, men and women differed in how they rated cigarette reward in response to the interaction of nicotine and dose instructions. For women, nicotine increased liking only in the presence of dose instructions and not in the absence of instructions, as hypothesized (see Fig. 1). Results were identical for the measure of satisfying (not shown). Unexpectedly, however, the opposite was seen for men, as nicotine increased each measure only in the absence of dose instructions and not in the presence of instructions. When examined separately by sex in follow-up tests, the interaction of nicotine × instructions significantly affected liking for women [$t(59)=2.45$, $p<.05$] and for men [$t(59)=4.26$, $p<.001$]. Results were similar for satisfying.

Perceptual characteristics Nicotine increased the rating of "similar to own brand," while sex interacted with dose instructions on this measure. In women, this rating was higher in the presence vs absence of instructions, regardless of nicotine, while the opposite was true in men (see Fig. 1). Nicotine also increased the rating of "how much nicotine" and interacted with instructions, such that the difference in ratings due to nicotine was greater in the presence vs absence of instructions (Fig. 1). There were no differences due to sex on this rating.

Mood and craving change

Baseline values (mean±SE) were not significantly different due to sex, nicotine, or instructions for negative affect (27.9±1.8), positive affect (43.5±1.8), and craving (76.6±1.7). As shown in Fig. 2, negative affect decreased from baseline to postexposure puffs [i.e., main effect of time; $F(1,112)=18.83$, $p<.001$], but was not significantly influ-

Table 1 ANOVA results (F ratios, df =1,112 for all) for self-reported cigarette ratings, mood and craving responses, and smoking behavior

Measure	Effect		
	Nicotine	Sex×nicotine×instructions	Other effects
Cigarette Ratings			
Reward			
Liking	21.66***	11.35***	
Satisfying	28.04***	10.50**	
Other Ratings			
Similar to own brand	21.09***		S×I, 4.03*
How much nicotine	61.02***		N×I, 6.98**
Mood and craving			
Negative affect			
Positive affect	4.42*		
QSU craving		4.85*	S, 6.06*
Smoking Behavior			
Puff number		3.92*	S×N, 3.98*
Latency to first puff		8.53**	I, 8.17**; S×N, 6.53*

Only significant results are presented
S Sex, *N* nicotine, *I* instructions
*$p<0.05$; **$p<0.01$; ***$p<0.001$

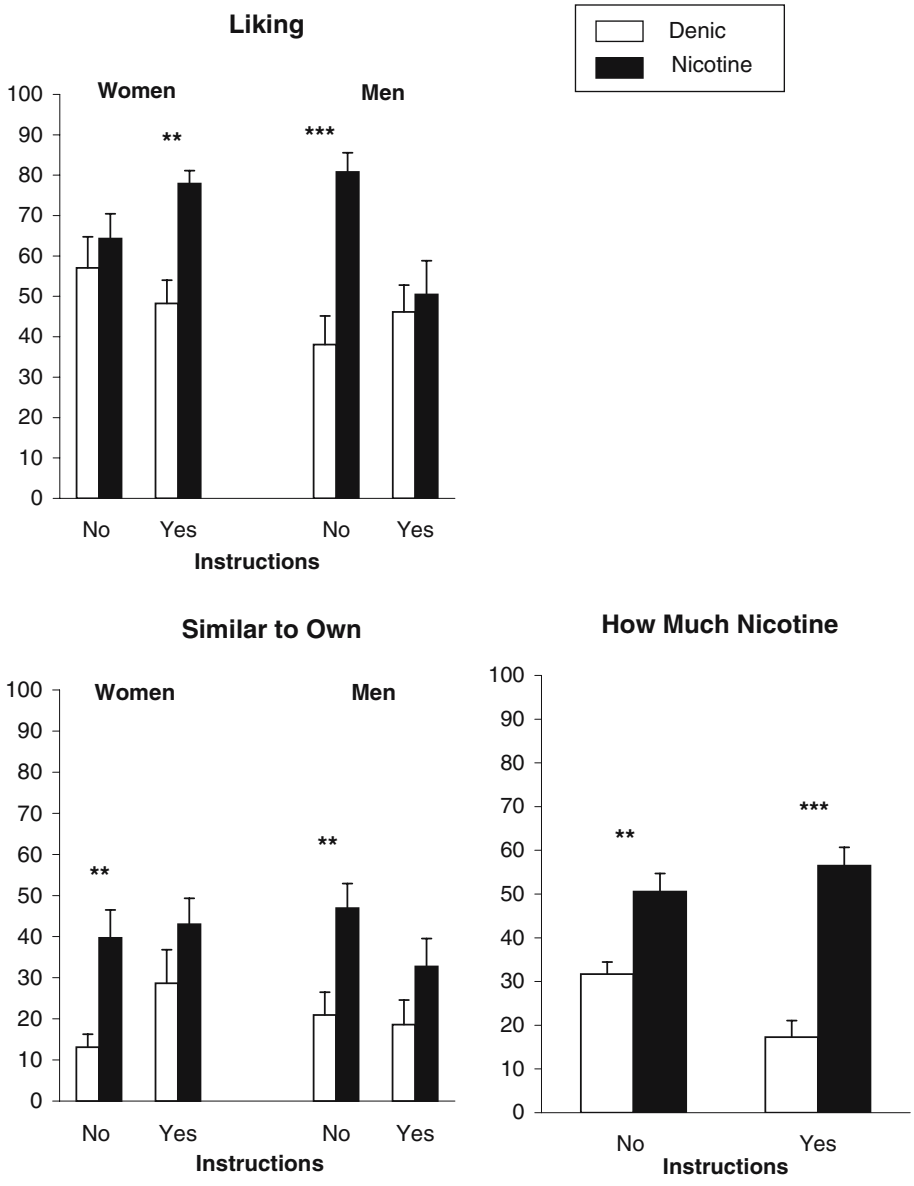


Fig. 1 Ratings of reward (liking; *top*) and cigarette perceptual characteristics (similar to own brand, how much nicotine; *bottom*) as a function of nicotine, the presence (yes) vs absence (no) of dose instructions and, where relevant, subject's sex. Those presented with dose instructions were accurately informed whether the cigarette contained a normal amount of nicotine or no nicotine. Each rating was made using a visual analog scale (range=0–100). *$p<0.05$; **$p<0.01$; ***$p<0.001$ for comparisons between nicotine and denicotinized cigarettes

enced by nicotine, sex, or instructions. Nicotine increased positive affect, but neither sex nor instructions had significant effects (see Fig. 2). Craving decreased from baseline to postexposure puffs [main effect of time; $F(1,112)=9.89$, $p<.005$], and this decrease was greater in men than women. Moreover, for men but not women, nicotine decreased craving in the presence of instructions but not in the absence of instructions (Fig. 2), opposite of the pattern of results for smoking reward (Fig. 1).

Smoking behavior

Similar to the results for cigarette reward ratings (above), smoking reinforcement during the ad lib smoking period differed between men and women in response to nicotine and dose instructions. As shown in Fig. 3, for women, nicotine increased the number of puffs and decreased latency to first puff only in the presence of dose instructions and not in the absence of instructions, as hypothesized, while these effects were not seen in men. Confirming this interpretation was the significant interaction of nicotine×instructions within the women alone for both puff number [$t(59)=2.12$, $p<.05$] and latency [$t(59)=4.30$, $p<.001$], but not within men [t's(59)=1.67 and 1.54, respectively, not significant]. Overall, smoking latency was slower in the presence vs absence of dose instructions, regardless of nicotine.

Covarying for cigarettes per day

Because men and women differed significantly on cigarettes per day, we repeated the analyses for the reward and reinforcement measures by conducting analyses of covariance (ANCOVA), with cigarettes per day as the covariate. Results were unchanged for the reward measures of liking and satisfying, and for the reinforcement measure of la-

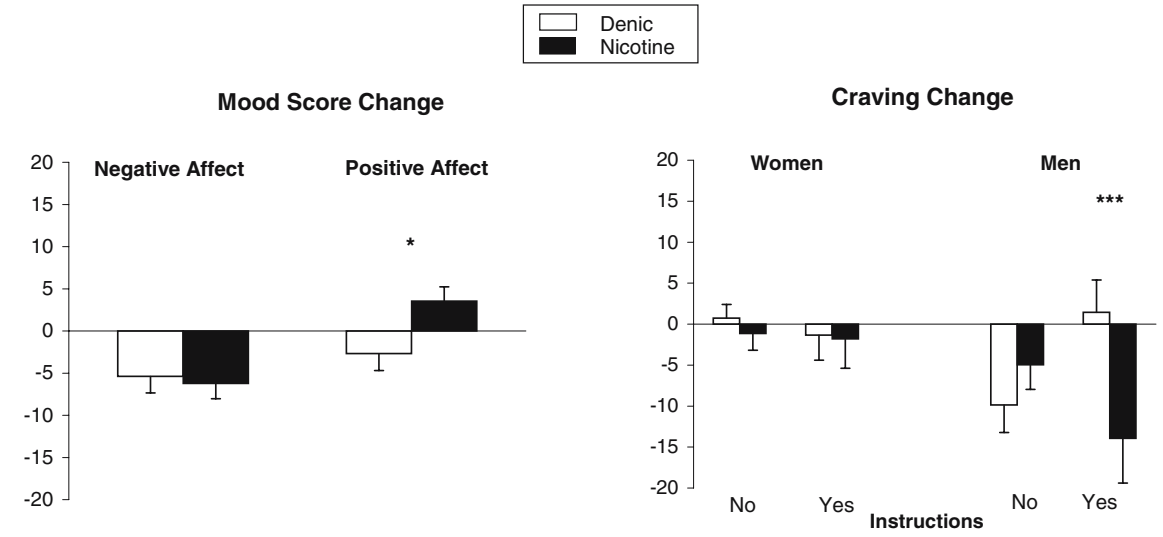


Fig. 2 Change from presmoking baseline to postexposure puffs in negative and positive affect scores and in craving as a function of nicotine and, where relevant, sex and dose instructions. *Asterisks* as in Fig. 1

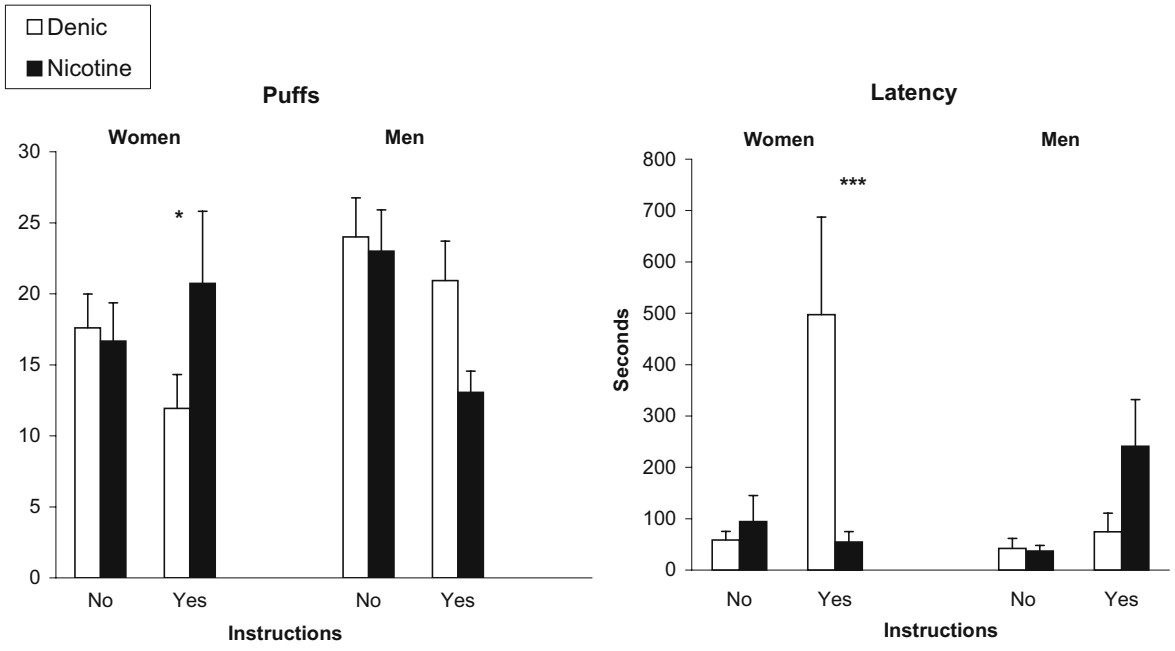


Fig. 3 Smoking reinforcement (number of puffs, latency to first puff) during the 30-min ad lib smoking period due to nicotine, dose instructions, and sex. *Asterisks* as in Fig. 1

tency to first puff. However, the interaction of sex×nicotine×instructions was no longer significant for number of puffs during the ad lib period [$F(1,111)=2.60$, $p=.11$, vs $p<.05$ without covarying cigarettes per day; see Table 1].

Discussion

In women, nicotine increased smoking reward and reinforcement only in the presence of dose instructions and not in the absence of instructions. In men, by contrast, instructions did not increase nicotine's effects on smoking reward and reinforcement. The greater responsivity of women vs men to nonpharmacological factors in smoking has previously been shown in research manipulating nonverbal drug information (e.g., cues; Perkins et al. 2001). The present study extends this potential sex difference to verbal drug information (i.e., dose instructions). The robustness of the effects of these instructions is perhaps surprising in light of their brevity, a single short sentence among other instructions given to all subjects. More extensive instructions, or instructions about the likely effects of the drug (i.e., manipulation of response expectancies; see Kirsch 1997), may have an even more pronounced influence on responding in women vs men.

In general, our findings confirm results from prior studies showing that nicotine increases smoking reward and reinforcement less in women vs men under blind

conditions (Perkins et al. 2002), while nicotine increases smoking reward (if not reinforcement) more in women vs men under conditions of accurate dose instructions (Perkins et al. 2004). However, these prior studies differed from each other in a number of ways (e.g., differences in samples, types of nicotine cigarettes, measures of reinforcement, within- vs between-subjects designs). By directly manipulating the presence vs absence of dose instructions within the same study, the current findings strengthen conclusions about these sex differences in response to dose instructions. Notably, because of the sex difference in response to nicotine as a function of dose instructions, the two-way interaction of nicotine × instructions was not significant for almost all measures (contrary to hypotheses). Thus, if we had not explicitly examined the possibility of sex differences in the influence of dose instructions, we may have concluded that providing dose instructions, a potentially important nonpharmacological factor in drug effects, has essentially no influence on the effects of nicotine during cigarette smoking. Clearly, such instructions do alter the effects of nicotine on smoking reward and reinforcement, but the influence differs markedly based on subject sex. Moreover, these results may have broader implications for pharmacology studies with women. As noted in the introduction, because most human pharmacology studies are conducted under blind conditions (without dose instructions), while drug users in the natural environment are not blind to drug, the results for women in pharmacology studies may not generalize well to drug effects for women in the natural environment.

In regard to possible explanations for these sex differences, differential perception of nicotine does not seem to be relevant, as men and women did not differ in ratings of "how much nicotine." Affect and craving responses also would not seem to explain these sex differences, since men and women generally did not differ on these responses, except for the greater craving relief due to nicotine in men given dose instructions (which was opposite of the observed sex differences in nicotine reward and reinforcement). When the analyses for reward and reinforcement were repeated using as a covariate cigarettes per day, which differed between men and women, the sex differences were unchanged for the reward and smoking latency measures but were no longer significant for number of puffs. Therefore, sex differences in the amount of puffing in response to the instructional and nicotine manipulations could be related to the higher daily smoking rate in men vs women, but this is unlikely to account for the other sex differences.

An unexpected observation was the apparent dampening influence of dose instructions on the effect of nicotine on smoking reward in men. Under no instructions (i.e., blind conditions), smoking reward in men differed markedly between the nicotine and denic cigarettes (see Fig. 1), as previously observed (Perkins et al. 2002). Yet, when given accurate instructions about dose, smoking reward in men no longer differed between the nicotine and denic cigarettes. In addition, smoking reinforcement (number of puffs, latency) in men was nonsignificantly reduced by nicotine under the dose instruction conditions, relative to no instructions (see Fig. 3). These observations suggest that dose instructions may dampen or reverse the typical effects of nicotine per se on smoking reward and reinforcement in men. Such findings are not unprecedented and are similar to the previously noted results for side effects and smoking abstinence reported by Hughes et al. (1989) in a clinical study with nicotine gum. On the other hand, nicotine alleviated craving in men only in the presence of instructions and not in the absence of instructions (Fig. 2), suggesting that instructions do enhance some effects of nicotine in men.

In contrast with most other responses, dose instructions had no effects on mood following the exposure puffs. Of interest, however, is the fact that smoking either the denic or nicotine cigarette (regardless of instructions) reduced negative affect, which was modestly elevated due to overnight abstinence, while only the nicotine cigarette increased positive affect (Fig. 2). Conceivably, withdrawal-related negative affect may be alleviated by mere smoking behavior, with or without nicotine, perhaps suggesting that relief of this type of negative affect is a conditioned response to smoking. On the other hand, increases in positive affect may be a direct influence of nicotine and largely unaffected by conditioned effects of smoking (see Rose et al. 2003).

As noted, these findings are consistent with research indicating broader sex differences in the relative influence of pharmacological (i.e., nicotine) and nonpharmacological factors on acute reinforcing effects of smoking in humans (see reviews by Perkins 1996; Perkins et al. 1999). Compared with men, the rewarding and reinforcing effects of smoking in women generally are less responsive to nicotine per se and more responsive to nonpharmacological manipulations such as verbal (instructions) and nonverbal (cues) information about drug. Moreover, nicotine self-administration behavior in female rats (vs male rats) is similarly more responsive to the presence of nicotine-associated stimuli, although female rats do not appear to be less sensitive than male rats to nicotine per se (Chaudhri et al. 2005). Research is more limited in regard to sex differences in pharmacological and nonpharmacological factors in the acute effects of other drugs. However, women may be more sensitive than men on some subjective responses to cocaine-related visual stimuli (Robbins et al. 1999) but less sensitive to cocaine per se (Lukas et al. 1996). Thus, sex differences in these influences may reflect very basic biological factors that cut across species and generalize across drugs, although far more research is needed to confirm the generalizability of these differences.

A strength of this study is that, to our knowledge, it is the first study to explicitly compare the influence of the presence vs absence of dose instructions on the acute subjective and reinforcing effects of cigarette smoking. It is also the first study with any drug to examine sex differences in response to this type of instructional manipulation. Limitations of this study include the very modest level of smoking exposure on which the reward and other ratings were based (two puffs). This amount was chosen because greater exposure could lead virtually any smoker to detect interoceptive effects of nicotine from the nicotine

cigarette, overwhelming the instructional manipulation. We also wanted subjects to remain sufficiently smoke-deprived as to allow an adequate test of smoking reinforcement during the subsequent ad lib smoking period. Another limitation is the relatively young sample of smokers; results with older and more dependent smokers may differ (see Brauer et al. 2001).

In conclusion, the presence of accurate dose instructions enhanced the effects of nicotine on smoking reward and reinforcement in women, but not in men. These findings are consistent with past research indicating that, compared to men, smoking behavior of women may be influenced less by nicotine and more by nonpharmacological factors. Previous studies showed that such factors included nonverbal information of smoking cues (sight and smell of smoke; Perkins et al. 2001), and this study extends these factors to include verbal information about nicotine dose. Future research should examine sex differences in the rewarding and reinforcing effects of other drugs following similar manipulations of verbal information.

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ORIGINAL INVESTIGATION

Association of *OPRM1* A118G variant with the relative reinforcing value of nicotine

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Abstract
Rationale The endogenous opioid system has been implicated in substance abuse and response to pharmacotherapies for nicotine and alcohol addiction. We examined (1) the association of the functional *OPRM1* A118G variant with the relative reinforcing value of nicotine and (2) the main and interacting effects of the mu-opioid receptor antagonist naltrexone on nicotine reinforcement.
Methods In a within-subject, double-blind human laboratory study, 30 smokers of each *OPRM1* genotype (A/A vs. A/G or G/G) participated in two experimental sessions following 4 days of orally administered naltrexone 50 mg or placebo. Participants completed a validated assessment of the relative reinforcing value of nicotine. This cigarette choice paradigm assesses self-administration of 0.6 mg nicotine vs. 0.05 mg (denicotinized) cigarettes after a brief period of nicotine abstinence.
Results The relative reinforcing value of nicotine (number of nicotine cigarette puffs) was predicted by a significant *OPRM1* by gender interaction. Among women, the low-activity G allele (A/G and G/G) was associated with a reduced reinforcing value of nicotine; among male smokers, there was no association with genotype. Smokers carrying a G allele were also significantly less likely to differentiate the nicotine vs. denicotinized cigarettes by subjective ratings of satisfaction and strength. No evidence for an effect of naltrexone on nicotine reinforcement was found in the overall sample or in the genotype or gender subgroups.
Conclusions This study provides initial evidence for an association of the *OPRM1* A118G variant with nicotine reinforcement in women.

Keywords Nicotine · Naltrexone · *OPRM1* · Addiction · Tobacco · Genetic

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Introduction

Despite decades of tobacco control efforts, about 22% of US adults (45.4 million Americans) are regular smokers (Centers for Disease Control and Prevention 2005).

Nicotine replacement therapy (NRT) and bupropion can double quit rates compared to placebo (Lerman et al. 2005). Varenicline, recently approved by the FDA for smoking cessation treatment, has shown greater efficacy than bupropion in recent trials (Gonzales et al. 2006). However, most smokers are unable to maintain long-term abstinence with any of these approaches. In order to develop more efficacious pharmacotherapies, a better understanding of the biological basis of different nicotine dependence phenotypes is needed.

Although much of the research on the neurobiology of nicotine dependence emphasizes the effects of nicotine on the dopamine reward pathway (Picciotto and Corrigall 2002), growing evidence supports the importance of the endogenous opioid system in nicotine reward. In preclinical models, nicotine administration increases release of beta-endorphin and enkephalin (Boyadjieva and Sarkar 1997; Houdi et al. 1991) which bind to the mu-opioid receptor (MOR). Pharmacologic blockade of the MOR by the opioid antagonist naloxone results in reduced nicotine reward and increased signs of nicotine withdrawal in animal models (Malin et al. 1993; Walters et al. 2005). In humans, some studies report reductions in smoking following acute or chronic treatment with naltrexone (Epstein and King 2004; Rukstalis et al. 2005), whereas others report no effect (Wong et al. 1999).

Recent studies suggest that the inconsistent findings for opioid antagonist effects on smoking behavior may be due, in part, to gender differences in effects of pharmacologic manipulation of the MOR. Compared to male rodents, females exhibit greater effects of opioid agonists on antinociception and reward (Cicero et al. 2000; Cook et al. 2000). Studies also show greater sensitivity to naltrexone among female smokers compared to male smokers (Covey et al. 1999; Epstein and King 2004).

Although the pharmacologic studies provide evidence for the role of the endogenous opioid system in nicotine dependence and related phenotypes, the specific mechanisms involved may be further elucidated by studies that examine how genetic variation in the MOR influences responses to nicotine. Compared to wild-type mice, MOR knock-out (KO) mice exhibit a diminished behavioral preference for nicotine in the conditioned place preference paradigm, reduced mecamylamine-precipitated nicotine withdrawal symptoms, and decreased antinociceptive effects of nicotine (Berrendero et al. 2002). In humans, the MOR gene (*OPRM1*) includes a common Exon 1 functional Asn40Asp (A118G) missense single nucleotide polymorphism (SNP). A recent study reported lower mRNA in human brain samples with the G (Asp40) allele, as well as lower protein levels in vitro, suggesting that the G allele is a partial loss of function allele (Zhang et al. 2005). Recent data support the role of *OPRM1* in therapeutic response to nicotine replacement therapy among smokers (Lerman et al. 2004) and the outcomes of naltrexone treatment for alcohol dependence (Oslin et al. 2003). To date, no other variants of *OPRM1* have been proven to alter protein levels or function. Furthermore, previous smoking cessation trials have found associations only for the A118G SNP and not haplotypes including other *OPRM1* SNPs of unknown function (Lerman et al. 2004). Therefore, the present study focused on the role of the functional *OPRM1* A118G SNP in the relative reinforcing value of nicotine.

Research on smoking-related "endophenotypes" may elucidate the biobehavioral mechanisms for genetic associations with smoking behavior. Such intermediate phenotype measures are considered to be more proximal biologically to their genetic antecedents than complex behaviors such as smoking cessation and therefore may provide a stronger and more reliable signal for genetic association studies (Munafo et al. 2005). One such measure, the cigarette choice paradigm, provides a behavioral measure of nicotine's relative reinforcing effects. Specifically, participants are given the opportunity to self-administer puffs from either a nicotine or denicotinized cigarette at fixed intervals over 2–3 h in a double-blinded fashion (Perkins et al. 1996). This model permits measurement of the relative reinforcing effects of nicotine, while controlling for some of the non-nicotine cigarette (sensory) effects (i.e., using a denicotinized cigarette as a control comparison). The number of nicotine vs. denicotinized puffs self-administered reflects the relative reinforcing value of nicotine, is sensitive to the effects of smoking deprivation, and predicts nicotine withdrawal and relapse following a clinical smoking cessation attempt (Perkins et al. 1996, 2001a, 2002a).

To elucidate the possible role of genetic variation in MOR in smoking behavior, this study examined (1) the association of the functional *OPRM1* A118G SNP with the relative reinforcing value of nicotine using a validated nicotine cigarette choice paradigm and (2) the influence of the MOR antagonist naltrexone on nicotine reinforcement, overall and by *OPRM1* genotype. Using a double-blind, within-subject design, participants in this study were tested for nicotine reinforcement following short-term (4 days) treatment with naltrexone and placebo (order counterbalanced across subjects). Based on evidence that the minor Asp40 (G) allele is associated with loss of function and presumably reduction in MOR density or activity (Zhang et al. 2005), one hypothesis was that carriers of this allele would exhibit less behavioral preference for nicotine in the cigarette choice paradigm (genotype main effect). We also hypothesized that naltrexone, compared to placebo, would reduce nicotine choices (medication main effect), particularly in participants with the *OPRM1* G allele (medication

by genotype interaction). However, it is also plausible that this low-activity allele would be associated with a reduced response to naltrexone. A third goal of this investigation was to test the hypothesis that effects of *OPRM1* genotype and of naltrexone would be greater among women than among men (gender by genotype and gender by treatment interactions).

Materials and methods

Study participants

Participants were recruited via local advertisements that invited non-treatment-seeking smokers to participate in a study on the effects of a medication on smoking behavior. Respondents were screened for eligibility in March 2004–August 2005, and the study was conducted according to the protocol approved by the university Institutional Review Board. Persons were eligible if they were current smokers aged ≥18 years who had smoked ≥10 nonmenthol cigarettes per day for the previous 12 months (only nonmenthol smokers were recruited because of the unavailability, at that time, of mentholated research cigarettes to conduct the study). All participants were of European ancestry to reduce the possible bias of ethnic admixture. Exclusion criteria included any psychotropic medication use, axis 1 psychiatric disorders, and other medical contraindications. Of the 63 eligible and consenting smokers who completed the phase 1 experimental session, 60 also completed the second experimental session.

Study procedures

Screening and pretreatment assessment

Following eligibility screening, all eligible participants provided a blood sample for genotyping of the *OPRM1* Asn40Asp (A118G) variant. Genotyping was performed using Assays-on-Demand SNP genotyping reagents (Applied Biosystems, Foster City, CA). Genotypes were called utilizing the Allelic Discrimination End-point Analysis on the ABI Prism 7900 Sequence Detection System. Five percent of the sample was repeated to validate the genotyping results. Because the minor allele frequency (Asn40; G allele) is 15–25% (Crowley et al. 2003) in individuals of European ancestry, we oversampled smokers with a G allele to obtain a 1:1 genotype ratio. The screening sample included 156 subjects, and the *OPRM1* allele frequencies were in Hardy–Weinberg equilibrium. The final sample included 30 participants with the AA genotype and 30 participants with AG or GG genotypes (only four individuals were homozygous for the G allele; for the purposes of analysis, AG and GG individuals were combined into a single group, as in previous research) (Lerman et al. 2004).

Medication phases

Participants completed two 4-day study medication phases, interspersed with a 5- to 7-day washout period. The order of study medications (naltrexone vs. placebo) was counterbalanced across participants and was double-blinded. During the naltrexone phase, participants received 12.5 mg (day 1), 25 mg (day 2), and 50 mg (days 3 and 4). During each medication phase, participants made daily visits to the research clinic to complete assessments.

Experimental sessions

On study day 4 from 8 a.m. to 2 p.m., participants attended a 6-h laboratory session to complete the cigarette choice paradigm. Participants maintained usual cigarette smoking patterns and were instructed to refrain from drinking alcohol or using recreational drugs or medications for 24 h prior to and 12 h following each session. To standardize the time since last cigarette, participants were asked to smoke one of their preferred/own brand of cigarettes, after which their CO was measured. Immediately following the baseline cigarette, participants ingested two opaque 25-mg naltrexone capsules (total of 50 mg) or two placebo capsules (Mallinckrodt/Tyco, St. Louis, MO).

Initial cigarette exposure Two hours after the baseline cigarette, participants were given an initial exposure to the research cigarettes. We used two brands of Quest® cigarettes (Vector Tobacco) containing genetically modified tobacco that varied the nicotine content: Quest® 1 (0.6 mg nicotine; 10 mg tar) and Quest® 3 (0.05 mg nicotine, essentially denicotinized; 10 mg tar). These cigarettes are virtually identical in all aspects other than nicotine yield when smoked by the Federal Trade Commission method. The cigarettes were color-coded, and for any given participant, this color scheme was maintained in all test cigarettes presented at the two sessions. Participants and research assistants were blinded to the relationship between cigarette nicotine level and cigarette color.

Cigarettes were smoked through sterilized mouthpieces of Clinical Research Support System (CReSS) smoking topography machines (Plowshare, Baltimore, MD). The duration of each puff was standardized by the use of a computer monitor that instructed participants when to initiate and discontinue each puff. Participants were instructed to take four puffs from the designated cigarette during each initial exposure trial. Following the initial exposure to the first cigarette, participants completed the

Cigarette Evaluation Scale (CES) and Sensory Questionnaire (SQ) (see "Measures" below). After a 20-min interval, the same initial exposure procedures were repeated with the other cigarette. The 20-min interval between exposure trials and the total of four puffs per trial were selected to prevent participants from becoming too satiated with nicotine during the course of the session.

Cigarette choice procedure Thirty minutes following the initial exposure to the research cigarettes, participants completed the first cigarette choice procedure. Participants were presented with two freshly lit color-coded cigarettes (same color coding as in the initial exposure) and were instructed to take four puffs from either cigarette (or any combination) every 30 min over a 3-h period (a total of 24 puffs).

Measures

Relative reinforcing value of nicotine

The primary outcome measure was the total number of times out of 24 that participants selected the 0.6-mg over the 0.05-mg nicotine cigarette during the six choice trials.

Subjective effects of initial exposure to cigarettes

The CES is a self-report instrument developed by Westman and colleagues (Westman et al. 1992) consisting of 11 items on which participants rate the cigarette they just puffed (e.g., "Was it satisfying") using response scales ranging from 0 (not at all) to 6 (extremely). Participants completed this measure after initial exposure to each cigarette. As in previous research (Blendy et al. 2005; Rukstalis et al. 2005), we utilized subscale scores for satisfaction (two items) and psychological relief (five items). The Cronbach's alphas for these subscales across sessions ranged from 0.71 to 0.90.

The SQ (Rose et al. 2000), which was administered at the same times as the CES, contains nine items on which participants rate the puffs they took from the cigarette they just smoked using response scales ranging from 0 (not at all) to 6 (extremely). The measure has two subscales—"liking" (four items, e.g., "How satisfying were the puffs you just took") and "strength" (five items, e.g., "Strength of puffs in chest"). The Cronbach's alphas for the subscales across sessions ranged from 0.76 to 0.89.

Withdrawal and craving

The Questionnaire of Smoking Urges (QSU) was administered prior to each cigarette choice session (after the 2-h deprivation period). The QSU consists of 32 Likert-scaled items that measure acute smoking craving and urges (Tiffany and Drobes 1991). The QSU has two factor-derived subscales. Factor 1 reflects desire to smoke for stimulation ("desire to smoke and anticipation of pleasure"); factor 2 assesses urge to smoke to relieve nicotine withdrawal ("anticipation of relief from negative affect and withdrawal"). The Cronbach's alphas for the QSU subscales across sessions ranged from 0.76 to 0.93.

A Withdrawal Symptom Checklist (WSC) was also administered at the same time point. The checklist consists of 18 common nicotine withdrawal symptoms. Participants rated the intensity of each symptom on a scale ranging from 0 (not present) to 3 (severe). Cronbach's alpha coefficients for the WSC total scale were 0.79 for the naltrexone session and 0.78 for the placebo session.

Side effects

The Symptoms Evaluation Checklist (SEC) was administered every day and prior to the initial exposure to the test cigarettes on test day (Rukstalis et al. 2005). The SEC consists of 17 items representing potential medication side effects (e.g., headache, nausea, fatigue) accompanied by response scales ranging from 0 (none) to 3 (severe). Participants rated the severity of each side effect in the SEC on each day of each medication phase, and a mean side effect severity score was created as the average of the responses to all items; these scores were then averaged across all 4 days of each medication phase.

Data analysis

The primary outcome variable of interest in the analysis was the relative reinforcing value of nicotine as measured by the number of nicotine puffs self-administered during the cigarette choice sessions. Secondary outcomes included subjective ratings of the nicotine and denicotinized cigarettes, withdrawal, smoking urges, and side effects. Analyses for all outcomes were performed using linear mixed-effects models (implemented via the MIXED procedure in SPSS). In addition to treatment phase (naltrexone vs. placebo), the models also included session (first vs. second) as a factor, to account for possible period effects; *OPRM1* genotype (A/A vs. A/G or G/G); gender; the interaction of gender by treatment; the interaction of *OPRM1* genotype by treatment; and the interaction of *OPRM1* genotype by gender. Other predictors considered for inclusion in the models were the nicotine level of the participant's regular cigarette brand, baseline nicotine dependence measured by the Fagerström Test for Nicotine Dependence (FTND) (Heatherton et al. 1991), cigarettes smoked per day at baseline, age, and BMI. These were

evaluated individually by adding each one to a model of cigarette choices using treatment and session as the other predictors. In each case, the interaction of the given predictor by treatment was also included and was allowed to drop out if nonsignificant. Of the predictors tested in this way, the only effect to reach significance was the main effect of baseline cigarettes per day (F=6.27, p=.015); it was included in all subsequent analyses.

Results

Study participants

Thirty-eight (63%) of the 60 participants were male, 25 (42%) were married, 23 (38%) were college graduates, and 28 (47%) were employed full-time. Ages ranged from 18 to 70 (mean=43.2, SD=15.0). Participants reported smoking between 10 and 40 cigarettes per day (mean=21.5, SD=9.4). The mean score on nicotine dependence (FTND) at baseline was 4.77 (SD=2.15).

By design, equal numbers of participants with *OPRM1* genotypes of A/A (n=30) and of A/G and G/G (n=30) were recruited into the study. Twenty-six participants had A/G genotypes, and four had G/G genotypes. Compared to those with a G allele, those with the A/A genotype were significantly older [mean (SD)=49.3 (11.8) vs. 37.1 (15.5); t=3.43, p=0.001], more likely to be married (60.0% vs. 23.3%; $\chi^2(3)$=9.85, p=0.020), and more likely to be employed full-time (63.3% vs. 30.0%; $\chi^2(2)$=6.84, p=0.033); however, none of these variables predicted the primary outcome measure of cigarette choice. The two genotype groups did not differ significantly in gender, educational level, baseline cigarettes per day, baseline nicotine dependence, nicotine level of own brand, or baseline Center for Epidemiological Studies-Depression (CESD) score. Male and female participants did not differ significantly from one another on any of the abovementioned variables.

Relative reinforcing value of nicotine

On average, participants with an *OPRM1* G allele took 16.2 (SD=6.2) puffs from the nicotine cigarette out of a possible maximum of 24, and participants with A/A genotypes took 18.5 (SD=4.2) nicotine puffs (t=1.68, p=.099). The results of the linear mixed-effects model of cigarette choice are shown in Table 1. There was a significant interaction of *OPRM1* genotype by gender [F(1, 55)=5.43, p=0.024], and the main effect of *OPRM1* genotype approached significance [F(1, 55)=3.09, p=0.084]. As shown in Fig. 1, among women, smokers with a G allele chose significantly fewer nicotine puffs than did those with the A/A genotype (about 50% from the nicotine cigarette vs. about 75%). There was no association between *OPRM1* genotype and cigarette choice among men.

Table 1 Mixed effects model of relative reinforcing value of nicotine

Predictor	F	p
Treatment (naltrexone=0, placebo=1)	1.74	0.19
Session (first=0, second=1)	0.04	0.83
OPRM1 genotype (A/A=0, G/*=1)	3.09	0.084
Gender (male=0, female=1)	1.49	0.23
Cigarettes per day at baseline	5.86	0.019
Treatment by gender	0.35	0.56
Treatment by *OPRM1* genotype	0.02	0.90
Gender by *OPRM1* genotype	5.43	0.024

As shown in Table 1, there was no evidence for main or interacting effects of treatment phase (naltrexone vs. placebo) on cigarette choices [mean (SD) nicotine puffs=17.03 (6.12) for the naltrexone phase and 17.73 (5.50) for the placebo phase; paired t=1.22, p=0.23].

Cigarette ratings

For each of the cigarette rating scales at each experimental session, we created difference scores (i.e., ratings of the nicotine cigarette minus ratings of the non-nicotine cigarette) and estimated linear mixed-effects models of the difference scores. There was a significant main effect of *OPRM1* genotype in the models of cigarette satisfaction [F(1, 55)= 4.33, p=0.042] and cigarette strength [F(1, 55=4.14, p=0.047] (Table 2). Smokers with the G allele reported significantly less difference (nicotine minus denicotinized) in satisfaction and strength than those with A/A genotypes. In the model of cigarette liking, there was a significant main effect of session (lower scores at session 1 vs. session 2, independent of treatment) [F(1, 56)=4.06, p=0.049]. There was also a significant interaction of *OPRM1* genotype by treatment phase [F(1, 56)=4.27, p=0.043]. In the naltrexone phase, but not in the placebo phase, smokers with the G allele had lower difference scores on liking than did other smokers. There were no significant effects of *OPRM1* genotype in the model of psychological relief, nor were there significant main effects of naltrexone vs. placebo phase on cigarette rating measures.

Smoking urges, withdrawal symptoms, and side effects

There were no significant main or interacting effects of *OPRM1* genotype or treatment phase on smoking urges or withdrawal at either treatment session or across treatment sessions (all ps>0.07). As expected, there was a significant effect of treatment phase on side effects scores (p<0.001); however, scores for both sessions were relatively low

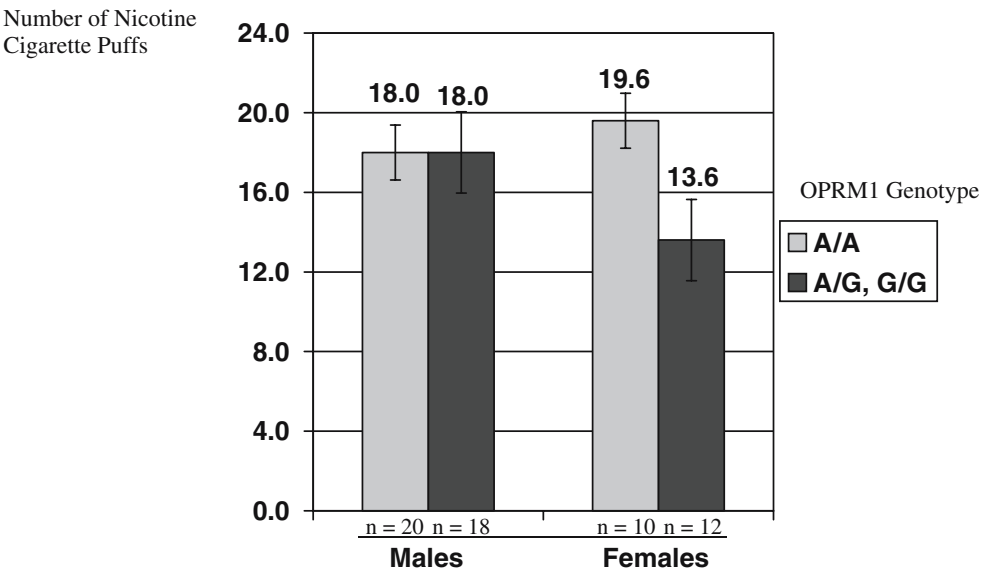


Fig. 1 Interacting effects of *OPRM1* genotype and gender on the relative reinforcing value of nicotine in the cigarette choice paradigm. Women with A/G or G/G genotypes took significantly fewer puffs from the nicotine (0.6 mg) cigarette than did women with A/A genotypes [$t(20)=2.36$, $p=.029$]. There was no difference in nicotine puffs by genotype among men [$t(36)=-0.02$, $p=.99$]

[mean (SD)=0.17 (0.16) for naltrexone, 0.10 (0.10) for placebo; paired $t(59)=4.54$, $p<0.001$]. The associations of *OPRM1* genotype with total side effects or individual common side effects (e.g., nausea, headache) were not significant.

Table 2 Cigarette rating scores by cigarette nicotine level and *OPRM1* genotype

Variable	Mean (SD) among participants with *OPRM1*=	
	A/A (n=30)	A/G or G/G (n=30)
Cigarette satisfaction[a]		
Nicotine	2.57 (1.07)	2.37 (1.04)
Non-nicotine	1.33 (0.80)	1.78 (1.05)
Liking[b]		
Nicotine	2.54 (0.87)	2.60 (0.80)
Non-nicotine	1.30 (0.61)	1.78 (0.99)
Strength[c]		
Nicotine	2.16 (0.79)	2.12 (0.75)
Non-nicotine	1.02 (0.63)	1.50 (0.76)

No significant association of genotype or treatment with relief was found.

[a] A significant main effect of *OPRM1* genotype on "satisfaction" difference scores (nicotine minus denicotinized cigarette) [$F(1,55)=4.33$, $p=0.042$].

[b] A significant interaction of *OPRM1* genotype by treatment condition on "liking" difference scores (nicotine minus denicotinized cigarette) [$F(1,56)=4.27$, $p=0.043$].

[c] A significant main effect of *OPRM1* genotype on "strength" difference scores (nicotine minus denicotinized cigarette) [$F(1,55)=4.14$, $p=0.047$].

Discussion

This human behavioral pharmacology investigation is the first to document a significant genetic association with a measure of the relative reinforcing value of nicotine in a validated nicotine cigarette choice paradigm (Blendy et al. 2005; Perkins et al. 2002a). The relative reinforcing value of nicotine was predicted by a significant interaction between the functional *OPRM1* A118G variant and gender. When given the opportunity to choose puffs from experimental cigarettes in a blinded fashion, women with the low-activity *OPRM1* G allele selected the nicotine cigarette approximately half of the time, whereas women with the *OPRM1* A/A genotype selected the nicotine cigarette in about 75% of all choices, about the same as for men regardless of genotype. Consistent with their reduced nicotine choices, smokers with the *OPRM1* G allele were significantly less likely than those with the A/A genotype to differentiate between the nicotine and denicotinized cigarette with respect to ratings of satisfaction and strength. These subjective measures suggest a possible pharmacologic mechanism for the differences in nicotine reinforcement between the genotype groups. However, these associations with *OPRM1* genotype should be considered preliminary given the modest effects and the fact that associations with the relative reinforcing value of nicotine were observed only in women.

Contrary to our hypothesis and to previous findings for effects of an acute dose of naltrexone on nicotine reward (Rukstalis et al. 2005), we found no evidence that 4 days of naltrexone treatment reduces nicotine cigarette choices in the total sample or in genotype or gender subgroups. This lack of effect cannot be attributed to low statistical power, as there was >80% power to detect a meaningful within-

subject medication effect (and the mean levels of the outcome were virtually identical for each medication phase). In cell culture and animal studies, chronic exposure to naltrexone produces upregulation of MOR protein levels in the ventral tegmental area and nucleus accumbens, regions important in nicotine addiction (Tempel et al. 1985). Previous laboratory investigations showing smoking reduction following naltrexone treatment have tested acute, single-dose administration, which would not be expected to produce upregulation in MOR (Epstein and King 2004; Hutchison et al. 1999; King and Meyer 2000; Rukstalis et al. 2005). However, other studies have documented reductions in smoking with short-term or long-term naltrexone treatment (Covey et al. 1999; Wewers et al. 1998) and with 100 mg naltrexone therapy (O'Malley et al. 2006), suggesting that tolerance to naltrexone may not be an adequate explanation for the lack of medication effects in the present study.

The suggestion of reduced nicotine reinforcement in female smokers with the low-activity *OPRM1* G allele is consistent with evidence from preclinical research. MOR KO mice exhibit reduced behavioral expression of preference for nicotine in a conditioned place preference paradigm (Berrendero et al. 2002). Furthermore, after pharmacologic blockade of MOR, effects of nicotine on phosphorylated CREB (P-CREB) are diminished, suggesting a possible biological mechanism by which reduced MOR activity may attenuate nicotine reward (Walters et al. 2005). Thus, several converging lines of evidence suggest that decreased MOR, resulting from either genetic or pharmacologic manipulation, is associated with diminished nicotine reward. Exploration of sex differences in these preclinical studies would be useful given the clinical findings in the present study.

Although the present study is the first to examine gender differences in *OPRM1* genotype associations, several preclinical studies have documented sex differences in opiate discrimination, self-administration, and reward (Craft et al. 1996; Cicero et al. 2003; Lynch and Carroll 1999). Human studies have also shown gender differences in opiate analgesic response (Aubrun et al. 2005); however, the direction of the association has not been consistent (Sarton et al. 2000). Interestingly, women have higher MOR receptor binding in cortical and subcortical areas of the brain compared to men, a difference that is reversed as women become postmenopausal (Zubieta et al. 1999). There is also evidence for estrogen-induced MOR regulation (binding and internalization) (Micevych et al. 2003; Mills et al. 2004).

The inference that estrogen may modulate MOR-mediated nicotine reward is also consistent with a wealth of evidence for gender differences in smoking behavior. Smoking behavior in women, relative to men, tends to be influenced more by conditioned reinforcing effects such as non-nicotine cues (e.g., sight and smell of a cigarette, expectations), and less by the pharmacologic effects of nicotine (Field and Duka 2004; Perkins et al. 2001b, 2005). Women are also less likely than men to discriminate different cigarette nicotine levels (Perkins et al. 2002b). Thus, we speculate that in women, reduced MOR activity associated with the *OPRM1* G allele may decrease the rewarding valence of nicotine and/or increase the salience and rewarding value of conditioned smoking cues. This hypothesis requires testing in future research.

Limitations of the present investigation should be considered. First, participants were required to choose between a nicotine and denicotinized cigarette and were not provided with the option not to smoke. Thus, the hypothesis that naltrexone reduces absolute smoking reinforcement, apart from effects on nicotine reward, could not be tested. Second, as suggested above, only one dosing regimen was tested for naltrexone effects, and other results might be obtained with different dosages or duration of treatment. Furthermore, the significant association of *OPRM1* with cigarette choices was observed only in women (n=22). Replication of this finding in an independent study is necessary in order to interpret the significance of *OPRM1* for nicotine reward. Finally, to reduce bias due to ethnic differences in allele frequencies, this study included smokers of European ancestry only.

The present study provides preliminary data supporting an association of the functional *OPRM1* A118G SNP with the relative reinforcing value of nicotine, adding to preclinical and clinical evidence for the role of the MOR in nicotine reward (Berrendero et al. 2002; Walters et al. 2005) and smoking behavior (Lerman et al. 2004). This investigation also provides initial evidence for gender heterogeneity in *OPRM1* effects that is consistent with previous findings of gender differences in effects of nicotine (Perkins et al. 1999) and in effects of opioid antagonists on smoking behavior (Epstein and King 2004). These findings lay the foundation for future research on the role of additional opioid pathway genes in nicotine reward and smoking behavior, as well as preclinical investigations that may elucidate the biological basis of estrogen effects on nicotine–opioid system interactions. Such research may ultimately reveal novel targets for nicotine dependence medication development and lead to tailored treatments based on smokers' individual needs.

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Expectancy and Pharmacology Influence the Subjective Effects of Nicotine in a Balanced-Placebo Design

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The expectancy and pharmacological effects of nicotine (0.60 mg) on memory and the subjective effects of cigarettes were examined by using a balanced-placebo design (i.e., expect either nicotine or no nicotine and receive either nicotine or no nicotine). A total of 120 college students who smoke were assigned to 1 of the 4 experimental groups, then rated the cigarettes on a number of dimensions and completed questionnaires on smoking urges, tension, and energy. Participants also completed tests of memory as well as predictions of memory. Pharmacology played a stronger role than expectancy in most ratings of the cigarettes, but significant effects of expectancy did emerge for feelings of increased wakefulness, concentration, calming, cigarette satisfaction, and hunger reduction. The presence of nicotine significantly reduced smoking urges, but expectancy alone reduced tension after smoking. Neither variable produced significant effects on memory or memory predictions. These findings demonstrate that nonpharmacological factors can play an important role in the self-reported effects of nicotine.

Keywords: balanced-placebo design, cigarette smoking, nicotine, stimulus expectancy

Many individuals who smoke may do so, at least in part, to induce cognitive changes in response to environmental demands. Consider college students who smoke while preparing for exams. Wesnes, Revell, and Warburton (1983) found that students smoked more cigarettes during exam weeks than during nonexam weeks. Moreover, 80% of students reported that smoking helped them concentrate, and 74% reported that smoking increased concentration. West and Lennox (1992) subsequently confirmed that college students smoked before exams to increase arousal during study, rather than to simply relieve stress or alleviate withdrawal. These studies suggest that nicotine's cognitive effects, or perhaps students' beliefs about nicotine's effects, may contribute to the maintenance of smoking behavior.

There is now increasing recognition in the field of nicotine and tobacco research that numerous factors, including nonpharmacological factors, can play important roles in maintaining smokers' addiction to cigarettes (Johnson, Bickel, & Kirshenbaum, 2004; Perkins, Sayette, Conklin, & Caggiula, 2003). Hundreds of articles have been published on the pharmacological effects of nicotine on human cognition (for reviews, see Heishman, Taylor, & Henningfield, 1994; Sherwood, 1993), and there is a substantial body of research pointing to cognitive improvements from cholinergic stimulation by specific nicotinic receptor agonists and cognitive impairments with nicotinic antagonists (Levin, 1992; Rezwani & Levin, 2001). In contrast, the role of nonpharmacological factors in participants' cognitive responses to smoking has been relatively understudied (Grunberg & Acri, 1991). The present research was designed to directly compare the effects of expectancy in smoking with the pharmacological changes induced by nicotine.

Stimulus expectancy (Kirsch, 1999; Perkins et al., 2004) can be manipulated through instructional sets, in which some participants are told to expect an active drug and others are told to expect an inactive drug. This type of expectancy is ignored in double-blind research, because in that research participants are not informed about which condition has been administered. Although double-blind research remains the standard protocol in clinical trials, it does have limitations (Kramer & Shapiro, 1984). Sutton (1991) has argued that continued reliance on double-blind designs is likely to create an overemphasis in the role of pharmacology and an underestimate of the role of stimulus expectancy in drug studies. He called for the increased use of a balanced-placebo design in nicotine and tobacco studies.

Two independent variables are included in a standard balanced-placebo design: type of substance expected (i.e., participants are told to expect either an active or an inactive substance) and type of substance received (i.e., participants receive either an active or an inactive substance). Thus, a 2 × 2 (Type of Instructional Set × Type of Substance Administered) factorial design is incorporated. Of the four conditions, two are deceptive in nature (some participants are told to expect an active substance but receive an inactive one, and other participants are told to expect an inactive substance but receive an active one). The major advantage

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of a balanced-placebo design is that the effects of stimulus expectancy can be directly compared with pharmacological effects. Despite calls by researchers to utilize the balanced-placebo design (Brandon, Juliano, & Copeland, 1999; Sutton, 1991), only two published studies have utilized it in research on cigarettes to date.

Juliano and Brandon (2002) examined whether the type of cigarette people expected and the type of cigarette they actually received influenced their levels of state anxiety, urge to smoke, and withdrawal symptoms. Participants abstained from smoking for 3 hrs before the study, and upon arrival they were placed in an anxiety-provoking situation. Not surprisingly, Juliano and Brandon found a main effect of nicotine: Cigarettes with nicotine reduced anxiety more than nicotine-free cigarettes. In addition, merely expecting to receive cigarettes with nicotine also reduced anxiety significantly, but only in smokers who believed nicotine would do so. Thus, response expectancy for anxiety seemed to moderate stimulus expectancy effects. A significant effect of stimulus expectancy also emerged for urge to smoke, though no significant effects were found for withdrawal measures.

In the Juliano and Brandon (2002) study, approximately one third of participants did not believe the deception. In general, it has been difficult to deceive people in the "told inactive substance but receive active substance" condition in balanced-placebo research, especially with alcohol (Martin & Sayette, 1993). Therefore, Perkins et al. (2004) conducted a smoking study using a modified balanced-placebo design. They told participants to expect either a regular dose or a low dose (instead of no dose) of nicotine in a cigarette, and they also manipulated the amount of nicotine actually administered. Instructional set did influence the perceived strength of the cigarette, as well as levels of liking and satisfaction. In contrast to Juliano and Brandon's findings, there was no reduction in cravings (although participants were not abstinent or in a high-stress situation as in the previous study). Unfortunately, Perkins et al. also found high levels of disbelief in their deceptive conditions, despite the modified instructions and the ingestion of only two puffs of the cigarette during smoking.

In the present research, we sought to extend the work of Juliano and Brandon (2002) and Perkins et al. (2004) using a standard balanced-placebo design with cigarette smokers. We tried to increase the effectiveness of the deception conditions in three ways. Following the suggestion of Juliano and Brandon, we used a lower dose of nicotine in the active drug condition (0.60 mg of nicotine compared with 1.10 mg in the Juliano & Brandon study and 0.90 mg in the Perkins et al. study) so that the pharmacological effects would be less likely to overshadow the stimulus expectancy effects. Second, we allowed participants to select their own cigarette from a package that contained a manufacturer's brand and labeling consistent with the instructional set (either nicotine or nicotine free) to increase the believability of the manipulation. Third, full debriefing of the participants as to the deceptive nature of the study was postponed until data were collected from all participants, in order to reduce the likelihood that smokers who completed the study would inform potential participants about the deception. We also sought to resolve previous inconsistencies on the effects of stimulus expectancy on cravings by administering the full Cigarette Evaluation Scale (CES; Westman, Levin, & Rose, 1992) immediately after participants smoked, along with additional measures of smoking urges, energy, and tension, on three separate occasions to track the time course of effects. Finally, we included measures of memory and memory predictions to assess whether expectancy versus pharmacology played a significant role in these areas.

We hypothesized main effects of instructional set (i.e., the type of cigarette participants were told they received) and nicotine (i.e., the presence or absence of nicotine in cigarettes) on several dependent measures. Consistent with past research, we expected to find a significant effect of instructional set on ratings of craving or urge reduction (Juliano & Brandon, 2002) and cigarette satisfaction (Perkins et al. 2004), as well as other dimensions of the CES. We sought to expand on these findings by assessing smoking urges, tension, and energy at three different points in the experiment. Next, we wanted to determine if the effects of instructional set and nicotine could be extended to more complex cognitive processes by including two distinct tests of memory and confidence. Finally, in cases where both variables produced statistically significant effects, we sought to compare the magnitude of effects in order to determine which variable produced the stronger effect.

Method

Participants

A total of 125 smokers were recruited from California State University, Long Beach, and were paid $30 each for their participation. Data from 5 participants were discarded due to errors during testing or failure to comply with instructions. Thus, 120 participants (30 women and 78 men, 12 missing gender data[1]) provided useable data for analyses. The age range of the participants was 18–57 years ($M = 22.2$, $SD = 6.3$). All participants completed a telephone screening interview to ensure their eligibility for participation: They all had smoked at least 10 cigarettes a day for at least 1 year, were not trying to quit smoking, and reported no major health concerns.

Participants were asked to abstain from using nicotine and caffeine products for 8 hr before the study. To ensure nicotine deprivation, we instructed all participants to complete an end tidal carbon monoxide (CO) breath analysis (Vitalograph, Lenexa, KS) upon arrival. Participants were not allowed to participate if their CO level exceeded 15 ppm.

[1] All participants responded to the demographic questionnaire, but these data were deleted inadvertently for 12 consecutive participants from the electronic handheld device.

Types of Cigarettes

Two types of cigarettes were used in this study: (a) Quest 1, which contains 0.60 mg of nicotine per cigarette by the Federal Trade Commission test method, and (b) Quest 3, which contains less than 0.05 mg nicotine per cigarette by the Federal Trade Commission test method. These nonmentholated cigarettes are matched for tar content (10 mg each) and size (85 mm each). Cigarettes were left in their packages until removed by a participant for smoking; in two of the four conditions, however, the types of cigarettes in each package had been switched before the experiment.

Questionnaires

Participants completed a total of 10 different questionnaires over the course of the experiment on an electronic handheld device (Palm Pilot Tungsten E; Palm, Sunnyvale, CA), including a demographic survey, a 6-item version of Fagerström Test for Nicotine Dependence (FTND; Heatherton, Kozlowski, Frecker, & Fagerström, 1991) and the 12-item Cigarette Dependence Scale (CDS; Etter, Le Houezec, & Perneger, 2003). They also completed a 20-item version of the Smoking Consequences Questionnaire–Adult (Copeland, Brandon, & Quinn, 1995) containing the most relevant subscales: (a) the Stimulation/State Enhancement subscale ($n = 7$ items), (b) the Boredom Reduction subscale ($n = 4$ items), and (c) the Negative Reinforcement/Negative Affect Reduction subscale ($n = 9$ items). The 10-item version of Questionnaire of Smoking Urges (QSU) developed by Cox, Tiffany, and Christen (2001) was included to measure cigarette cravings in participants three times during the course of the experiment. The 20-item version of Activation-Deactivation Adjective Checklist (AD-ACL; Thayer, 1986) also was used to measure participants' self-reported levels of arousal (i.e., tension and energy) three times during the course of the experiment. The 12-item Cigarette Evaluation Scale (CES; Westman et al., 1992) was administered immediately after participants smoked. One item was added to this scale as a manipulation check, which asked participants to indicate what type of cigarette they had just smoked (nicotine vs. nicotine free). Participants then answered two questions about how the cigarette they smoked would influence their performance on the upcoming memory tests (i.e., "Please rate the effect the cigarette will have on your ability to learn 40 pairs of unrelated nouns" and "Please rate the effect the cigarette will have on your ability to remember the answers to a series of trivia questions"). The same questions were asked again after the tasks (rephrased to reflect past performance), along with a second manipulation check, which asked participants to report the strength of the cigarette they smoked previously. Finally, the 33-item Social Desirability Scale (SDS; Crowne & Marlowe, 1960) was administered at the end of the study to assess the degree to which participants attempted to present themselves in a favorable manner.

Memory Tasks

Study and prediction task. Participants were asked to study 40 concrete, two-syllable, English nouns (e.g., market), presented on a computer monitor. The order of presentation was randomized for each participant, and all nouns appeared for two study trials of 3 s each. Immediately following the second trial, participants were asked to predict how likely they were to recall each noun (i.e., immediate prediction), on a scale of 0 (*definitely will not recall*) to 100 (*definitely will recall*). After studying and providing judgments for all 40 nouns, participants were asked to make a prediction of how many total words (0–40) they would recall (i.e., aggregate prediction).

Prospective remembering task. This task was used as a cognitive test as well as a distracter activity before the memory recall task. Participants were asked to answer 200 general knowledge questions (e.g., "What is the capital city of Norway?"), which appeared for 6 s on the monitor followed by a 3-s interval to select one of four alternatives ("Voss," "Oslo," "Trondheim," "Bergeb"). After each question, participants were asked to indicate their degree of confidence in their answer on a scale of 0–100. Within the same task, participants also were instructed to press the *a* key each time they saw the name of an animal in the question or the alternatives (i.e., prospective memory test). A total of six animal questions were presented, and *a* responses were scored as correct if they occurred during the animal question and response sequence or during the very next question and response sequence.

Memory recall task. Immediately after the prospective memory task, the participants were asked to write down as many of the English words as they could remember, with no time limitation. Misspellings were counted as correct if the first three letters of the word matched those of the actual word.

Design and Procedures

A 2×2 between-subjects factorial design was used. The two independent variables were type of instructions that participants received (told nicotine vs. told no nicotine) and type of cigarette that participants received (contained nicotine vs. contained no nicotine). We randomly assigned participants to one of four conditions based on their order of appearance: told nicotine–received nicotine ($n = 31$), told nicotine–received no nicotine ($n = 29$), told no nicotine–received nicotine ($n = 30$); told no nicotine–received no nicotine ($n = 30$). All participants completed the 2-hr experiment individually.

All procedures were reviewed and approved by the California State University Institutional Review Board. Participants signed an informed consent form upon arrival, which stated that they may or may not receive nicotine during the experiment, and completed a CO breath analysis to verify cigarette abstinence. Then, participants were asked to complete six questionnaires on the Palm Pilot, in the following order: demographics, FTND, CDS, Smoking Consequences Questionnaire, baseline QSU, and baseline AD-ACL. Next,

participants were informed about which type of cigarette (nicotine vs. nicotine free) they would receive, although in two conditions the participants were deceived.

Several steps were taken to enhance the effectiveness of our deception. First, participants reviewed a small information leaflet provided by the manufacturer in each package of cigarettes explaining that cigarettes with different amounts of nicotine (including cigarettes free of nicotine) are now available commercially. Participants were then offered an open package of cigarettes from one of two cartons, both of which were labeled by the manufacturer, and asked to select a cigarette to smoke. We determined which of four conditions each participant received and arranged for the correct package of cigarettes (half of which had deceptive labels) to be presented. Participants were tested by one of three other experimenters who were not informed of the deceptive nature of the study. Thus, both the experimenter and the participant should have believed they received the type of cigarette shown on the package.

The experimenter accompanied participants to an open area outside the building to smoke the entire cigarette and to complete four questionnaires immediately afterward: the CES, two questions on the predicted effects of the cigarette on memory performance, and the QSU and AD-ACL questionnaires for the second time each. Upon returning to the lab, participants completed the study and prediction task as well as the prospective remembering task on the computer and then the memory recall test. After the memory recall phase, participants were asked to complete another questionnaire on the effects of the cigarette on the memory tasks, a final measure of QSU, and a final measure of AD-ACL. Lastly, participants were asked to complete the SDS. To control for the influence of caffeine, we asked participants to estimate the number of hours since they last consumed caffeine and the amount ingested. After all these procedures, participants were paid and partially debriefed, excluding the deceptive feature. After all testing was completed, letters explaining the four conditions and a summary of the main results were mailed to all participants.

Results

All tests of statistical significance were conducted with an alpha level set at .05 unless otherwise noted. Measures of effect size are reported for all statistically significant effects using eta squared (η^2), following Clark-Carter's (1997) criteria: η^2 of .010 is a small effect, η^2 of .059 is a medium effect, and η^2 of .138 or above is a large effect.

Instructional Set Manipulation Checks

In order to test whether participants believed the instructions about the type of cigarette they received, we performed two manipulation checks. Immediately after smoking, participants rated the cigarette on 12 dimensions of the CES. A 13th question was added: "What type of cigarette did you just smoke?" and participants chose either "Nicotine free" or "Nicotine." A total of 9 participants (7.5% of the sample) failed to select the type of cigarette that they were told they received: Five participants in the told nicotine–received no nicotine condition reported that they did not receive nicotine, 2 participants in the told no nicotine/received nicotine condition reported that they received nicotine, and 1 participant in each of the nondeceptive conditions reported that they received the opposite type of cigarette. Thus, a relatively small portion of the sample disbelieved the instructions based on the first manipulation check.

Near the end of the experiment, participants completed a second manipulation check. They were asked "How would you rate the amount of the nicotine that your cigarette contained?" and were given four alternatives: "nicotine free" (scored as –2), "moderately weak amount" (scored as –1), "moderately strong amount" (scored as + 1), and "very strong amount" (scored as + 2). The mean rating for all participants in the told no nicotine condition was −1.54 (SD = 0.89), and the mean in the told nicotine condition was −0.66 (SD = 0.83). A 2 × 2 analysis of variance (ANOVA) revealed a large, statistically significant effect of instructional set $F(1, 111) = 29.92$, $\eta^2 = .212$. Neither the presence or absence of nicotine nor the interaction between instructions and presence or absence of nicotine had a significant effect. To evaluate the influence of the 9 participants who disbelieved the instructions, we conducted the same ANOVA on cigarette strength omitting these individuals. The outcome of the three F tests did not change, but the effect size of instructional set increased by over 36% (i.e., η^2 increased from .212 to .290). Thus, including the 9 disbelievers in the analysis dampened the effect of instructional set considerably.

There has been some debate in the balanced-placebo literature as to how to treat data from participants whose behavior is inconsistent with the instructional set (e.g., Martin & Sayette, 1993). In our data, a relatively small number of participants produced a substantial reduction in effect size for cigarette strength, and so we decided to report all subsequent analyses excluding these participants. In most cases, this omission did not change the outcome of statistical significance testing. For completeness, we note the cases where tests of significance did differ in the Appendix.

Demographic Information

Participants' mean age, level of cigarette dependence (as assessed by the FTND and the CDS), and CO levels are shown in Table 1. A one-way ANOVA was conducted for each variable to test for significant differences across groups. None of the ANOVAs were significant.

Evaluation of Cigarettes

Immediately after smoking, participants rated their cigarette on the 12 dimensions of the CES (Westman et al., 1992). The mean ratings for each dimension across all four conditions are shown in Table 2, along with the outcome of inferential statistical tests and corresponding effect sizes. The expectancy of nicotine had a significant main effect in 5

Table 1
Mean Scores on the Demographic Variables Across Groups

Dependent measure	Condition (expected/received)							
	Nic/Nic		Nic/No nic		No nic/Nic		No nic/No nic	
	M	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*
Age (years)	23.12	1.68	22.78	1.53	22.30	1.17	20.96	0.55
Carbon monoxide level	5.37	0.48	6.37	0.63	6.21	0.61	6.76	0.46
Fagerström Test for Nicotine Dependence	10.10	0.42	9.29	0.38	9.00	0.32	8.86	0.27
Cigarette Dependence Scale	42.72	1.18	42.58	1.35	39.03	1.23	39.79	1.27
Smoking Consequences Questionnaire-Adult								
Negative Reinforcement/Negative Affect Reduction desirability	24.93	3.48	26.12	3.72	19.75	4.70	18.31	3.85
Negative Reinforcement/Negative Affect Reduction likelihood	56.07	3.62	58.58	4.15	53.25	3.37	49.25	3.33
State Enhancement/Stimulation desirability	6.10	3.38	11.50	3.49	2.41	3.56	2.44	3.02
State Enhancement/Stimulation likelihood	27.47	2.43	30.42	3.03	28.54	2.06	24.69	2.40
Boredom Reduction desirability	6.93	2.02	7.42	2.44	5.32	2.42	8.03	2.02
Boredom Reduction likelihood	22.87	1.91	26.38	1.93	23.81	1.67	22.38	1.74

Note. One-way analyses of variance confirmed that none of these dependent measures varied significantly across condition. Nic = nicotine.

of 12 cases (wakefulness, concentration, hunger reduction, satisfaction, and calming). The presence of nicotine had a significant main effect in 8 of 12 cases (satisfaction, calming, taste, dizziness, irritability reduction, craving reduction, nausea, and strength). Thus, the presence of nicotine produced a greater number of statistically significant findings with generally larger effect sizes. However, the effect sizes of instructional set were actually larger than pharmacological effect sizes for the dimensions of wakefulness, concentration, and hunger reduction.

Correlations between total scores on the SDS and each of the 12 dimensions were computed for each level of instructional set. If participants were responding untruthfully in order to appear more favorably, then significant correlations should have emerged. None of the correlations were reliably nonzero. In the told nicotine condition, which should have been most susceptible to social desirability, the correlations ranged from −.02 to +.15. Thus, none of the significant effects of instructional set can be accounted for by mere demand characteristics.

Table 2
Mean Ratings, F Ratios, and Effect Sizes by Condition for the Cigarette Evaluation Scale

Dimension	Condition (expected/received)								Effect					
	Nic/nic		Nic/No nic		No nic/Nic		No nic/No nic		Expected		Received		Expected × Received	
	M	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*	*F*	η^{2a}	*F*	η^{2a}	*F*	η^{2a}
Made me feel awake	3.77	0.36	3.71	0.33	2.89	0.37	2.55	0.28	8.98[b**]	M				
Helped me concentrate	3.67	0.37	3.46	0.36	3.18	0.35	2.38	0.24	5.46[b*]	S				
Reduced my hunger	3.50	0.36	3.54	0.43	3.14	0.31	2.45	0.30	4.28[b*]	S				
Was satisfying	5.40	0.33	3.17	0.38	4.21	0.37	2.66	0.24	6.51[b*]	S	32.53[b***]	L		
Was calming	4.90	0.37	3.83	0.37	4.07	0.31	3.03	0.28	5.94[b*]	S	9.91[b**]	M		
Tasted good	4.57	0.31	2.50	0.36	4.07	0.38	2.34	0.25			33.16[b***]	L		
Made me dizzy	3.10	0.42	1.63	0.24	2.36	0.27	1.62	0.16			13.67[b***]	M		
Reduced my irritability	4.27	0.33	2.96	0.38	3.54	0.34	3.21	0.30			5.98[b*]	S		
Reduced my cravings	4.07	0.40	3.50	0.36	3.64	0.31	2.76	0.28			4.49[b*]	S		
Made me feel nauseous	1.33	0.14	1.71	0.30	1.32	0.14	1.86	0.26			4.57[b*]	S		
Was very strong[c]	2.47	0.31	1.38	0.14	1.64	0.16	1.54	0.20			7.20[d***]	M	4.86[d*]	S
Gave me throat and chest sensations	2.53	0.31	2.13	0.31	2.96	0.37	2.24	0.28						

Note. *F* ratios reflect the outcomes of statistical comparisons using two-way (Expected × Received) analysis of variance; only significant *F* ratios are provided. Nic = nicotine.
[a] S = a small effect size; M = a medium effect size; L = a large effect size.
[b] $df = 1, 107$.
[c] Analyses of simple main effects were conducted to follow up on the significant interaction in this case: The expected nicotine-received nicotine group scores were significantly higher than the others, and no other significant differences emerged.
[d] $df = 1, 106$.
$^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$.

Smoking Urges, Tension, and Energy

Participants' levels of smoking urges were assessed with the QSU before smoking, immediately after smoking, and near the end of the experiment. A 3 × 2 × 2 (Time of Assessment × Instruction Set × Nicotine) mixed ANOVA revealed a statistically significant main effect of time and a statistically significant interaction between instructional set and time, $F(2, 212) = 63.87$, $\eta^2 = .376$, and $F(2, 212) = 6.32$, $\eta^2 = .056$, respectively. The pattern of means is illustrated in Figure 1A, collapsed across the effect of nicotine (which was not statistically significant). Participants who were told to expect nicotine began with slightly higher levels of smoking urges compared with participants who were told to expect no nicotine; immediately after smoking, however, this pattern was reversed. Both groups finished the experiment with equivalent levels of smoking urges. The simple main effects of time were significant for both levels of instruction set, Fs > 16.38, ps $< .05$. Post hoc paired t-tests showed that all comparisons over time were significant for the told nicotine group (ts > 5.47, ps $< .05$). For the group told no nicotine, the reduction in urges from before to immediately after smoking and after smoking to the end of the experiment (ts > 3.76, ps $< .05$) were statistically significant. These results were not due to demand characteristics, because correlations between SDS scores and QSU scores were nonsignificant for both levels of instructional set. Overall, being told that cigarettes contained nicotine did significantly reduce the participants' urge to smoke, but the effect was short lived.

Participants' levels of self-reported tension and energy over time were assessed with the two subscales of the AD-ACL. For the sum of the 10 items corresponding to tension, a three-way ANOVA revealed a statistically significant main effect of time and a statistically significant interaction between nicotine and time, $F(2, 214) = 10.67$, $\eta^2 = .091$, and $F(2, 214) = 4.30$, $\eta^2 = .039$, respectively (see Figure 1B); no other main effects or interactions were significant. Analysis of the simple main effects of time showed a significant effect when nicotine was administered $F(2, 114) = 15.35$, $\eta^2 = .212$, but no significant effect of time when no nicotine was administered. In the former case, post hoc paired t-tests showed a significant decrease in tension from before to after smoking, followed by a significant increase in tension comparing postsmoking scores with scores at the end of the experiment, (ts > 4.59, ps $< .05$). In regard to scores for the 10-item energy subscale, only the effect of time was significant, $F(2, 212) = 16.23$, $\eta^2 = .131$, and no follow-up analyses were conducted. Thus, cigarettes with nicotine did reduce participants' levels of tension immediately after smoking, but they did not influence self-reported levels of energy. These results contrast those reported for smoking urges, where instructional set (but not nicotine itself) played a significant role.

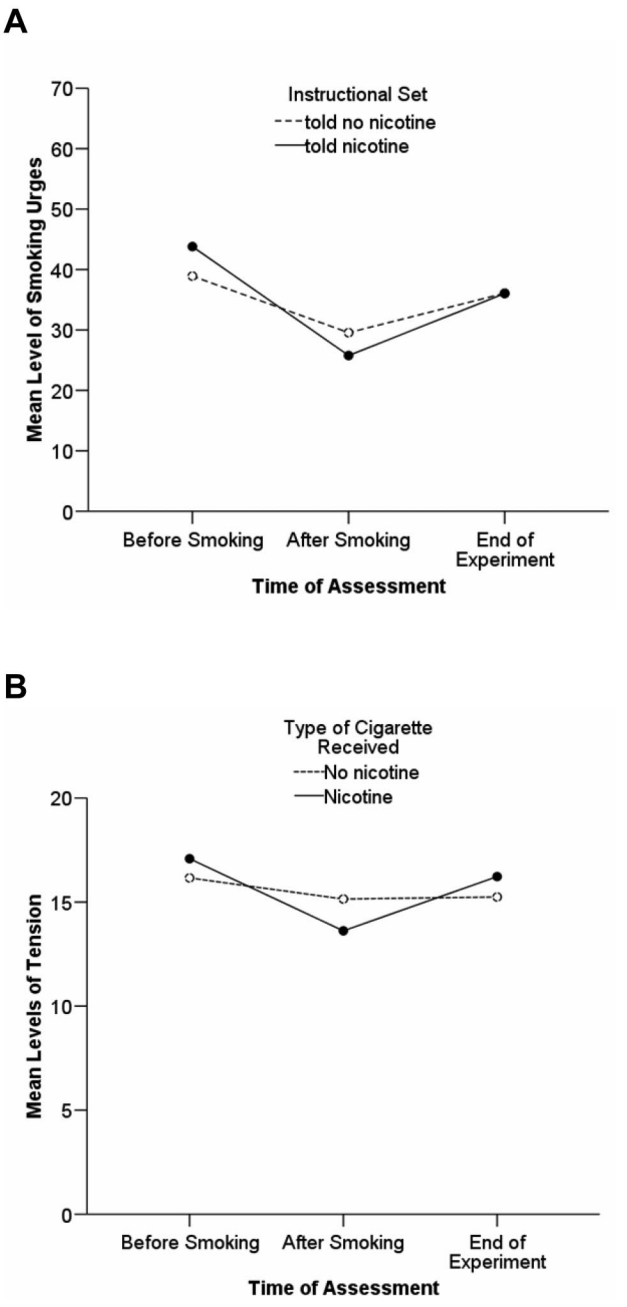


Figure 1. A: Mean responses on the Questionnaire of Smoking Urges over time collapsed across the presence or absence of nicotine. B: Mean responses on the Tension subscale of the Activation-Deactivation Adjective Checklist over time collapsed across instructional set.

Memory Performance

Study and prediction task. Participants provided two types of predictions regarding their future memory performance while studying the 40 English nouns: 40 item-by-item predictions (ranging from 0 to 100 each) and a single aggregate prediction ranging from 0 to 40 at the end of the task. Both types of judgments are shown as proportions in Table 3 for consistency. A 2 × 2 (Instruction Set × Nicotine Presence or Absence) ANOVA revealed no significant

Table 3
Mean Scores on the Computerized Tests of Memory and Confidence

Dependent measure	Condition (expected/received)							
	Nic/nic		Nic/ No nic		No nic/ Nic		No nic/ No nic	
	M	*SEM*	*M*	*SEM*	*M*	*SEM*	*M*	*SEM*
Noun-learning task								
Immediate prediction	.54	.04	.53	.04	.56	.04	.47	.03
Aggregate prediction	.50	.04	.49	.04	.51	.04	.46	.04
Actual memory score	.30	.02	.32	.04	.36	.04	.28	.03
Prospective remembering								
General knowledge accuracy	.59	.01	.61	.02	.59	.02	.59	.01
General knowledge confidence	.65	.03	.64	.02	.68	.02	.65	.02
Prospective remembering accuracy	.43	.06	.53	.06	.46	.06	.47	.06

Note. Nic = nicotine.

effects. Thus, neither the nicotine nor the expectancy of nicotine influenced participants' predictions of the memory performance.

Memory recall task. The third row of Table 3 lists mean recall performance across the four conditions. A 2 × 2 (Instruction Set × Nicotine Presence or Absence) ANOVA revealed no significant effects.

Prospective remembering task. Three aspects of the prospective remembering task were analyzed: general knowledge accuracy, confidence in the correctness of general knowledge answers, and prospective remembering accuracy. Mean scores are shown in the bottom half of Table 3. A 2 × 2 (Instruction Set × Nicotine Presence or Absence) ANOVA was conducted on each dependent measure, and no statistically significant effects emerged.

Discussion

We conducted this research to directly compare the pharmacological effects of nicotine in cigarettes with the effects of instructional sets on cigarette evaluation, smoking urges, tension, energy, and memory performance using a balanced-placebo design. The strongest effects were noted with the CES: 11 out of 12 dimensions were influenced by at least one variable, and two dimensions were influenced by both. Instructional set alone was sufficient to produce significant positive changes in response to the items "Made me feel awake," "Helped me concentrate," and "Reduced my hunger." Instructional set also produced significant effects along with nicotine for the items "Was calming" and "Was satisfying." These data provide strong evidence for the role of nonpharmacological factors such as stimulus expectancy in variables that may contribute to the maintenance of smoking behavior. Why only these particular items, and not the other seven dimensions, were influenced by expectancy is uncertain, especially because the factor structure of the CES has not been explored. It is tempting to speculate that cognitive factors (i.e., concentration, wakefulness, and calming) may be more susceptible to expectancy, but in contrast to this notion we found no statistically significant effects on our computerized memory and confidence measures.

The effects of instructional set and nicotine on craving reduction have been inconsistent in two previous studies using a balanced-placebo design. Juliano and Brandon (2002) used a 3-hr abstinence interval and found that both instructional set and nicotine reduced the urge to smoke. In contrast, Perkins et al. (2004) found no reduction in cravings in minimally abstinent smokers. We asked our participants to abstain for 8 hrs and found an effect of nicotine on the craving item of the CES as well as an effect of instructional set over time on the QSU. Thus, our findings of significant effects for both variables resemble those of Juliano and Brandon, which suggests that at least moderate deprivation may be necessary to uncover effects of these variables on participants' ratings of smoking urges.

Overall, the 12-item CES tended to show the greatest number of significant effects for both independent variables, even though some of the same constructs (e.g., smoking urges and tension) were assessed with reliable instruments that were administered several times during the experiment. One possible explanation is that the CES may be more susceptible to demand characteristics because each dimension is assessed with only one item. However, participants' responses to the SDS were not significantly correlated with any of the responses to the CES. Another possibility is that the CES showed the largest effects because it was the questionnaire administered immediately after smoking, when the influence of nicotine should be greatest. Mendelson, Sholar, Goletiani, Siegel, and Mello (2005) have shown that nicotine plasma levels can peak within 12–14 min after smoking, although lower nicotine cigarettes showed a longer time course. Most participants would have been just beginning the memory tasks about 15 min after smoking. Unfortunately, the plasma nicotine levels obtained after smoking were not measured in this study, although these data have been reported for low nicotine cigarettes in past research (Rose & Behm, 2004). The low dose of nicotine we used (0.60 mg, which would be considered a "light" or "ultralight" cigarette) combined with the short window available to detect changes due to nicotine may explain why we found no significant effects on our memory and confidence tasks. Lastly, the null findings on the memory tasks

may have been affected by differences in baseline performance across groups, which was not assessed in this study.

A final consideration concerns the integrity of the balanced-placebo design. We observed only 9 participants out of 120 (i.e., 7.5%) who reported that they received a different type of cigarette than we instructed. This rate of disbelief is substantially lower than the rates reported by Juliano and Brandon (2002) and Perkins et al. (2004). The difference could be due to the modifications to our procedures noted earlier or perhaps to the lower dose of nicotine used, making it easier to convince participants in the told no nicotine–received nicotine condition. This antiplacebo condition has proven especially troublesome in past investigations using the balanced-placebo design (Perkins et al., 2004). In our study, however, only 2 participants disbelieved the antiplacebo condition. Thus, it may be possible to successfully execute a standard balanced-placebo design using commercially available cigarettes with varying levels of nicotine. The dosage may prove critical however, in that researchers can find themselves in a catch-22: Higher doses of nicotine would likely produce measurable changes in complex cognitive processes (such as memory), but using higher doses might challenge the validity of a standard balanced-placebo design.

We hope that researchers will continue to investigate factors related to the response expectancy and stimulus expectancy effects of nicotine. For example, Perkins et al. (2006) recently showed that dose instruction played a stronger role in nicotine reinforcement and reward in women compared with men. The present study has shown substantial effect sizes of stimulus expectancy for characteristics of cigarettes that may be important for maintaining smoking behavior, such as feelings of calmness, hunger reduction, wakefulness, improved concentration, and satisfaction. Increased use of the balanced-placebo design may continue to uncover expectancy effects that match, or even exceed, the pharmacological effects studied with more traditional designs.

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Appendix

Outcomes of Statistical Tests Including All Participants That Differed From Those Reported in the Main Text

Demographic Information

Including the 9 participants who disbelieved the instructions resulted in the one-way ANOVA for FTND total reaching statistical significance, $F(3, 116) = 3.29$, $p < .05$. Tukey's post hoc test revealed that the told nicotine–received nicotine group had significantly higher FTND scores compared with both groups that expected no nicotine.

Evaluation of Cigarettes

Including the 9 participants who disbelieved the instructions produced the following changes to tests of statistical significance: (a) for "Helped me concentrate," the main effect of instructional set became nonsignificant ($p = .06$); (b) for "Was satisfying," the main effect of instructional set became nonsignificant ($p = .06$); (c) for "Was calming," the main effect of instructional set became nonsignificant ($p = .10$); (d) for "Made me feel nauseous," the main effect of nicotine became nonsignificant ($p = .05$); and (e) for "Gave me throat and chest sensations," the main effect of nicotine became significant ($p < .05$).

Smoking Urges, Tension, and Energy

None of the tests of statistical significance differed from those reported in the main text.

Cognitive Performance

For the study prediction task, memory recall task, and prospective remembering task, none of the tests of statistical significance differed from those reported in the main text.

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fMRI Imaging of the Effects of Smoked Tobacco and Placebo Tobacco

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Tobacco is the most frequently abused drug worldwide. Despite the high prevalence of abuse, little is known about the acute effects of smoking on brain. To address this question, experiments were performed in human smokers using functional magnetic resonance imaging (fMRI) during tobacco cigarette and placebo tobacco smoking. A nonmetallic smoking device was constructed and evaluated for its ability to deliver smoke and nicotine to subjects using a paced smoking paradigm. Subjects smoked either a Marlboro Red brand tobacco cigarette or a Quest "Nicotine Free" cigarette (containing only 0.03 mg nicotine) during fMRI scanning with concurrent assessment of heart rate and changes in subjective mood state. Visual analog scales for mood states were presented every 2.5 minutes throughout the functional scan. Using General Linear Modeling techniques, changes in subjective sensation of "high" after smoking tobacco were found to be most highly correlated with BOLD signal change in the bilateral anterior nucleus of the thalamus, bilateral caudate head and tail, bilateral posterior cingulate (these and all subsequent correlations were found to be significant at p£0.0001). These findings are consistent with both the reinforcing properties of nicotine and the anatomical distribution of terminals emanating from regions of high nicotinic acetylcholine receptor density. Changes in subjective sensation of "high" after smoking placebo tobacco were most highly correlated with BOLD signal change in the orbitofrontal cortex, and dorsolateral prefrontal cortex. These findings are consistent with the cognitive components of reward mediated by conditioned reinforcers only in the absence of active drug. This study is the first to assess and compare changes in BOLD signal and its relationship to reported subjective effects during active tobacco and placebo smoking.

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Selectively reduced responses to smoking cues in amygdala following extinction-based smoking cessation: results of a preliminary functional magnetic resonance imaging study

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ABSTRACT

Preliminary studies suggest an extinction-based smoking cessation treatment using reduced nicotine content (RNC) cigarettes decreases self-report craving for cigarettes prior to quitting and may be an effective smoking cessation treatment. The aims of this study was to evaluate the effect of an extinction-based smoking cessation treatment on brain responses to smoking cues using blood-oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI). Sixteen ($n = 16$) dependent smokers were scanned using BOLD fMRI at baseline, following 2–4 weeks of smoking RNC cigarettes while wearing a 21-mg nicotine patch, and 2–4 weeks following quitting smoking. During scanning, participants viewed smoking-related pictures (e.g. lit cigarette) and pictures of people engaged in everyday activities (e.g. using a stapler). Event-related BOLD responses to smoking and control cues were analyzed in regions of interest (ROIs) known to subserve reward, attention, motivation and emotion. The extinction-based treatment simultaneously attenuated responses to smoking cues in amygdala while potentiating responses to control cues. Exploratory analysis indicated that this pattern was also observed in the thalamus of future abstinent but not relapsing smokers. The results of this preliminary study suggest that an extinction-based treatment for smoking cessation alters brain responses to smoking and control cues in amygdala—a region previously associated with drug cue reactivity and extinction.

Keywords Amygdala, cigarette craving, cue reactivity, extinction, fMRI, tobacco smoking.

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INTRODUCTION

Although nicotine is considered the primary psychoactive component of tobacco smoke, there is a growing appreciation of the role of non-pharmacological factors (e.g. conditioned gustatory and environmental cues) in the maintenance of, and relapse to, cigarette smoking (Caggiula *et al.* 2002a; Rose & Behm 2004b; Rose 2006). Conditioned nicotine cues have been shown to enhance nicotine reinforcement and self-administration in rats (Caggiula *et al.* 2002b), and reinstate nicotine seeking behavior following extinction (LeSage *et al.* 2004; Liu *et al.* 2006). In smokers, exposure to conditioned smoking cues has been shown to induce drug craving (Carter & Tiffany 1999) and reactions to smoking cues can predict smoking relapse (Waters *et al.* 2003; Payne *et al.* 2006). Finally, administration of the sensory aspects of smoking without usual doses of nicotine (i.e. denicotinized smoke) has been shown to significantly reduce *ad libitum* smoking of one's usual brand of cigarettes (Rose *et al.* 2003), suggesting that these conditioned sensory cues alone may be reinforcing.

Despite evidence indicating an important role for conditioned drug cues in maintaining tobacco addiction, smoking cessation treatments designed to extinguish responses to these cues have been largely unsuccessful. This is likely due, in part, to a lack of incorporation of modern learning principles of extinction (Conklin & Tiffany 2002). For instance, the renewal effect (Bouton 1994)—in which extinction in one context is not conferred to other contexts—may explain why treatments in which cue exposure occurs in only one context (e.g. the clinic) do not generalize to contexts in which the drug is commonly used (e.g. at home, in the car, etc.). Studies demonstrate that the renewal effect does occur in human drug users (Collins & Brandon 2002; Thewissen *et al.*

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2006) and that extinction can be enhanced in animals by conducting trials in multiple contexts (Gunther, Denniston & Miller 1998).

Recently, an extinction-based treatment in which smokers switch to reduced nicotine content (RNC) cigarettes prior to quitting has shown promise as a new and potentially effective smoking cessation treatment (Rose *et al.* 2006). The rationale behind this approach is that the smoking of RNC cigarettes prior to quitting extinguishes the link between smoking cues and the delivery of bolus nicotine to the brain, thus rendering smoking less reinforcing and less difficult for the smoker to quit altogether. This treatment has a potential advantage over previous extinction-based strategies in that extinction training can occur in multiple contexts (i.e. in all of the contexts the smoker typically smokes), in the face of a wide range of ecologically valid cues, and over many trials.

The aim of the present study was to preliminarily evaluate the effects of an extinction-based smoking cessation treatment on self-report craving, smoking cessation outcomes, and brain responses to visual smoking cues using blood-oxygen level-dependent (BOLD) functional magnetic resonance imaging (fMRI). Human neuro-imaging studies have demonstrated that smokers exhibit increases in brain activity in response to smoking cues in regions subserving attention, emotion, reward and motivation (Brody *et al.* 2002; Due *et al.* 2002; David *et al.* 2005; McClernon *et al.* 2005). Moreover, responses to smoking cues in frontal brain regions have been shown to be modulated by abstinence-induced craving (McClernon *et al.* 2005) and by the expectation that smoking will occur (Wilson *et al.* 2005; McBride *et al.* 2006), whereas cue-provoked craving has been associated with the magnitude of responses to cues in limbic structures including the amygdala (Smolka *et al.* 2006). While the effect of any type of extinction-based treatment on physiological responses to smoking cues has not been evaluated, extinction of fear conditioned responses has been shown to attenuate brain responses to conditioned stimuli (LaBar *et al.* 1998; Phelps *et al.* 2004).

Continued exposure to conditioned drug cues in the absence of drug reward results in an extinction of behavior to obtain the drug. This phenomenon, and the effects of factors that can reinstate drug seeking behavior, have been evaluated using animal behavioral (Chiamulera *et al.* 1996; LeSage *et al.* 2004; Liu *et al.* 2006) and neuropharmacological (See *et al.* 2003) studies. Although the neurobiological basis or correlates of drug extinction have not been studied extensively, it is reasonable to hypothesize that just as extinction results in a decrease in drug seeking behavior, it should also result in a decrease in brain activation in regions provoking and supporting drug-seeking behavior. Stated another way, to the degree that drug seeking involves brain regions associated with assigning value to cues associated with reward and motivated behavior, activation in these regions in response to cues following extinction should be diminished. Thus, we hypothesized that an extinction-based treatment would result in a diminution of responses in regions previously shown to be active in response to smoking cues.

MATERIALS AND METHODS

Sample

Twenty ($n = 20$) adult dependent smokers were recruited from the community. Participants had to be interested in quitting smoking, smoke at least 15 cigarettes per day for at least 2 years, have an afternoon breath carbon monoxide (CO) level greater than 15 p.p.m., be right-handed, be free of serious health problems and medications altering central nervous system functioning, test negative for illicit drug use, and not have any conditions making magnetic resonance imaging (MRI) research unsafe. Female participants of child bearing potential were required to test negative for pregnancy at screening and in the 72 hours prior to each MRI. Potential participants were scheduled for a screening visit at which they read and signed an Institutional Review Board-approved informed consent form, provided a urine sample that was analyzed for illicit drugs, and provided information regarding health and smoking history. Participants entered a mock scanner in order to screen out potentially claustrophobic participants and facilitate habituation to the scanning environment.

Procedure

Prior to treatment, participants were instructed to smoke their usual brand of cigarettes for 2 weeks (Fig. 1). During this baseline phase, participants attended one laboratory visit (V1) and completed an fMRI scanning session. At the lab visit, as in all following visits, breath and saliva samples were acquired, measures of smoking withdrawal and mood were administered, and daily diaries of smoking behavior were collected. Participants were also provided with study cigarettes and medications, instructions for using them and a copy of Quit Smart™ smoking cessation guide. Advice on quitting and minimal support were provided as requested.

During the treatment phase, laboratory visits 2–6 (V2–6) were scheduled 1 week apart and during this phase participants were instructed to switch from their usual brand of cigarettes to the provided RNC cigarettes. They were also instructed to wear a 21 mg/day nicotine patch during this 4-week period. After the end of the second week of smoking RNC cigarettes and wearing the nicotine patch, a second fMRI scanning session was

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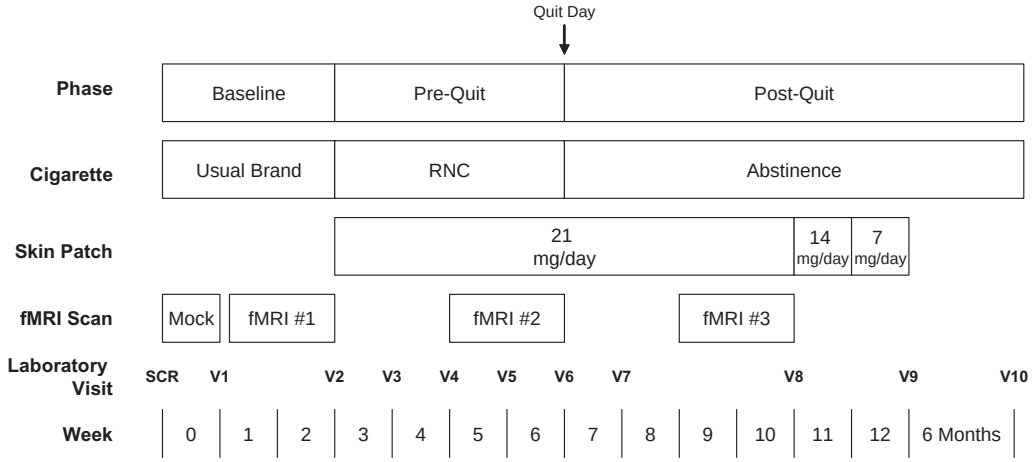


Figure 1 Timeline of experimental phases, procedures, functional magnetic resonance imaging (fMRI) scans and laboratory visits. RNC = reduced nicotine content; SCR = screening

conducted. Participants were instructed to quit smoking abruptly the morning after V6 but to continue wearing the nicotine patch. During the quit phase, additional return visits were held 1 (V7), 11 (V8) and 12 (V9) weeks after quitting. Following V8 participants were instructed to wear the 14 mg/day nicotine patch for 1 week followed by the 7 mg/day nicotine patch for an additional week. A third fMRI scanning session was conducted 2–4 weeks following the quit day.

RNC cigarettes

Reduced nicotine content cigarettes (mentholated and non-mentholated) were obtained from Vector Tobacco Co. (Richmond, VA, USA), which markets these cigarettes under the brand name 'Quest-3'. The nicotine delivery per cigarette (Federal Trade Commission (FTC) method) is <0.05 mg, tar delivery is approximately 8.5 mg and the CO delivery is 12.5 mg. A previous study showed little or no compensatory smoking behavior with RNC cigarettes, in contrast with low tar and nicotine ventilated filter cigarettes (Rose & Behm 2004a). Participants were given enough packs of cigarettes to match their daily cigarette intake and instructed to start smoking them exclusively starting the morning following V3. At V3 through V5 they were given additional packs of RNC cigarettes.

Nicotine patch

Transdermal nicotine skin patches (Nicoderm CQ®, GlaxoSmithkline, Middlesex, UK) were used to provide nicotine replacement during smoking of RNC cigarettes and post quitting. We proposed that transdermal nicotine would attenuate nicotine withdrawal symptoms brought on by smoking RNC cigarettes but because nicotine is delivered in steady state, would not be contingent on smoking behavior or cues. Participants were instructed to begin wearing a 21 mg/day nicotine patch beginning the morning following V2 and to wear one patch each day until 1 month after quitting smoking (through V8). Following V8 they were weaned off the 21 mg/day patch, using the 14 mg/day patch during week 11 and the 7 mg/day patch during week 12.

Smoking behavior and biochemical verification

Smoking behavior and treatment compliance were characterized with smoking and medication diaries and the analysis of salivary cotinine and expired CO levels (Vitalograph, Inc., Lenexa, KS, USA) collected at the beginning of each laboratory visit.

fMRI sessions

Participants were instructed not to smoke in the 2 hours prior to each scanning session to induce mild (but not severe) craving. This was done in order to avoid potential ceiling or floor effects in brain responses to smoking cues. At the beginning of each scanning session expired CO was measured. Total time in the scanner was approximately 1.5 hours.

Cue exposure task

Photographic smoking, control and target cues were presented during functional imaging using previously established methods (McClernon *et al.* 2005). Smoking cues ($n = 60$) consisted of pictures of smoking-related objects and people smoking cigarettes. Control cues ($n = 60$) consisted of pictures of everyday objects (e.g. stapler, keys) and people engaged in everyday activities (e.g. using the phone). Target cues ($n = 15$) were pictures of animals. Participants were asked to press a button whenever they saw a target. Stimuli included pictures from a variety of sources (Lang, Bradley & Cuthbert 1995; Gilbert & Rabinovich 1999; McClernon *et al.* 2005).

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All visual stimuli were projected onto a screen behind the participant's head, which the participant viewed using mirrored goggles. During each of the nine functional runs, 15 images were presented with approximately half being smoking cues, half control cues, and one to two targets. Thus, a total of 135 cues were presented during each session, and while the same set of images was viewed at each, the order of presentation varied across sessions. Picture cues were presented for 4 seconds with a variable 18- to 22-second stimulus onset asynchrony. Total task time was 52.2 minutes (5.8 minutes × 9 runs).

Imaging parameters

A 1.5 T GE NVi SIGNA scanner with 41 mT/m gradients was used for image acquisition. Each participant's head was held in place using a vacuum-pack system to minimize head motion. Following a localizer series, BOLD functional images were collected for 28 contiguous slices (4 mm thick) parallel to the horizontal plane connecting the anterior and posterior commissures. A spiral-out gradient-echo pulse sequence sensitive to BOLD contrast was used, with TR = 2000 ms, TE = 40 ms, FOV = 25.6 cm, matrix = 64 × 64, flip angle = 90° and in-plane resolution = 4 mm^2. After completion of nine runs of the functional data collection, T1-weighted structural imaging was conducted on the same slices as the functional images (i.e. 28 slices, 4 mm thick) with TR = 450 ms, TE = 20 ms, FOV = 25.6 cm, matrix = 256 × 256 and in-plane resolution = 1 mm^2. At the end of one of the sessions a high-resolution spoiled gradient recalled (SPGR) anatomical image (124 slices, 1 mm thick) was acquired with TR = 12.4 ms, TE = 5.3 ms, FOV = 25.6 cm, matrix = 256 × 256 and in-plane resolution = 1 mm^2.

Imaging data processing and analysis

Prior to data analysis, functional data underwent preprocessing using SPM 99 and analysis using custom MATLAB (Mathworks, Inc., Natick, MA, USA) scripts. Data were corrected via sinc interpolation for time of acquisition of each slice within the imaging volume and were realigned to correct for head motion over the course of the session using SPM 99. Custom MATLAB scripts were used to extract epochs surrounding individual events from the overall time series. For each event, we calculated the percent change in BOLD signal over an 18-second peristimulus epoch (10 TRs: −4 seconds to +14 seconds), baseline corrected to the mean value of the first three timepoints in the epoch. This provided the mean BOLD response to each trial type for each voxel in each participant at each session.

Anatomical regions of interest (ROIs) were manually drawn bilaterally on the SPGR images of each participant using two atlases as guides (Duvernoy 1999; Mai, Assheuer & Paxinos 2003) for the following brain structures: ventral and dorsal anterior cingulate gyrus (ACG), superior frontal gyrus (SFG), middle frontal gyrus (MFG), inferior frontal gyrus (IFG), nucleus accumbens (nAC), thalamus, caudate, putamen, amygdala, hippocampus and insula. As with a previous imaging study (McClernon *et al.* 2005), given the relatively small number of voxels making up the nucleus accumbens, a 'ventral striatum' ROI was constructed consisting of the nucleus accumbens and the ventral-most slice of the caudate and putamen. Within each slice of each ROI, we calculated the mean BOLD response across all voxels for the epochs described in the previous paragraph. We then calculated a mean BOLD response for each bilateral ROI by averaging across slices weighted by the number of functional voxels in each slice. These mean ROI responses (for each participant) formed the basis for our data analysis.

In order to assess the effects of treatment on responses to smoking and control cues, percent signal values from the 4, 6 and 8 seconds timepoints (i.e. those around the predicted maximum of the BOLD response) were averaged together and entered into a 3 (Scan: baseline phase, treatment phase, quit phase) × 2 (Stimulus: smoking, control) repeated-measures ANOVA for each ROI using SPSS 11.5. Greenhouse–Giesser correction was applied to effects involving Session, although uncorrected degrees of freedom are reported for clarity. Because targets were included to control for participants' attention to the task, and thus have different cognitive and response requirements from the other cues, we excluded targets from the ANOVA analyses.

RESULTS

Sample characteristics

Two participants ($n = 2$) dropped out because of scheduling constraints while one ($n = 1$) dropped out because of illness unrelated to the study protocol. Data for one participant were unusable due to noisy signal and were thus removed from analyses. The final sample consisted of 16 smokers (14 females, 4 minority) with a mean age of 39.13 (SD = 9.89). They reported smoking a mean of 22.63 (SD = 8.05) cigarettes per day with 0.98 mg (SD = 0.32) FTC nicotine delivery for 20.41 (SD = 8.98) years and had a mean baseline Fagerstrom Test for Nicotine Dependence (FTND) score of 6.50 (SD = 1.67). Mean baseline plasma cotinine level was 252.50 ng/ml (SD = 107.88).

Smoking behavior and abstinence

Smoking behavior was assessed by self-reported counts of cigarettes smoked per day, expired air CO readings and salivary cotinine levels. During the baseline phase,

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participants reported smoking a mean of 21.1 (SD = 8.35) per day of their usual brand of cigarettes. Mean CO and salivary cotinine levels were 22.25 ppm (SD = 10.88) and 169.59 ng/ml (SD = 133.90), respectively. Participants were largely treatment compliant. Following the instruction to switch to smoking RNC cigarettes exclusively, participants reported smoking a mean of 13.48 (SD = 10.22) per day of the RNC cigarettes and a mean of 2.01 (SD = 2.72) per day of their usual brand. During the treatment phase, mean CO and salivary cotinine levels were 15.09 ppm (SD = 10.21) and 110.45 ng/ml (SD = 97.58), respectively.

FMRI results: treatment effects

Shown in Table 1 are the results from the statistical analysis of activation to smoking and control cues for each ROI. Across scans, activation was significantly less negative over the peak 4- to 8-second interval in response to smoking cues than control cues in the ventral anterior cingulate gyrus (ACG). A Scan × Stimulus interaction was observed in the amygdala ROI (Fig. 2). *Post hoc* analyses of this interaction indicated that brain responses to smoking cues were of significantly higher amplitude ($P < 0.05$) than control cues at the baseline scan but not during the treatment or post-quit scans. Finally, a main effect of Scan was observed for the caudate ROI (not shown). *Post hoc* analyses of this main effect indicated that across stimulus types, activation was of significantly higher amplitude during baseline scan compared with the post-quit scan ($P < 0.05$). The amplitude of activation during the treatment-phase scan did not differ from the baseline or post-quit scans.

Exploratory analysis of treatment outcome effects

Four participants were continuously abstinent at 1 month as defined by having a CO level <9 at all

Table 1 Functional magnetic resonance imaging region of interest ANOVA results.

Region	Mean No. of voxels [a]	BA	Scan (df = 2, 30)		Stimulus (df = 1, 15)		Stimulus × Scan (df = 2, 30)	
			F	P	F	P	F	P
Ventral ACG	29	32/33	1.93	NS	13.30	0.002[c]	0.02	NS
Dorsal ACG	101	32/24/33	0.98	NS	1.28	NS	0.01	NS
IFG	138	9/44/45	2.00	NS	0.15	NS	0.35	NS
MFG	291	8/9/46	1.57	NS	0.33	NS	0.88	NS
SFG	549	6/8/9/10	2.81	NS	0.42	NS	0.33	NS
Amygdala	41		0.76	NS	0.01	NS	4.53	0.023[d]
Hippocampus	56		0.42	NS	1.23	NS	0.18	NS
Caudate	66		3.82	0.038[b]	0.03	NS	0.57	NS
Putamen	64		1.04	NS	0.27	NS	0.18	NS
Thalamus	101		1.68	NS	0.05	NS	0.03	NS
Ventral striatum	38		3.32	NS	0.70	NS	0.26	NS
Insula	102		0.36	NS	0.32	NS	0.50	NS

[a]Number of voxels was averaged across both hemispheres. [b]Baseline scan > post-quit scan, irrespective of stimulus class. [c]Smoking stimuli > control stimuli, irrespective of scanning session. [d]Smoking stimuli > control stimuli at baseline scan; no significant difference at other scans. ACG = anterior cingulate gyrus; BA = Brodmann's Area; IFG = inferior frontal gyrus; MFG = middle frontal gyrus; SFG = superior frontal gyrus.

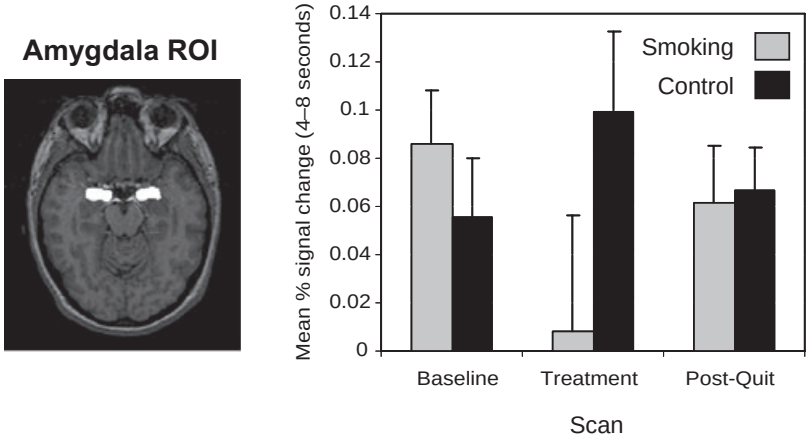


Figure 2 Mean of percent signal change in amygdala following stimulus onset during baseline, treatment and post-quit phase scans. A Stimulus × Phase interaction was observed, $P = 0.023$. Details of statistical results can be found in the text. ROI = region of interest

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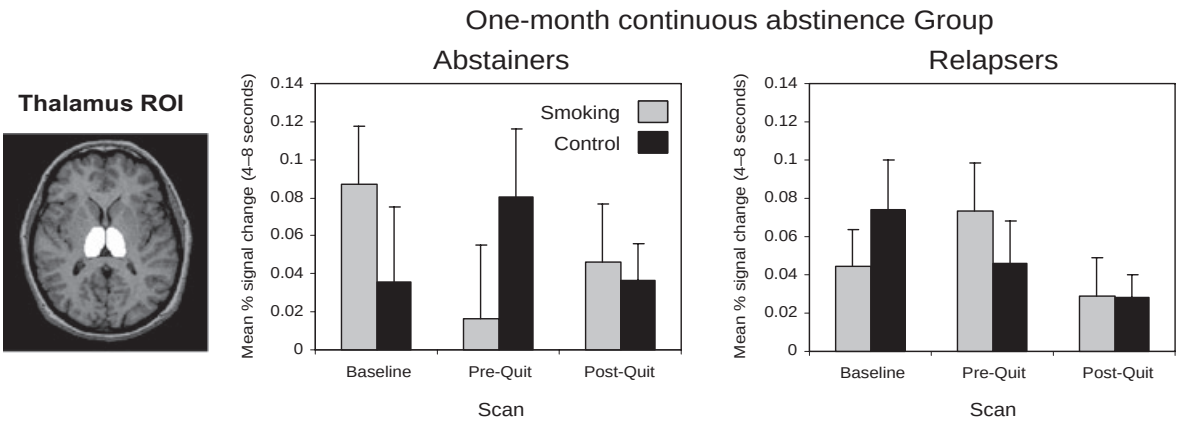


Figure 3 Mean of percent signal change in thalamus following stimulus onset during baseline, treatment and post-quit phase scans in 1-month abstinence and relapsing participant groups. A Stimulus × Phase × Group interaction was observed, $P = 0.032$. Additional detail regarding statistical analyses can be found in the text. ROI = region of interest

post-quit visits inclusive of the 1 month visit and reports of no cigarette smoking since the quit day. In order to evaluate in a preliminary way whether treatment outcome was a significant factor effecting brain responses, we included 1-month abstinence group as a between-subject factor in our analyses. Significant interactions were observed in two ROIs. In thalamus, a Stimulus × Scan × Group ($F = 3.89$, d.f. = 2, 28, $P = 0.032$) interaction was observed. As can be see in Fig. 3, the amplitude of responses to smoking cues was larger than responses to control cues at baseline among future abstainers, but this pattern was reversed following treatment. In contrast, for relapsers differences between responses to smoking and control cues were negligible at all three scans. A trend for a similar Stimulus × Scan × Group interaction was also observed in the ventral striatum ROI (not shown; $F = 3.41$, d.f. = 2, 28, $P = 0.052$). As with thalamus, in the group that would later be abstinent, responses to smoking cues were larger than responses to control cues at baseline but this pattern was reversed following treatment.

No differences in baseline characteristics or smoking behavior were observed between future relapsers and abstainers (all $P > 0.05$). However, participants who complied with treatment (i.e. no usual brand cigarettes during the treatment phase) had a 67% success rate while only 9% of non-compliant participants were continuously abstinent at 1 month ($\chi^2 = 6.2$, d.f. = 1, $P = 0.013$).

DISCUSSION

The findings of this preliminary study suggest that an extinction-based treatment for smoking cessation can alter brain responses to conditioned drug cues. Following 2–4 weeks of a treatment designed to extinguish smoking behavior (Rose *et al.* 2006), responses to visual smoking cues were attenuated coincident with potentiation of responses to control cues. This pattern of changes in brain cue reactivity was observed in the amygdala which has been shown previously to support drug cue reactivity (Grant *et al.* 1996; Childress *et al.* 1999; Bonson *et al.* 2002; Due *et al.* 2002; Okuyemi *et al.* 2006) and also to be sensitive to extinction effects (Phelps *et al.* 2004). A similar pattern was observed in thalamus among 1-month abstinence, but not relapsing participants.

In the present study, smokers underwent an extinction-based treatment that required them to smoke RNC cigarettes while wearing a nicotine patch for 1 month before quitting smoking (Rose *et al.* 2006). The rationale behind this approach is that the smoking of RNC cigarettes prior to quitting extinguishes the link between smoking cues and the delivery of bolus nicotine to the brain, thus rendering smoking less reinforcing and less difficult for the smoker to quit altogether. Combining the smoking of RNC cigarettes with transdermal nicotine administration allows extinction to occur as nicotine is still non-contingent with smoking cues while preventing nicotine withdrawal. This approach has been shown to result in abstinence rates that are comparable to, and possibly higher than, conventional smoking cessation strategies (Rose *et al.* 2006).

Although conceptualized as an extinction-based treatment, it is worth noting that other mechanisms might explain the efficacy of the current treatment approach and, in turn, the effects observed in the present study. Participants in the present study decreased their daily number of smoked cigarettes during the treatment phase. Moreover, despite being given nicotine replacement, plasma cotinine levels dropped by nearly a third from the baseline to the treatment phase. Thus, the treatment phase may have served as a pre-quit weaning period during which smoking behavior and nicotine administration were decreased. By decreasing these two factors, the

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reward salience of cues associated with smoking may have been devalued independently of any extinction effects and resulted in the observed brain responses. Moreover, the observed effects of the treatment on brain cue reactivity may have been due to placebo or expectancy effects as all participants knew they were receiving a treatment designed to help them quit smoking. Future studies that impose pre-treatment smoking frequency and nicotine levels during treatment and also include control groups that undergo active, but not extinction-based treatments, will be able to fully evaluate these potential alternative explanations and confounds.

Current models of the neurobiological basis of drug cue reactivity propose that cue reactivity phenomena are mediated by many of the same neural circuits underlying reward learning and emotional information processing (Volkow *et al*. 2004). The acquisition of nicotine (unconditioned stimulus)–smoking cue (conditioned stimulus) associations is thought to be largely mediated via the mesolimbic dopamine 'reward pathway' (Di Chiara 1999; Balfour *et al*. 2000). According to these accounts, dopaminergic neurons in the ventral tegmental area, stimulated directly and/or indirectly by nicotinic action, release dopamine into the shell of the nAC; the resulting reward signal leads to an increased incentive salience of cues associated with the delivery of nicotine. Thus, otherwise neutral (or even aversive) cues (e.g. ashtray, taste of cigarette smoke) become conditioned nicotine reinforcers—maintaining regular use and causing lapse or relapse following quitting smoking.

Various formulations of conditioned drug reinforcement hypothesize that subsequent exposure to conditioned cues results in activation of multiple brain pathways including those involved in the evaluation of the emotional or reward content of stimuli (e.g. amygdala, nAC); attention (e.g. anterior cingulate, thalamus); and motivation and executive functions (e.g. prefrontal cortex, orbito-frontal cortex) (Chiamulera 2005). Human fMRI (Due *et al*. 2002) and positron emission tomography (PET) (Brody *et al*. 2002) studies have confirmed these hypotheses and have also shown that both nicotine abstinence (McClernon *et al*. 2005) and the expectation that smoking will take place (Wilson *et al*. 2005; McBride *et al*. 2006) can further modulate these responses. In the present study, the observed larger amplitude responses to smoking cues in the ventral ACG across all sessions and amygdala during the baseline session are consistent with these earlier findings.

Continued exposure to conditioned drug cues in the absence of drug reward results in an extinction of behavior to obtain the drug. This phenomenon, and the effects of factors that can reinstate drug-seeking behavior, have been evaluated using animal behavioral studies (Chiamulera *et al*. 1996; LeSage *et al*. 2004; Liu *et al*. 2006). As noted above, while the neurobiological basis or correlates of drug extinction have not been studied extensively, it is reasonable to hypothesize that just as extinction results in a decrease in drug-seeking behavior, it should also result in a decrease in brain activation in regions supporting drug-seeking behavior.

The region in which we observed attenuation of responses to smoking cues—the amygdala—has been shown to be critically involved in drug conditioning and extinction in animal models (See *et al*. 2003). See *et al*. (2003), for instance, demonstrated that the basolateral amygdala plays a critical role in the acquisition of drug–cue associations and in the ability of cues to reinstate drug-seeking behavior (an animal model of relapse). Moreover, this region has been activated during conditioning and extinction in human neuro-imaging studies (LaBar *et al*. 1998; Knight *et al*. 2004; Phelps *et al*. 2004). In one of these studies (Phelps *et al*. 2004), colored blocks were either paired (CS+) or not paired (CS–) with electric shocks to the wrist. During conditioning, presentation of the CS+ was associated with greater amygdala responses compared with CS– presentation. During early extinction, not only did responses to the CS+ diminish, but there was a greater response to the CS– compared with the CS+. During later extinction responses to all stimuli were diminished. Also observed were decreases in responses to the CS+ by extinction in frontal regions including the ventral ACG and medial frontal gyrus. While Phelps *et al*. (2004) and other previous imaging studies of extinction have examined fear extinction rather than reward-related extinction as in the present study and have evaluated only laboratory-based conditioning and extinction, they add support to the notion that the patterns observed in the present study represent extinction-related processes.

Exploratory analyses revealed that in smokers who would be continuously abstinent 1 month following quitting smoking, treatment resulted in attenuation of responses to smoking cues and potentiation of, or no change in, responses to control cues in thalamus. In future relapsers, this pattern was not observed. The thalamus subserves sensory, attentional and arousal processes and has been shown to be active in fMRI studies of sensory gating (Kumari *et al*. 2007) and alerting (Fan *et al*. 2005). Moreover, the thalamus is a region rich in nicotinic cholinergic receptors (Kimes *et al*. 2003; Gallezot *et al*. 2005) and has been shown to be active in response to drug cues (George *et al*. 2001; Due *et al*. 2002). In a recent PET study, thalamic metabolic activity was related to the arousal modulating effects of nicotine (Rose *et al*. 2007). In the present study, a trend for a treatment outcome effect was also observed in the ventral striatum, a region known to subserve reward signaling (Galvan *et al*. 2005; Knutson & Gibbs 2007), to be active

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in response to drug cue presentation (Kilts *et al.* 2001; David *et al.* 2005) and to be associated with increased dopamine release (Brody *et al.* 2004) and neural activity (Stein *et al.* 1998) following nicotine administration. Together these findings suggest that the treatment implemented in the present study modulated brain cue reactivity in regions playing an important role in the modulation and processing of external cues.

The finding of greater baseline cue reactivity among those smokers with better smoking cessation outcomes may be counterintuitive, particularly in light of studies that have shown that enhanced cue reactivity is predictive of poorer outcomes (Payne *et al.* 2006). However, one *post hoc* explanation is that as the treatment was designed to devalue the reward value of conditioned drug cues, the treatment worked best for those smokers who were most sensitive to these effects in the first place. Conversely, smokers whose smoking behavior was less driven by conditioned cues were not helped by the treatment. Regardless of the mechanism, findings relating thalamus and ventral striatum activity to cessation outcome are highly interesting but, given the limitations of the study, should be viewed as highly preliminary and in need of replication in larger and better-controlled studies.

A number of important limitations of the present study should be noted. While the observed results are consistent with the predicted effects of an extinction-based treatment, the inclusion of a control group that did not receive extinction-based treatment would aid in interpretation of findings. For instance, without a control group it is unclear whether participants would have shown decreases in brain responses to smoking cues over time, independent of any kind of treatment. Although the inclusion of control cues mitigates this limitation somewhat by helping to rule out habituation as the reason for declines in smoking cue reactivity, a control group receiving no treatment or treatment devoid of extinction-based components could further rule out effects of time and habituation. In addition to the above, the present study is also limited by a relatively small sample size, consisting primarily of female participants. Though still requiring further research, women have been shown to be more sensitive to the conditioned, sensory aspects of smoking (Perkins *et al.* 2001) and it is unclear whether our findings would generalize to male smokers.

Finally, the study may have been limited by its exclusive use of ROI-based fMRI data analytic techniques. ROI-based approaches have several strengths including the fact that they do not make assumptions about the shape of the hemodynamic response and reduce the risk of Type I error by substantially reducing the number of statistical comparisons overall (Huettel, Song & McCarthy 2004). However, as an ROI-based approach evaluates the average response across voxels of a pre-defined region, it assumes, usually incorrectly, that activation within an ROI is homogenous. The ROIs examined in the present study were chosen a priori based on previous analyses of data using the same stimuli and they were not exhaustive of all regions that have been shown to be active in response to drug cues. For these reasons, we cannot be sure where the largest activations in response to smoking cues occurred. Moreover, it remains unknown if the observed significant activations would have been considered significant if a standard voxel-wise analysis had been conducted. Analyses of future, larger trials will incorporate both ROI and voxel-wise approaches to fully characterize the effects of treatment on brain cue reactivity.

In summary, the results of this study suggest that an extinction-based smoking cessation treatment can alter brain responses to smoking cues and that these changes may be associated with treatment outcome. We observed an extinction-based treatment to alter brain responses to smoking cues in the amygdala among all smokers, and in the thalamus among smokers who achieved 1-month continuous abstinence. Future studies will evaluate the effects of other smoking cessation treatments on brain physiology during cue reactivity in larger samples.

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Want to improve your chances of getting your research published in peer-reviewed journals? You are invited to a one-day workshop on writing for publication run jointly by the Society for the Study of Addiction and the International Society of Addiction Journal Editors. The course is suitable for doctoral students and researchers working in the social, behavioural and biomedical fields.

Building on last year's successful workshop, study topics include how to choose the right journal, how to prepare your manuscript and respond to referees' comments, and awareness of good citation practices. Small group discussion based around case studies will tackle authorship disagreements, ethical decision-making, and the seven deadly sins of scientific publishing and how to avoid them. Participants will get an inside look at how editors work and how they make decisions about what material to publish.

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The workshop will run between 1000-1530 hours, with a light lunch and refreshments on arrival and departure. Cost of attendance will be £69. A special combined price will apply if you are also attending the Society for the Study of Addiction Annual Symposium on 15 and 16 November: please visit the Society website for programme information:
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To reserve your place, please contact Graham Hunt, Leeds Addiction Unit, 19 Springfield Mount, Leeds, LS2 9NG (graham.hunt@leedsmh.nhs.uk) no later than Friday 26 October. Enrolled participants will receive further instructions before the workshop.

Before coming to the workshop, participants are recommended to read *Publishing Addiction Science: A Guide for the Perplexed*. Free downloads from www.parint.org and www.isaje.net

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Stimulus and Response Expectancies Influence the Cognitive Effects of Cigarettes

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The pharmacological, stimulus expectancy, and response expectancy effects of light cigarettes (0.60 mg yield of nicotine) compared with virtually nicotine-free cigarettes (less than 0.05 mg yield of nicotine) were examined using a between-subjects design. A total of 103 college student smokers completed tests of cognition before and after smoking one type of cigarette, which was evaluated on a number of dimensions. Cigarettes with nicotine were preferred on most dimensions, and stimulus expectancy partially mediated the relationship between nicotine and subjective effects of the cigarettes. Stimulus expectancy also mediated the effect of nicotine on tension reduction immediately after smoking, but not near the end of the experiment. Response expectancy effects of nicotine were related to predicted and actual recall performance, such that participants who performed well tended to attribute the effect to the cigarette they smoked. Implications for smoking cessation and research studies using non-nicotine cigarettes are discussed.

Keywords: memory, metacognition, stimulus expectancy, sustained attention, response expectancy

There is growing consensus that changes in cognitive processing contribute to the reinforcing effects of cigarettes (e.g., Brandon, Herzog, Irvin, & Gwaltney, 2004; Kassel, 1997; Levin, 1992). In college students, for example, smoking behaviour increases substantially during periods of intense study (Wesnes, Revell, & Warburton, 1983), and students report increased smoking specifically to increase their level of cognitive arousal (West & Lennox, 1992). Whereas a large, established body of research has focused on the cognitive effects of nicotine (for reviews, see Heishman, Taylor, & Henningfield, 1994; Sherwood, 1993), there is now increasing recognition that numerous factors, including nonpharmacological factors, can play important roles in maintaining smokers' addiction to cigarettes (Johnson, Bickel, & Kirshenbaum, 2004; Perkins, Sayette, Conklin, & Caggiula, 2003; Rose, 2006). The focus of the present study was on the role of expectancy in nicotine's cognitive effects.

The term expectancy denotes two different phenomena in nicotine research. First, participants may hold certain beliefs about the effects of a drug on their own internal state, which can be based on past use of the drug or information received from others. This type of expectancy is more precisely termed 'response expectancy' and it has been shown to influence a variety of outcomes, including mood states, memory and drug use (Kirsch, 1999). Response expectancies related to cigarettes have been shown to be a causal factor in smoking motivation (e.g., Copeland & Brandon, 2000). In addition, Hendricks and Brandon (2008) showed that smokers had different expectancies for cigarettes, which were primarily associated with negative health consequences, compared with nicotine, which was more closely associated with addiction. Both cigarettes and nicotine were equally associated with arousal.

Expectancy can also refer to whether participants expect an active or inactive substance in a study. This variable is more precisely termed 'stimulus expectancy' (Kirsch, 1999; Perkins et al., 2004). Stimulus expectancy can be manipulated through instructional sets, in which some participants are told to expect an active drug and others are told to expect an inactive control. In a balanced placebo design, the type of substance expected and received are completely crossed, creating four conditions (two of which are deceptive). To date, four published studies have used a balanced placebo design with cigarettes (Juliano & Brandon; 2002; Kelemen & Kaighobadi, 2007; Perkins et al., 2004; Perkins et al.,

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2008), and all have uncovered significant stimulus expectancy effects with cigarettes independent of nicotine itself. However, one difficulty in using balanced placebo designs with cigarettes is maintaining the deception. In the four studies mentioned, participants' rates of disbelief ranged from 7% to 33%.

Stimulus expectancy typically is ignored in double-blind research designs, because participants are assumed to be naive as to which condition has been administered. The utility of double-blind procedures has been challenged, however (e.g., Kramer & Shapiro, 1984; Roth et al., 1991; Sutton, 1991). For example, Mooney, White, and Hatsukami (2004) reviewed the literature on nicotine replacement therapy and they found that the majority of studies did not report any data on the integrity of the blindness procedures. In the small number of studies that did report such data, participants judged the type of substance they received at levels significantly greater than chance in over 70% of cases (12 out of 17 studies). Fortunately, there are procedures to evaluate data from ineffective double-blind designs (e.g., Hughes & Krahn, 1985), although such procedures are rarely applied.

The present study was designed as a double-blind investigation of nicotine (0.60 mg) versus no nicotine (less than 0.05 mg) in cigarettes on cognition. The initial focus was on response expectancies for cigarettes regarding cognitive performance. All participants completed a baseline assessment of cognitive tasks, followed by questions about how the cigarette they received would influence their performance on a second round of the same tasks. We also collected confidence ratings during the recall tasks to allow more fine-grained analysis of response expectancy. The main hypothesis was that positive correlations would emerge between participants' response expectancies and their predicted or actual performance. We assessed the effectiveness of our double-blind design by offering a financial incentive to participants to who correctly identified the type of cigarette they received. To anticipate the results, participants were able to correctly indentify the presence or absence of nicotine at levels exceeding those predicted by chance. Therefore, additional analyses were conducted to test for stimulus expectancy effects, especially as a potential mediator of nicotine's cognitive effects.

Method

Participants

Useable data were obtained from 103 students — 41 women and 62 men — at a large urban university in the United States. Data from 5 other participants were discarded due to experimenter errors or because the participants failed to follow instructions. The mean age of participants was 21.6 years old ($SD = 4.7$). Participants were paid $30 USD each, with a bonus of $5 USD awarded at the end of the study to those who correctly identified the type of cigarette (nicotine vs. non-nicotine) they had smoked. All participants reported smoking at least 10 king-sized nonmentholated cigarettes per day for the last year and they were not trying to quit smoking. The mean FTND score (Heatherton, Kozlowski, Frecker, & Fagerström, 1991) of participants was 3.3 ($SD = 1.9$) and the mean score on the Cigarette Dependence Scale (Etter, Le Houezec, & Perneger, 2003) was 41.8 ($SD = 8.2$).

Participants were instructed to abstain from smoking for at least 5 hours before the experiment. Upon arrival for testing, all students were tested for abstinence by end tidal carbon monoxide (CO) analysis. Students were informed in advance that they would be excluded if their expired CO exceeded 15 parts per million. The mean CO level was 6.1 ($SD = 4.0$). Two students slightly exceeded 15 but they were allowed to participate because subtracting the baseline reading of a nonsmoker tested at the same time resulted in a reading of 15 or less.

Design

A pretest–posttest design was used. The between-subjects independent variable was type of cigarette. The major dependent variables included subjective ratings of cigarettes, levels of smoking urges, tension and energy, sustained attention performance, and predicted as well as actual memory performance.

Materials

Types of cigarettes. Two types of cigarettes were used: (a) Quest 1, which delivered 0.6 mg of nicotine per cigarette and (b) Quest 3, which delivered less than 0.05 mg nicotine per cigarette. These cigarettes were identical in tar content (10 mg each), size (85 mm each), and type of filter. The cigarettes were removed from their packages and coded by the author, who had no direct contact with the participants.

Study and prediction task. Participants studied 40 pairs of Swahili–English vocabulary items (e.g., Zulia — Carpet) presented twice, in random order, for 5 seconds each. During the second study trial, participants were asked to predict how likely they were to remember each English word given the Swahili word about 20 minutes later, using a scale of 1–100. Half of the ratings were made immediately after the pair was studied the second time (i.e., an immediate prediction) and the other half of the items were rated after all 40 items had been restudied (i.e., a delayed prediction; for rationale see Kelemen & Weaver, 1997; Nelson & Dunlosky, 1991). At the end of this study session, participants predicted how many total words (0–40) they would remember (i.e., make an aggregate prediction). Overall, this task required approximately 10 minutes.

Sustained attention task. After the study and prediction task, participants completed a test of sustained attention for 12 minutes. Participants were shown a series of digits on the screen, one at a time, at the rate of 100 per minute

(i.e., 100 ms each with an interstimulus interval of 500 ms). Participants were asked to press the space bar whenever three consecutive odd or even digits appeared, which occurred an average of eight times per minute.

Memory recall task. Immediately after finishing the sustained attention task, participants completed a cued-recall memory test of the Swahili–English word pairs studied earlier. Each Swahili word appeared at the top of the screen one at a time, and participants were asked to type in the English equivalent. Misspellings were counted as correct if the first three letters matched those of the target word.

Procedure

Upon arrival, participants provided informed consent and completed a CO breath analysis. Participants were informed that later in the study they would be given a cigarette to smoke, which may or may not contain nicotine. They were offered an additional $5 USD if they could guess whether or not it contained nicotine after smoking it. Then participants completed several questionnaires on an electronic handheld device, including the FTND, cigarette dependence scale and a smoking consequences questionnaire (responses on this latter questionnaire did not predict performance on any of the dependent measures so it is not discussed further). Next, a baseline measurement of smoking urges was obtained using the questionnaire on smoking urges (QSU; Cox, Tiffany, & Christen 2001), and a baseline measure of energy and tension also was obtained using the activation–deactivation adjective checklist (AD-ACL; Thayer, 1986). Participants then completed baseline assessment of cognitive performance (i.e., the study and prediction phase of the memory test, followed by a test of sustained attention and then the memory recall phase).

Next, the research assistant escorted participants outside of the building to smoke the cigarette and neither person knew which type of cigarette was provided. After finishing their cigarette, participants completed the 12-item cigarette evaluation scale (CES; Westman, Levin, & Rose, 1992), guessed whether or not their cigarette contained nicotine and assessed how it would influence their subsequent cognitive performance. They also completed the QSU and AD-ACL again. Finally, participants returned to the lab to complete the second round of computerised cognitive testing using different stimuli. After the second round of testing was completed, participants completed a posttest measure of smoking urges (QSU), arousal (AD-ACL) and perceived cognitive effects. Following debriefing procedures, participants were paid and dismissed. The entire set of procedures required approximately 2 hours.

Results

All tests of statistical significance were conducted at $p < .05$ unless otherwise noted.

Blindness Check

Participants correctly identified the type of cigarette they smoked with a high degree of accuracy in both the nicotine condition (M = 71% correct) and non-nicotine condition (M = 88% correct). Both levels of performance were significantly greater than the 50% level expected by chance using one-sample t tests, $t(50) = 3.20$ and $t(51) = 8.60$, respectively. Only 21 of the 103 participants misidentified the type of cigarette received: 6 people who received a non-nicotine cigarette thought it did contain nicotine and 15 people who received nicotine thought the cigarette did not.

Because the blindness aspect of the design was compromised, it was difficult to interpret subsequent analyses on the role of nicotine in isolation because those effects could be confounded by stimulus expectancy. To address this concern, two additional types of analyses were included for each dependent measure: (a) a direct test of stimulus expectancy using a subset of the data, that is, when participants received nicotine and reported that it did (n = 36) or did not (n = 15)[1]; and (b) mediation analyses using the full dataset, examining whether the relationship between type of gum received and a dependent measure was mediated statistically by participants' stimulus expectancy.

Subjective Effects of Cigarettes

Participants rated the subjective effects of their cigarette on 12 dimensions using the CES. Cigarettes with nicotine were rated significantly higher than cigarettes without nicotine in 10 of 12 dimensions using one-way ANOVAs (see leftmost data in Table 1). To test the role of stimulus expectancy, follow-up one-way ANOVAs were conducted on data from the 51 participants who received nicotine, using stimulus expectancy as the independent variable. Participants who believed that they received nicotine rated their cigarettes as significantly higher compared with participants who believed they did not (see rightmost data in Table 1). Stimulus expectancy played a statistically significant role in 80% (8 of 10) of the dimensions that were influenced by nicotine itself. Further, the pattern of means across dimensions is quite clear: participants tended to rate cigarettes highest when they received nicotine and believed they did, lower when they simply received nicotine (regardless of belief), still lower when they received nicotine but thought they did not, and lowest when they simply did not receive nicotine (regardless of belief).

Several statistical techniques have been proposed to test for simple mediation effects, including the use of regression equations (Baron & Kenny, 1986), a Sobel test and a bootstrap method for assessing indirect effects (Preacher & Hayes, 2004). In this study, all three types of analyses yielded identical results, so only the Sobel test outcomes are reported here. Using the 12 subjective ratings as dependent measures, Sobel tests confirmed a

Table 1

Mean Ratings on the Cigarette Evaluation Scale by Type of Cigarette and Participants' Judgments about the Type of Cigarette

	Type of Cigarette					Nicotine Cigarette Expectancy				
	Nicotine		No nicotine			Nicotine		No nicotine		
Item	*M*	*SEM*	*M*	*SEM*	η^2	*M*	*SEM*	*M*	*SEM*	η^2
The cigarette was satisfying.*†	4.79	0.24	1.98	0.16	0.48	5.42	0.23	3.33	0.43	0.31
The cigarette was calming.*†	4.90	0.24	2.33	0.19	0.41	5.44	0.26	3.53	0.34	0.26
The cigarette reduced my irritability.*†	4.35	0.23	2.31	0.18	0.32	4.78	0.26	3.20	0.39	0.18
The cigarette helped me concentrate.*†	3.23	0.22	1.77	0.13	0.25	3.56	0.26	2.40	0.36	0.11
The cigarette tasted good.*†	3.79	0.25	2.00	0.19	0.24	4.17	0.30	2.93	0.38	0.10
The cigarette reduced my cravings.*†	4.02	0.23	2.40	0.20	0.22	4.33	0.27	3.20	0.42	0.10
The cigarette made me feel awake.*	3.69	0.26	2.17	0.17	0.19	4.03	0.32	3.00	0.39	0.06
The cigarette made me dizzy.*†	2.60	0.26	1.42	0.12	0.14	3.06	0.33	1.53	0.27	0.14
The cigarette was very strong.*†	2.29	0.22	1.35	0.14	0.11	2.67	0.29	1.40	0.19	0.13
The cigarette reduced my hunger.*	3.48	0.28	2.44	0.24	0.07	3.75	0.35	2.73	0.38	0.06
The cigarette made me feel nauseous.	1.56	0.18	1.50	0.15	N/A	1.58	0.23	1.47	0.29	N/A
The cigarette gave me throat and chest sensations.	2.63	0.23	2.21	0.20	N/A	2.61	0.29	2.73	0.43	N/A

Note: *M* indicates ratings; *SEM* indicates standard error of the mean. Eta^2 was included as a measure of effect size when the difference between conditions was statistically significant.
* indicates a statistically significant difference due to the amount of nicotine using an ANOVA, $p < .05$, $N = 103$.
† indicates a statistically significant difference in nicotine cigarettes due to participants' judged presence or absence of nicotine using an ANOVA, $p < .05$, $N = 51$.

significant mediation effect of stimulus expectancy on nicotine for the first 9 of the 12 dimensions listed in Table 1 (i.e., ratings of cigarette satisfaction, calming, irritability reduction, enhanced concentration, taste, craving reduction, enhanced awakeness, dizziness reduction, and strength; all Zs > 1.98, all ps < .05). No mediation was detected for hunger reduction, nausea induction, or throat and chest sensations. Thus, the outcome of mediation analyses on the full dataset was very consistent with the outcome of the one-way ANOVAs noted in Table 1 using only participants who received nicotine; both sets of analyses suggested a strong role of stimulus expectancy in the subjective effects of nicotine in cigarettes.

Smoking Urges, Tension and Energy

Participants' smoking urges were assessed before smoking (baseline), immediately after smoking and near the end of the experiment. Two difference scores (i.e., levels immediately after smoking minus baseline and levels near the end of the experiment minus baseline) were analysed using one-way ANOVAs. As expected, nicotine significantly reduced smoking urges immediately after the cigarette (difference score $M = –13.06$, $SEM = 1.76$) compared with non-nicotine cigarettes (difference score $M = –4.33$, $SEM = 1.36$), $F(1, 101) = 15.62$, $\eta^2 = 0.14$; the difference near the end of the experiment did not reach statistical significance, $F(1, 101) = 3.31$, $p = .07$. Thus, cigarettes with nicotine did significantly reduce the urge to smoke, but the effect was short-lived. As before, the influence of stimulus expectancy was examined in two ways. First, the type of cigarette participants believed they had did not influence QSU scores when participants received nicotine, Fs < 1, ps > .30. Second, the Sobel test of mediation was likewise non-significant, $Z = –1.72$, $p = .09$.

Participants' levels of tension and energy also were assessed at baseline, immediately after smoking and near the end of the experiment. One-way ANOVAs revealed significant tension reduction in the presence of nicotine (difference score immediately after smoking: $M = –2.20$, $SEM = 0.51$; difference score near end of experiment: $M = –2.47$, $SEM = 0.51$) compared with non-nicotine cigarettes (difference score immediately after smoking: $M = –0.88$, $SEM = 0.31$; difference score near end of experiment: $M = –0.96$, $SEM = 0.43$), $F(1, 102) = 4.88$, $\eta^2 = 0.05$ and $F(1, 102) = 5.20$, $\eta^2 = 0.05$, respectively. Surprisingly, mediation analyses using the Sobel test confirmed a significant effect of stimulus expectancy immediately after smoking, ($Z = –2.46$, $p < .05$) but not near the end of the experiment ($Z = –0.83$, $p > .40$). The identical pattern was observed with the sample of participants who receive nicotine: those who believed they received nicotine had greater tension reduction ($M = –2.92$, $SEM = 0.59$) compared with those who believed they did not ($M = –0.47$; $SEM = 0.87$) immediately after smoking, $F(1, 50) = 5.20$, $\eta^2 = 0.10$, but not near the end of the experiment. For energy, no significant difference due to nicotine was evident and therefore no mediation analyses were conducted.

Cognitive Performance

Difference scores were computed for attention, recall, as well as immediate, delayed, and aggregate predictions of recall by subtracting participants' baseline data from their postcigarette data (see Table 2). For attention, participants were significantly faster by approximately 50

Table 2

Difference Scores (Post-Cigarette Minus Pre-Cigarette) for Cognitive Performance by Nicotine Condition

	Type of Cigarette			
	With Nicotine		Without Nicotine	
Dependent Measure	*M*	*SEM*	*M*	*SEM*
Sustained Attention Accuracy (*d'*)	0.18	0.04	0.19	0.04
Sustained Attention Response Latency (ms)*	–53.75	16.32	–2.13	13.60
Immediate Prediction Magnitude	–2.85	1.81	–5.05	1.52
Delayed Prediction Magnitude	0.86	1.77	–1.10	1.94
Aggregate Prediction Magnitude	–0.02	0.71	–0.39	0.54
Recall	0.00	0.02	–0.01	0.02

Note: Main entries are mean difference scores.
* indicates a statistically significant difference due to the amount of nicotine using an ANOVA, $p < .05$.

ms after nicotine compared with non-nicotine cigarettes, $F(1, 102) = 5.90$, $\eta^2 = 0.06$; both the ANOVA on participants who received nicotine and the Sobel test on the full sample showed that this effect was not mediated by stimulus expectancy. For sustained attention accuracy (as operationalised by *d prime*), no significant differences were observed. In addition, no significant differences were obtained when comparing difference scores for recall and all predictions of performance across nicotine conditions.

Response Expectancies about Nicotine's Cognitive Effects

Pearson correlations were computed to examine the relationship between individuals' beliefs about the effects of smoking (i.e., response expectancies) and their predicted as well as actual performance. Participants were asked to rate the effect of the cigarette on each cognitive task twice after smoking: once before the second round of cognitive tasks (i.e., pretasks) and again near the end of the experiment (i.e., posttasks). An example of the question for a pretask rating concerning recall was, 'Please rate the effect the cigarette will have on your ability to recall the Swahili–English word pairs on a test'. For all questions, responses ranged from –10 ('*Highly impaired*') to 10 ('*Highly improved*'), with 0 labelled '*No effect*'. Because the questions asked about the effects of the cigarette, and not nicotine per se, mean correlations were computed across all participants. As before, difference scores were used for the dependent measures to control for baseline performance.

In general, correlations between the expected effect of the cigarette and cognitive performance were nonsignificant before the tasks (see Table 3). In contrast, the correlations were larger and statistically significant in four of five cases (*r*s ranging from 0.25–0.32), when the cigarette effects were evaluated after the tasks. Thus, participants who perceived more positive effects of the cigarette near the end of the experiment tended to show better cognitive performance.

Table 3

Correlations between the Expected Effects of Smoking and Several Measures of Cognitive Performance

Difference Scores	Pretask	Posttask
Sustained Attention Accuracy (d')	0.00	0.32**
Aggregate Prediction Magnitude	0.01	0.25*
Immediate Prediction Magnitude	0.03	0.09
Delayed Prediction Magnitude	0.16	0.23*
Recall	0.01	0.31**

Note: Main entries indicate Pearson product–moment correlation coefficients (*r*).
* $p < .05$
** $p < .01$

Discussion

The results of this study demonstrated significant effects of both stimulus expectancy and response expectancy in the subjective and cognitive effects of cigarettes. Stimulus expectancy typically is ignored in research intended to be double-blind, such as the present study, but this practice is questionable because it has proven difficult to implement effective double-blind procedures in nicotine research (Mooney et al., 2004). We conducted a strong test of our own procedures by offering financial incentives for participants to correctly identify their condition, and smokers were able to determine the presence or absence of nicotine in their cigarettes with a high level of success. Thus, it was essential to examine the role of stimulus expectancy, which did in fact play a substantial role in the subjective effects of the cigarettes.

Consistent with past research (Cohn, Cobb, Ali, & Juliano, 2004; Strasser, Lerman, Sanborn, Pickworth, & Feldman, 2007), nicotine cigarettes were preferred on a number of dimensions. However, participants' expectancies about the type of cigarette they received partially mediated these drug effects. This finding was multifaceted for smokers' tension levels, where stimulus expectancy mediated the effects of nicotine immediately after smoking but not approximately 30 minutes later (near the end of the experiment). From a theoretical perspective, these results represent a small step toward the larger goal of establishing direct versus indirect (i.e., mediated) effects of nicotine on behaviour, which has been identified as a major goal of basic research in this area (Waters & Sutton, 2000). From an applied perspective, these findings have implications for some cessation studies of reduced-nicotine cigarettes, especially recent research on the effectiveness of such cigarettes administered with various nicotine replacement therapies (e.g., Becker, Rose, & Albino, 2008; Rezaishiraz, Hyland, Mahoney, O'Connor, & Cummings; 2007; Rose, Behm, Westman, & Kukovich, 2006). For example, Becker et al. (2008) examined a combined treatment of reduced-nicotine cigarettes

and nicotine patches in a clinical trial; they concluded that this combination 'offers promise as a new smoking cessation treatment' (p. 1139). Because that study used a double-blind design, the role of stimulus expectancy was not examined. The present results, however, suggest the possibility that smokers' beliefs about their actual condition could have contributed to the perceived benefits of the treatment. If so, the efficacy of the combined treatment might be reduced in the real world when participants are aware that their cigarettes contain little or no nicotine.

The present study also examined the role of response expectancy in cigarette smoking, that is, whether or not individuals who believed nicotine had positive cognitive effects would show better performance or more confidence in their performance. The results here also were affirmative, but qualified. Significant positive correlations were observed between memory performance (both predicted and actual) and response expectancies assessed near the end of the experiment. The nonsignificant correlations between pretask response expectancies and the cognitive measures cast doubt on the possibility that response expectancies played a causal role in performance. Still, the possibility that participants who performed well on the tasks might have attributed their performance to the effects of the cigarettes posthoc is troubling, especially because nicotine itself affected attention but not memory. Thus, these data might illustrate the formation of new response expectancies in regard to these laboratory cognitive tasks, especially because the tasks may have been novel to the participants. Smokers do have established expectancies about cigarettes on other aspects of behaviour, and such response expectancies are important in smoking motivation (Copeland & Brandon, 2000).

Several limitations were present in this study. First, the role of stimulus expectancy was assessed entirely posthoc and the sample sizes of groups who expected one type of cigarette but received the other were small. Because only 6 people reported that their cigarette contained nicotine when it did not, it was impossible to look at the role of stimulus expectancy in the non-nicotine condition. Still, the finding of stimulus expectancy effects in the group that received nicotine cigarettes is noteworthy: it demonstrates that expectancy can operate even in the presence of an active drug. A benefit of examining stimulus expectancy posthoc is that no deceptive conditions existed, which have been problematic in balanced-placebo studies (Juliano & Brandon; 2002; Kelemen & Kaighobadi, 2007; Perkins et al., 2004; Perkins et al., 2008).

Another limitation was that a low dose of nicotine was administered in the 'active' cigarette condition. The current dose (0.60 mg) was quite low by most smokers' standards: the most popular type of cigarette in the United States yields approximately 1.2 mg of nicotine per cigarette (U.S. Centers for Disease Control and Prevention, 2005). This low dose may explain why no significant changes emerged for predicted or actual memory. The problem of administering a dose of nicotine sufficient to induce pharmacological changes while still maintaining double-blind procedures is well-known (Hughes & Krahn, 1985), and this study was not able to completely surmount it. Finally, the cognitive effects of nicotine in cigarettes may be somewhat transient. Sakurai and Kanazawa (2002) have shown the levels of nicotine concentration in blood return to baseline within 10 minutes of smoking a cigarette in nondeprived smokers. In the present study, participants would have just finished their questionnaires and been starting their cognitive tasks at the time when the nicotine levels began to wane. Both the issues of dose and timing present continuing challenges for future researchers. Possible solutions include a comparison of high versus low nicotine levels (instead of low levels versus nonnicotine) and the use of repeated smoking bouts during long experimental procedures.

The examination of individual differences in responses to smoking using effective placebo cigarettes has been identified as an important area in need of research (Perkins et al., 2003). The present study showed that both stimulus expectancy and response expectancy can influence the cognitive effects of nicotine in cigarettes. Based on these results, reliance on double-blind administration procedures to control nicotine expectancies clearly is not sufficient. Fortunately, the effects of both types of expectancy can be assessed, but the results of studies that fail to do so must be interpreted with caution.

Endnotes

1 Stimulus expectancy was not examined in the non-nicotine condition because only 6 participants believed that non-nicotine cigarettes contained nicotine

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Original Investigation

Effects of acute nicotine administration on ratings of attractiveness of facial cues

Angela S. Attwood, Ian S. Penton-Voak, & Marcus R. Munafò

Abstract

Introduction: It has been suggested that one mechanism by which nicotine may exert an influence over behavior is by enhancing the reinforcing properties of other stimuli. We therefore sought to test the hypothesis that nicotine enhances the hedonic impact of behaviors performed in the presence of nicotine, using ratings of facial attractiveness, as we considered these to have considerable ecological validity in the context of the social environment within which cigarette smoking takes place.

Methods: Male and female participants attended a single testing session and were randomized to smoke either a nicotine-containing or a denicotinized cigarette, after which they completed ratings of attractiveness of 20 male and 20 female faces. Participants were required to have abstained from smoking for 24 hr prior to testing, and the nicotine manipulation was conducted double blind.

Results: A 2 × 2 × 2 mixed-model repeated measures analysis of variance indicated a significant main effect of cigarette, reflecting higher attractiveness ratings in the nicotinized compared with the denicotinized condition.

Discussion: Our data indicate that nicotine increases ratings of attractiveness of facial cues. We did not observe any evidence that these effects differed between males and females. We also did not observe effects on subjective ratings of mood, suggesting that the effects we observed on ratings of attractiveness may not simply reflect global hedonic effects, or a positivity bias in questionnaire responding, in the nicotine condition.

Introduction

Despite the widely acknowledged addiction potential of tobacco, due primarily to the actions of nicotine, the psychoactive and hedonic effects of nicotine are modest (Balfour, 2004). For example, nicotine presented intravenously in doses comparable with those obtained from a single cigarette puff produces only modest ratings of liking and satisfaction (Rose, Behm, Westman, & Johnson, 2000). While dependent smokers experience considerable negative reinforcement through the relief of withdrawal symptoms, in particular after overnight abstinence, the role of positive reinforcement, in particular among young, non-dependent smokers at an early stage in the progression to regular smoking and dependence, is unclear.

One mechanism by which nicotine may exert an influence over behavior is by enhancing the reinforcing properties of other stimuli (Donny et al., 2003). For example, nicotine appears to increase responding for a concurrently available, reinforcing, nonpharmacological stimulus in animal models, even when the nicotine is neither temporally nor causally associated with the behavior (Donny et al., 2003). In other words, in addition to its action as a primary reinforcer, nicotine may serve to potentiate the reward value of other stimuli (Balfour, 2004). Other evidence from animal models indicates increased responding for food, alcohol, and cocaine (Bechtholt & Mark, 2002; Clark, Lindgren, Brooks, Watson, & Little, 2001; Popke, Mayorga, Fogle, & Paule, 2000) and decreased thresholds for brain reward stimulation (Kenny & Markou, 2006) following the administration of nicotine.

The mechanism proposed to account for this effect (Balfour, 2004) is the increase in extracellular dopamine evoked in the medial shell of the nucleus accumbens by the administration of nicotine (e.g., by smoking a cigarette). In particular, any activity or stimulus experienced while dopamine overflow remains elevated will have increased hedonic impact (Balfour, 2004). As a result, an increase in dopamine overflow resulting from nicotine administration could exert a more general positive influence on other behaviors performed concurrently. This, in turn, might contribute to the pleasure associated with the habit and, therefore, to the addiction potential of nicotine, independently of any direct hedonic effects of nicotine administration (Balfour, 2004). To date, the majority of evidence for these effects comes from animal models of nicotine administration and self-administration.

While negative reinforcement processes are important in maintaining tobacco use and strengthening dependence, they cannot explain why people begin to use tobacco or continue to

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do so until dependence has developed. Therefore, positive reinforcement models are likely of particular importance in the early stages of tobacco use and in the development of dependence (Glautier, 2004). The potentiation by nicotine of the reward value of other stimuli may comprise part of the process whereby tobacco use is initiated and maintained prior to the development of dependence. Furthermore, as tolerance to the effects of nicotine develops with increasing dependence, negative reinforcement processes may come to dominate over positive reinforcement processes, so that this potentiating effect of nicotine may decline in relative (or absolute) strength as dependence develops.

We sought to test the hypothesis that nicotine enhances the hedonic impact of behaviors performed in the presence of nicotine compared with the same behaviors performed in the absence of nicotine. We used ratings of facial attractiveness, as we considered these to have considerable ecological validity in the context of the social environment within which cigarette smoking takes place. Also, evidence suggests that facial attractiveness is correlated with activity in the reward pathways such as the orbitofrontal cortex and striatum (Aharon et al., 2001; Kampe, Frith, Dolan, & Frith, 2001; O'Doherty et al., 2003). We recruited young, nondependent adults at a relatively early stage in their smoking career since we would expect the effects of nicotine on the hedonic aspects of other behaviors to be greatest prior to or during the development of dependence. Moreover, we tested participants when they were nicotine free (i.e., after at least overnight abstinence) to maximize the effects of nicotine administration and because we expected withdrawal effects to be negligible in nondependent smokers. Finally, we included male and female participants to explore possible sex differences, given a broad literature that suggests differential effects of nicotine in men and women (Perkins, Donny, & Caggiula, 1999).

Methods

Design and overview

Male and female participants attended a single testing session and were randomized to smoke either a nicotine-containing or a denicotinized cigarette, after which they completed ratings of attractiveness of 20 male and 20 female faces. The experimental design therefore consisted of two between-subjects factors of cigarette (nicotinized, denicotinized) and sex (male, female) and one within-subjects factor of target sex (male, female). Participants were required to have abstained from smoking for 24 hr prior to testing, and the nicotine manipulation was conducted double blind.

Participants

Male and female (n = 48, 50% male) nondependent smokers (with a score of less than 3 on the Fagerström Test for Nicotine Dependence [FTND; Heatherton, Kozlowski, Frecker, & Fagerström, 1991]) were recruited from the staff and students at the University of Bristol and the general population. Participants received either £5 or course credit for participation, as appropriate. The study was approved by the Faculty of Science Research Ethics Committee.

After providing informed consent, participants completed a screening process, consisting of an interview conducted by a trained researcher, to ensure good physical and psychiatric health. Exclusion criteria included drug dependence (excluding caffeine) and significant current or past medical or psychotic illness. Participants also were required to be free from medication and illicit substances, indicated by self-report, and were required to abstain from smoking for 24 hr prior to the study, with recent abstinence confirmed by exhaled carbon monoxide (CO) breath test (<5 ppm).

Materials

Cues for the attractiveness task comprised 40 photographic facial stimuli (20 male and 20 female, mean age 21 years). The facial photographic images were collected using a Canon EOS300D at a focal length of 50 mm under standardized lighting conditions provided by two lateral diffuse Portaflash DL1000 studio lights. Images were captured at a resolution of 2,048 × 3,072 pixels in full color. All images had a constant background consisting of a gray cloth. The photographic subjects wore no overt facial jewelry, and cues to clothing were masked with a neutral smock. Participants were asked to pose, standing, with a neutral expression and photographed at a distance of approximately 150 cm. The camera lens was at the same horizontal plane as the eyes for each photograph. This procedure provides replicable images of the same individual and provides images that are equivalent to experience of faces in day-to-day interaction (Stephan, Clement, Owen, Dobrostanski, & Owen, 2004). All the photographic subjects were University of Bristol undergraduate students of European ancestry in 2005, and each individual was the current heterosexual partner of one other individual in the opposite-sex face set.

The questionnaire measures used included the FTND, the Spielberger State-Trait Anxiety Inventory (STAI state anxiety subscale only; Spielberger, Gorusch, Lushene, Vagg, & Jacobs, 1983), and Visual Analog Scales (VAS) of mood, anxiety, and craving, comprising the items "happy," "drowsy," "depressed," "anxious," "energetic," "irritable," and "craving a cigarette" rated on a 100-mm scale from "not at all" to "extremely."

Procedures

On test days, participants were randomized to receive either a nicotinized (0.6 mg nicotine) or a denicotinized (0.005 mg nicotine) cigarette. Cigarettes were administered between subjects, as previous studies in our laboratory have demonstrated that the use of a within-subjects design can lead to an unblinding of the cigarette condition among participants. Cigarettes were commercially available reduced-nicotine Quest cigarettes (Vector Tobacco Ltd., Durham, NC) that had been labeled independently by a third party so that the procedure was conducted double blind.

Baseline ratings included self-report measures of mood and craving (FTND, STAI state anxiety, VAS). Participants were then given their cigarettes to smoke and completed the ratings of facial attractiveness. After smoking the entire cigarette, participants completed self-report measures of mood and craving (STAI state anxiety, VAS).

For the ratings of facial attractiveness, facial stimuli were presented in a blocked format (male and female), with the order of blocks randomized across participants. The testing was self-paced, and participants were required to rate each face for

attractiveness, using a 7-point Likert scale anchored at "very unattractive" and "very attractive." Responses were entered using numeric keys on the testing computer. The presentation of facial stimuli was controlled by the E-Prime (version 1.2) software package.

After completing the attractiveness rating task, participants completed an awareness check to test whether they were aware of whether their cigarettes contained nicotine. Participants were then briefed as to the purpose of the study.

Data analyses

Data were analyzed within a 2 × 2 × 2 mixed-model repeated measures analysis of variance (ANOVA) framework, with cigarette (nicotinized, denicotinized) and sex (male, female) as between-subjects factors. For the analysis of attractiveness rating data, target sex (male, female) was included as a within-subject factor, and for the analysis of questionnaire data, time (pre-cigarette and post-cigarette) was included as a within-subjects factor.

An alpha level of .05 was maintained throughout. All analyses were performed using SPSS version 12.0.

Results

Characteristics of participants

Four participants were excluded based on high exhaled CO readings, so that the final sample for analysis consisted of 44 participants (52% male). Participants were aged on average 23 years ($SD = 5$), began testing at 14:01 ($SD = 89$ min), smoked 33 cigarettes/week ($SD = 18$), had smoked for 7 years ($SD = 4$), and had an average FTND score of 0 ($SD = 0.5$), indicating that they were nondependent.

A series of 2 × 2 ANOVAs, with cigarette (nicotinized, denicotinized) and sex (male, female) as between-subjects factors, were conducted to assess the matching of participants in the four cells of the experimental design on a number of variables. We found no significant differences in cigarettes per week, years smoking, FTND score, or baseline STAI state anxiety ($p > .20$), but significant differences in age ($p = .012$) and start time ($p = .032$) were observed. We therefore included age and start time as supplementary covariates in subsequent analyses.

Questionnaire measures

A series of 2 × 2 × 2 mixed-model repeated measures ANOVAs, with cigarette (nicotinized, denicotinized) and sex (male, female) as between-subjects factors and time (pre-cigarette, post-cigarette) as a within-subjects factor, were used to examine STAI state anxiety and VAS data.

State anxiety

For STAI state anxiety data, we found marginal main effects of sex, $F(1, 40) = 2.88$, $p = .097$, and cigarette, $F(1, 40) = 3.05$, $p = .088$. No other main effects or interactions approached statistical significance ($p > .20$).

Visual Analog Scales

For VAS data, we found that there was a significant main effect of time for ratings of "irritable," $F(1, 40) = 10.39$, $p = .003$, reflecting a decrease from pre-cigarette to post-cigarette. In addition, we found significant time × cigarette, $F(1, 40) = 5.43$, $p = .025$, and time × sex, $F(1, 40) = 4.22$, $p = .047$, interactions, the former reflecting a greater decrease in the nicotinized compared with the denicotinized condition and among females compared with males on ratings of "irritable." We also found a significant main effect of time for ratings of "craving a cigarette," $F(1, 40) = 24.57$, $p < .001$, reflecting a decrease from pre-cigarette to post-cigarette, but this was not qualified by a significant time × cigarette interaction ($p = .47$). No other main effects or interactions were significant ($p > .05$). In particular, no time × cigarette interactions, which would reflect a differential effect of cigarette over time, were significant ($p > .17$).

Attractiveness rating task

A 2 × 2 × 2 mixed-model repeated measures ANOVA, with cigarette (nicotinized, denicotinized) and sex (male, female) as between-subjects factors and target sex (male, female) as a within-subjects factor, was used to examine attractiveness rating data. We found a significant main effect of cigarette, $F(1, 40) = 5.37$, $p = .026$, reflecting higher attractiveness ratings in the nicotinized condition compared with the denicotinized condition, and a significant main effect of target sex, $F(1, 40) = 12.23$, $p < .001$, reflecting higher attractiveness ratings for female faces compared with male faces, as typically observed in studies of facial attractiveness (Bashour, 2006).

No other main effects or interactions were significant ($p > .12$). Removing three participants of non-European ancestry did not alter these results nor did including as covariates age, start time, or VAS ratings of "irritable" and "craving a cigarette" post-cigarette. In all cases, the main effect of cigarette remained significant ($p = .018$–$.047$).

Awareness check

At the end of testing, participants were asked whether they thought their cigarettes contained nicotine. Participants were accurate in 57% of cases, which was not significantly different from chance, $t(43) = 0.90$, $p = .37$.

Discussion

Our data indicate that, among nondependent smokers who have not smoked recently, nicotine increases ratings of attractiveness of facial cues. This finding is consistent with current theories of nicotine addiction, which suggest that part of the addiction potential of nicotine stems from its ability to noncontingently enhance the positively reinforcing properties of other stimuli. We did not observe any evidence that these effects differed between males and females. Also, we did not observe any effects of nicotine on subjective ratings of mood, indicating that the effects we observed on ratings of attractiveness may not have simply been a consequence of global hedonic effects, or a positivity bias in questionnaire responding, in the nicotine condition. We did observe effects on ratings of "irritable," reflecting a greater decrease in the nicotinized compared with the denicotinized condition, but when measure was included as a covariate in the analyses of ratings of attractiveness, our results were not altered substantially. Moreover, although we observed a decrease of ratings of "craving a cigarette" over time, this did not differ between cigarette conditions, suggesting that it was not a result of withdrawal relief.

These data are important because they complement and extend evidence from the animal literature that suggests that nicotine exerts both direct and indirect effects that may act synergistically to produce nicotine dependence with repeated exposure. In particular, in addition to increasing the incentive salience of cues explicitly paired contingently with nicotine presentation, it may also potentiate the impact of moderately pleasurable or reinforcing cues to which the individual is exposed when nicotine is noncontingently present. In particular, the prolonged hypersensitivity to reward that persists after acute nicotine intake has terminated may serve as a powerful interoceptive discriminative stimulus (Donny et al., 2003; Kenny & Markou, 2006) and may contribute to relapse even after long periods of abstinence.

Several limitations of the present study should be considered when interpreting these results. First, we did not include a set of control stimuli to ascertain whether the observed effects of nicotine were specific to facial cues or were more general. Although the general lack of effects on self-reported mood ratings suggests that our results do not simply arise from global hedonic effects or reporting biases, future studies should include a range of categories of stimuli as control conditions. In particular, careful delineation of stimuli that might be considered to be broadly positively reinforcing or to encourage approach behavior (such as facial cues) and those that might not (such as household objects) would be valuable. Investigation of the effects of nicotine on facial cues projecting positive and negative emotional expressions also would be informative.

Second, we restricted our recruitment to light, nondependent smokers to maximize the acute effects of nicotine and model their impact in the development of dependence. We further required participants to be nicotine free to maximize the effects of nicotine administration. Although we expected negligible withdrawal effects in nondependent smokers, the effects of nicotine administration on ratings of "irritability" over time suggest that this was not entirely the case. Future studies should include dependent smokers, both satiated and in withdrawal, to explore whether the effects we report here are likely to be of greatest importance in the development of dependence or are a consequence of withdrawal relief. In addition, the use of more detailed and sensitive measures of withdrawal symptoms and positive and negative affect should be included in future studies. It also might be valuable to test differing doses of nicotine, across both nondependent and dependent smokers.

Third, our sample size was relatively small due to practical constraints on data collection and may have lacked power to detect sex differences in the effects we observed. Although our current data suggest that any sex differences are likely to be negligible, these results should be regarded as preliminary and require replication (in larger samples) and extension (by the inclusion, e.g., of a control condition).

Fourth, we did not control for familiarity effects (whereby study participants may have known the photographic subjects) or the ancestry of participants and target faces. However, all photographic subjects were no longer at the University of Bristol, so we consider it unlikely that study participants, who were primarily current undergraduate students, would have been familiar with them, and removing participants of non-European ancestry did not alter our results.

In conclusion, our data suggest that nicotine increases ratings of attractiveness of facial cues, consistent with evidence that nicotine potentiates the reinforcing properties of rewarding stimuli. These effects do not appear to be due to global hedonic effects or reporting bias following nicotine administration, although further research, including the use of more sensitive measures of withdrawal symptoms and positive and negative affect, as well as appropriate control conditions, is needed to exclude this possibility. If these findings prove robust, they provide support in humans for evidence of the effects of nicotine on brain reward systems that has, to date, been drawn largely from research using animal models.

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Declaration of Interests

None declared.

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Ventral Striatal Dopamine Release in Response to Smoking a Regular *vs* a Denicotinized Cigarette

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Prior studies have demonstrated that both nicotine administration and cigarette smoking lead to dopamine (DA) release in the ventral striatum/nucleus accumbens. In tobacco-dependent individuals, smoking denicotinized cigarettes leads to reduced craving, but less pleasure, than smoking regular cigarettes. Using denicotinized cigarettes and ^{11}C-raclopride positron emission tomography (PET) scanning, we sought to determine if nicotine is necessary for smoking-induced DA release. Sixty-two tobacco-dependent smokers underwent ^{11}C-raclopride PET scanning, during which they smoked either a regular or denicotinized cigarette (double-blind). Change in ^{11}C-raclopride binding potential (BP) in the ventral striatum from before to after smoking was determined as an indirect measure of DA release. Cigarette craving, anxiety, and mood were monitored during scanning. Smoking a regular cigarette resulted in a significantly greater mean reduction in ventral striatal ^{11}C-raclopride BP than smoking a denicotinized cigarette. Although both groups had reductions in craving and anxiety with smoking, the regular cigarette group had a greater improvement in mood. For the total group, change in BP correlated inversely with change in mood, indicating that greater smoking-induced DA release was associated with more smoking-related mood improvement. Thus, nicotine delivered through cigarette smoking appears to be important for ventral striatal DA release. Study findings also suggest that mood improvement from smoking is specifically related to ventral striatal DA release.
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Keywords: dopamine; nicotine; tobacco; ventral striatum; positron emission tomography; ^{11}C-raclopride

INTRODUCTION

Many research groups have demonstrated that nicotine administration or cigarette smoking results in dopamine (DA) release in the ventral striatum/nucleus accumbens (VST/NAc). In studies of rodents, nicotine-induced DA release in the VST/NAc was shown using microdialysis (Di Chiara and Imperato, 1988; Damsma *et al*, 1989; Pontieri *et al*, 1996; Sziraki *et al*, 2001), whereas nicotine-induced DA release was disrupted by lesioning of dopaminergic neurons prior to nicotine administration (Corrigall *et al*, 1992). In non-human primates, DA release in response to intravenous nicotine administration has been demonstrated (indirectly) by displacement of ^{11}C-raclopride (a ligand that binds to D2/D3 DA receptors) during positron emission tomography (PET) scanning (Dewey *et al*, 1999; Marenco *et al*, 2004). Similarly, studies of human smokers have demonstrated DA release in response to cigarette smoking (Brody *et al*, 2004, 2006; Scott *et al*, 2007) and nicotine gum administration (Takahashi *et al*, 2008) using ^{11}C-raclopride and PET, though not all studies using these (and similar) methods have shown ^{11}C-raclopride displacement (Tsukada *et al*, 2002; Barrett *et al*, 2004; Montgomery *et al*, 2007). Both nicotine administration (in doses comparable to human cigarette smoking) (Cumming *et al*, 2003; Marenco *et al*, 2004; Takahashi *et al*, 2008) and cigarette smoking itself (Brody *et al*, 2004, 2006; Scott *et al*, 2007) result in DA release in the range of 5–10%, indicating that nicotine intake is an important factor in DA release from smoking. Significant associations between DA release (as measured by ^{11}C-raclopride displacement) and behavioral responses to smoking, such as reduction in craving (Brody *et al*, 2004, 2006) and enhancement of pleasure (Barrett *et al*, 2004; Montgomery *et al*, 2007), have also been demonstrated in human smokers.

In prior research, smoking denicotinized cigarettes has been compared to smoking regular cigarettes, to examine the effect of the presence or absence of nicotine on tobacco-related symptoms. In tobacco-dependent smokers, smoking denicotinized cigarettes has been found to result in a degree

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of withdrawal relief that is similar to smoking regular cigarettes (Pickworth *et al*, 1999; Buchhalter *et al*, 2001; Dallery *et al*, 2003; Donny *et al*, 2007); however, denicotinized cigarettes provide less satisfaction (Butschky *et al*, 1995; Gross *et al*, 1997), reward (Naqvi and Bechara, 2005; Donny *et al*, 2007), and anxiety reduction (Pomerleau *et al*, 1984; Buckley *et al*, 2007) than regular cigarettes. Denicotinized cigarettes also do not elevate heart rate like regular cigarettes (Butschky *et al*, 1995; Pickworth *et al*, 1999; Schuh *et al*, 2001; Buchhalter *et al*, 2001), but both cigarette types increase skin conductance responses (Naqvi and Bechara, 2006). Furthermore, a recent study directly demonstrated that the airway sensation from nicotinized cigarette puffs were more rewarding than such sensations from denicotinized cigarette puffs (though both cigarette types produced greater reward than unlit puffs) (Naqvi and Bechara, 2005). Taken together, these studies indicate that sensory factors associated with smoking are important for relief of withdrawal, but that nicotine may be necessary for the rewarding properties of smoking.

Using denicotinized (and regular) cigarettes and ^{11}C-raclopride PET scanning, the primary goal of the study presented here was to determine if nicotine is necessary for smoking-induced DA release. Given the extensive scientific literature linking reward/pleasure with DA release (Koob, 1992; Leshner and Koob, 1999) and the aforementioned studies of denicotinized cigarettes indicating less of a rewarding effect from these cigarettes, we hypothesized that smoking a denicotinized cigarette during scanning would result in less DA release than smoking a regular cigarette. Given that prior studies demonstrate associations between DA release and hedonic responses and craving reduction with smoking, and that smoking reduces anxiety in the presence of a stressor (Jarvik *et al*, 1989) and when a distractor is present (Kassel and Shiffman, 1997), we also sought to examine associations between DA release and change in these symptoms with smoking.

METHODS

Research Participants

Two hundred and forty-three potential research participants were screened during a telephone interview, in which medical, psychiatric, and substance abuse histories were obtained without personal identifiers. Qualified participants who wished to participate received a complete description of the study in person and gave written informed consent, using forms approved by the local IRB. Participants were then evaluated further using screening questions from the Structured Clinical Interview for DSM-IV to verify eligibility (First *et al*, 1995). Inclusion/exclusion criteria were the same as in our previous reports (Brody *et al*, 2004, 2006), with the central inclusion criteria being DSM-IV nicotine dependence and smoking 10–40 cigarettes per day and exclusion criteria being any history of an Axis I psychiatric or substance abuse/dependence diagnosis other than nicotine dependence, current use of medications or medical conditions that might affect brain function, and pregnancy. Participants were all treatment seeking and were either enrolled in a larger smoking cessation treatment study or offered open-label treatment following study participation.

Experimental Design

Sixty-two otherwise healthy adult (21–65 years of age) cigarette smokers completed the following study. Participants underwent bolus-plus-continuous-infusion ^{11}C-raclopride PET scanning, during which they smoked either a regular or denicotinized cigarette (double-blind). Demographic and other clinical variables were collected at baseline, and symptoms of tobacco withdrawal were monitored during the scanning session. A structural magnetic resonance imaging (MRI) scan was also obtained within 1 week of the PET session.

Demographic and Rating Scale Measures

Demographic and rating scale measures were obtained immediately prior to PET scanning. Demographic data included age, sex, number of cigarettes per day, number of years smoking, and highest level of education. Rating scale data included the Fagerström Test for Nicotine Dependence (FTND) (Fagerström, 1978; Heatherton *et al*, 1991), Hamilton Depression (HAM-D) (Hamilton, 1967) and Anxiety (HAM-A) (Hamilton, 1969) rating scales, and the Shiffman–Jarvik Withdrawal Scale (Shiffman and Jarvik, 1976). Exhaled carbon monoxide (CO) levels were also obtained prior to PET scanning using a MicroSmokerlyzer (Bedfont Scientific Ltd, Kent, UK) as an estimate of recent smoking levels.

During the PET session, the Urge to Smoke (UTS) Scale for measuring craving (Jarvik *et al*, 2000), state anxiety questions from the Spielberger State-Trait Anxiety Index (Spielberger, 1983), and an analog scale for mood rating (from 'sad' to 'happy') (Wild *et al*, 2001; Bowen *et al*, 2006; Mitterschiffthaler *et al*, 2007) were administered immediately before and after smoking.

PET and MRI Scanning

Positron emission tomography and MRI scanning sessions followed the same general protocol as in our previous reports (Brody *et al*, 2004, 2006). PET sessions were performed using a bolus plus continuous infusion of ^{11}C-raclopride. Participants were instructed to smoke as per their usual habit on the morning of the PET session and to smoke a cigarette immediately prior to testing, which began at noon. Between noon and 1345 hours, participants were interviewed and filled out rating scale questionnaires as described above. At 1345 hours, each participant had a 20-gauge intravenous catheter placed and then was positioned on the PET scanning bed (for acquisition of planes parallel to the orbitomeatal line). Scanning began at 1410 hours with a slow bolus injection of 5 mCi ^{11}C-raclopride in 20 ml normal saline over a 60-s period, followed by continuous infusion of the tracer (5 mCi, corrected for decay) for the remainder of the testing session. This bolus-plus-continuous-infusion method was performed as described in prior studies (Carson *et al*, 1997; Ito *et al*, 1998; Brody *et al*, 2006). Brain scans were acquired continuously for the next 50 min.

At 1500 hours, participants were removed from the scanner with the infusion still running and had a 10-min break in an outdoor area adjacent to the PET scanning room, at which time they smoked either a regular or

denicotinized cigarette (described below). A 3-h period of abstinence prior to the smoke break in scanning was chosen based on the finding that craving starts to peak at this time in smokers (Schuh and Stitzer, 1995), though it is acknowledged that DA release may be different if smokers are cigarette-deprived for longer periods of time. After the break, participants were quickly repositioned (<1 min) in the scanner using a preplaced mark on their forehead and a laser light from the scanner. Scanning then resumed for 30 min more.

Positron emission tomography scans were acquired with a GE Advance NXi PET tomograph (General Electronic Medical Systems, Milwaukee, WI) with 35 slices in 3-dimensional mode. ^{11}C-raclopride was prepared by an established procedure (Farde *et al*, 1986; Ehrin *et al*, 1987). PET scans were acquired as 10 prebreak and six postbreak 5-min frames. Attenuation correction scanning for both emission scans was performed using a 5-min transmission scan obtained with the germanium rotating rod source built into the GE scanner at the end of the scanning session.

An MRI scan of the brain was obtained within 1 week of the PET session with the following specifications: three-dimensional Fourier transform spoiled gradient-recalled acquisition with TR = 30 ms, TE = 7 ms, 30° angle, two acquisitions, 256 × 192 view matrix. The acquired volume was reconstructed as 90 contiguous, 1.5-mm thick, transaxial slices.

Cigarette Smoking during the Break in Scanning

During the break in scanning, subjects smoked either one of their own cigarettes (mean 1.0 ± 0.03 mg nicotine, 14.2 ± 0.5 mg tar; $n = 46$) or a standard denicotinized cigarette (0.05 mg nicotine, 10 mg tar; Quest 3 brand; $n = 16$). Three-fourths of the participants were assigned to the regular cigarette group because this study was part of a larger treatment study that examined responses to regular cigarettes before and after treatment, and only those who smoked a regular cigarette were eligible for the larger study. To maintain a blind, all subjects gave one of their cigarettes to a research assistant who de-identified it by covering any brand markings. This de-identified regular brand cigarette or a de-identified denicotinized cigarette was then given to the study investigator (ALB, REO, or ZAM) who gave it to the participant to smoke during the break in scanning. Participants were observed continuously during the break. For the regular cigarette condition, subjects smoked one of the following cigarettes: Marlboro Regular ($n = 9$), Marlboro 100 ($n = 2$), Marlboro Light ($n = 1$), Camel Regular ($n = 6$), Camel Light ($n = 3$), Kool Regular ($n = 3$), Newport Regular ($n = 6$), Newport 100 ($n = 5$), Parliament Light ($n = 9$), or Benson and Hedges Regular ($n = 2$). Eleven of the cigarettes smoked for the regular cigarette condition were menthol cigarettes, whereas the denicotinized cigarettes were not menthol cigarettes.

To monitor smoking topography during scanning, cigarettes were smoked through a Clinical Research Support System device (Plowshare, Baltimore, MD). Although all standard recordings were obtained with this device, the primary measure of interest was total puff volume of the cigarette (ml), which indicated the amount of each cigarette that was smoked.

PET Image Analysis

PET-to-MRI coregistration was performed using an automated image registration method (Woods *et al*, 1993) within MEDx 3.3 (Sensor Systems Inc., Sterling, VA). Prebreak (0–50 min) and postbreak (60–90 min) scans were coregistered to the MRI separately, and the individual 5-min time frames were coregistered to MRI scans using the transformation matrix parameters from the coregistration of the summed images.

Regions of interest (ROIs) were drawn on MRI and transferred onto the coregistered PET scan frames. ROI placement on each frame was verified by visual inspection by the region drawers (MRC and DS) and the PI. The ROI value used for statistical analysis was the volume-weighted decay-corrected mean of the left and right ventral caudate (VCD) ROIs (including NAc), consisting of three slices each, resulting in a single value for each 5-min frame of PET scanning (Brody *et al*, 2006). The cerebellum was drawn as a reference region. These regions were similar to those drawn in prior ^{11}C-raclopride PET studies (Mawlawi *et al*, 2001; Martinez *et al*, 2003). Binding potential (BP) for VCD/NAc was calculated using the simplified reference tissue model (Lammertsma and Hume, 1996), with $BP = B'_{max}/K_d = C_{VCD/NAc}/C_{cerebellum} - 1$, where C is radioligand concentration. BP was determined for each 5-min PET frame, and mean BP values were compared between the frames before (40–50 min) and after (60–90 min) the break in scanning. These time frames are almost identical to those recommended as optimal for maximizing signal-to-noise ratio with the ^{11}C-raclopride bolus-plus-continuous-infusion method (Watabe *et al*, 2000; Mawlawi *et al*, 2001). This method assumes 40 min for the radiotracer to reach an approximate steady state (Ito *et al*, 1998; Watabe *et al*, 2000; Mawlawi *et al*, 2001). It also assumes that intrasynaptic DA concentration will remain elevated throughout the 30-min postsmoking scan, as indicated by animal microdialysis and imaging studies cited above.

The single PET data variable for this study was percent change in BP from before to after smoking, defined as $100 \times (BP_{after\ smoking} - BP_{before\ smoking})/BP_{before\ smoking}$. A single BP measure was used here, based on prior reports demonstrating nearly identical BP changes for the left and right VCD/NAc (Brody *et al*, 2004, 2006), to minimize the risk of type I error.

Statistical Analyses

Demographic and rating scale data means (± SEMs) were determined for the study groups. To verify baseline similarities in demographics between groups, unpaired Student's t-tests and a χ^2 test (for gender) were performed for these variables. To determine similarities and differences in clinical responses to smoking the two cigarette types, unpaired Student's t-tests were performed between groups for changes in the three withdrawal symptom measures (craving/UTS, anxiety/Spielberger State-Trait Anxiety Index (STAI), and mood analog scale scores) from before to after smoking. To further characterize pre- to postsmoking changes in withdrawal symptoms, within-group paired t-tests and examinations of means were also performed.

For the PET data, changes in BP were compared between groups with an ANCOVA, with percent change in BP as the dependent measure, group (regular *vs* denicotinized cigarette) as the between-subject factor, and age, gender, and total puff volume as covariates. Even though the groups did not have significant differences in these covariates (see below), these variables were included in the analysis, based on prior reports linking them with differences in DA system function (Wang *et al*, 1995; Pohjalainen *et al*, 1998; Kaasinen *et al*, 2000; Staley *et al*, 2001).

For the total group, correlation coefficients (controlling for group) were determined between percent change in BP and percent change in craving (UTS), state anxiety (STAI), and mood analog scores, to determine relationships between DA concentration change and effects of smoking on withdrawal symptoms. All statistical tests were performed with SPSS version 15.0 (SPSS, Chicago, IL).

RESULTS

Clinical Results

The total subject sample was middle-aged (40.5 ± 1.5 years old), mostly male (67.7%), smoked moderately (24.2 ± 0.9 cigarettes/day) and for many years (22.5 ± 1.5), was moderately nicotine-dependent (FTND scores—6.4 ± 0.2), and had moderate CO levels prior to the scanning session (17.0 ± 1.1 p.p.m.). The groups assigned to smoking either a regular or a denicotinized cigarette did not differ significantly in any of these variables (Table 1) or on any of the subscales from the Shiffman–Jarvik Withdrawal scale (all unpaired Student's *t*-tests, NS).

The groups also did not differ in smoking topography. For the regular and denicotinized cigarette groups, total puff volume (886 ± 44 *vs* 941 ± 81 ml, respectively), number of puffs (17.3 ± 1.2 *vs* 19.3 ± 1.8, respectively), and average puff volume (57.3 ± 2.7 *vs* 52.6 ± 4.5 ml) were similar between groups (all unpaired Student's *t*-tests, NS).

From before to after smoking during scanning, the two groups did not differ significantly in craving or anxiety alleviation (both unpaired Student's *t*-tests, NS), but did differ in mood improvement. For craving, both groups had significant within-group reductions in craving/UTS score (−76.0 (± 3.5)% for the regular cigarette group, paired Student's *t*-test, $p < 0.0001$ and −71.8 (± 6.9)% for the denicotinized cigarette group, paired Student's *t*-test, $p < 0.0001$), indicating that craving was reduced significantly with both cigarette types. Similarly, both groups had comparable reductions in STAI anxiety scale scores (−26.1 (± 19.5)% for the regular cigarette group and −21.7 (± 16.3)% for the denicotinized cigarette group). In contrast, the regular cigarette group had significantly more mood improvement (+15.2 (± 6.0)%) than the denicotinized cigarette group (−0.5 (± 4.4)%) (unpaired Student's *t*-test, $p = 0.04$).

PET Results

The group that smoked regular nicotine-containing cigarettes had a greater mean reduction in ^{11}C-raclopride BP (−8.4 (± 1.5)%) than the group that smoked denicotinized cigarettes (−1.2 (± 2.5)%) (ANCOVA, d.f. = 1, 57, F = 6.8, $p = 0.01$) (Figure 1), indicating that those who smoked regular cigarettes had greater DA release.

For the total group, there was a significant negative correlation between change in ^{11}C-raclopride BP and change in mood scale scores (correlation coefficient, $r = -0.33$, $p = 0.01$) (Figure 2), indicating that greater DA release is associated with more mood improvement. There were no significant correlations between change in ^{11}C-raclopride BP and change in UTS scale scores (correlation coefficient, $r = 0.13$, NS) or STAI anxiety scale score (correlation coefficient, $r = -0.01$, NS).

Table 1 Demographic and Rating Scale Variables

Variable	Total group ($n = 62$)	Regular nicotine cigarette ($n = 46$)	De-nicotinized cigarette ($n = 16$)	p-value (Nic *vs* De-Nic groups)
Age	40.5 (± 1.5)	41.6 (± 1.7)	37.3 (± 3.4)	NS
Percent female	32.3%	34.8%	25.0%	NS
Cigs/day	24.2 (± 0.9)	24.9 (± 1.0)	22.3 (± 1.6)	NS
Years of smoking	22.5 (± 1.5)	23.5 (± 1.7)	19.8 (± 3.3)	NS
Education (yrs)	13.8 (± 0.2)	13.8 (± 0.3)	13.9 (± 0.3)	NS
FTND	6.4 (± 0.2)	6.4 (± 0.3)	6.4 (± 0.4)	NS
HAM-D	1.8 (± 0.3)	1.5 (± 0.3)	2.4 (± 0.6)	NS
HAM-A	2.0 (± 0.3)	1.7 (± 0.3)	2.6 (± 0.8)	NS
CO level (p.p.m.)	17.0 (± 1.1)	17.5 (± 1.5)	15.3 (± 1.7)	NS
% Change in UTS (craving) with smoking	−74.9 (± 3.1)	−76.0 (± 3.5)	−71.8 (± 6.9)	NS
% Change in STAI with smoking	−25.0 (± 15.0)	−26.1 (± 19.5)	−21.7 (± 16.3)	NS
% Change in Mood with smoking	7.6 (± 5.4)	15.2 (± 6.0)	−0.5 (± 4.4)	0.04

Values are stated as mean (± SEM); *p*-values are for unpaired Student's *t*-tests between the groups that smoked either a regular nicotine or a denicotinized cigarette. Cig, cigarette; CO, exhaled carbon monoxide; De-Nic, denicotinized; FTND, Fagerström Test for Nicotine Dependence; HAM-A, Hamilton Anxiety Rating Scale; HAM-D, Hamilton Depression Rating Scale; Mood, mood analog rating scale score; Nic, nicotine; NS, nonsignificant; p.p.m., parts per million; STAI, Spielberger State-Trait Anxiety Index state anxiety questions total score; UTS, Urge to Smoke Scale; yrs, years.

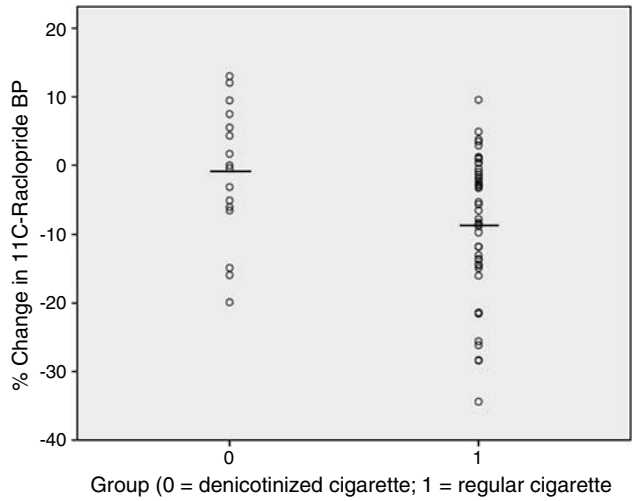


Figure 1 Scatterplot showing percent change in ^{11}C-raclopride binding potential (BP) for the group that smoked a denicotinized cigarette (−1.2 (±2.5)%; $n=16$) and the group that smoked a regular cigarette (−8.4 (±1.5)%; $n=46$). The group that smoked a denicotinized cigarette had less of a decrease in ^{11}C-raclopride BP than the group that smoked a regular cigarette, indicating that nicotine inhalation is important for dopamine release.

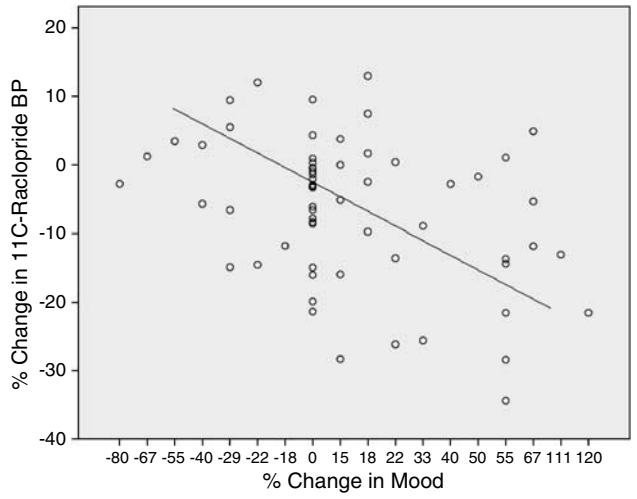


Figure 2 Scatterplot showing the negative correlation ($r=-0.33$, $p=0.01$) between percent change in ^{11}C-raclopride binding potential (BP) (an indirect measure of dopamine release) and percent change in mood analog rating scale score for the total group of tobacco-dependent smokers ($n=62$).

DISCUSSION

In tobacco-dependent smokers, smoking regular *vs* denicotinized cigarettes resulted in different patterns of both clinical and PET scanning findings. Smoking regular cigarettes resulted in significantly decreased craving and anxiety, improved mood, and DA release, whereas smoking denicotinized cigarettes resulted in decreased craving and anxiety, but no evidence of mood improvement or significant DA release. In addition, for the total group, there was an association between increasing DA release and mood improvement. These findings indicate that nicotine is necessary for smoking-induced DA release and support prior research demonstrating that denicotinized cigarettes relieve craving (Eid *et al*, 2005; Buchhalter *et al*, 2005), but that regular nicotine-containing cigarettes provide greater withdrawal relief (Pickworth *et al*, 1999; Buchhalter *et al*, 2001; Dallery *et al*, 2003; Donny *et al*, 2007) and more satisfaction (Butschky *et al*, 1995; Gross *et al*, 1997) and reward (Naqvi and Bechara, 2005; Donny *et al*, 2007) (measured here as mood improvement) than denicotinized cigarettes. The central finding of this study (smoking a regular cigarette resulting in greater displacement of ^{11}C-raclopride BP than smoking a denicotinized cigarette) is consistent with animal microdialysis (Imperato *et al*, 1986; Di Chiara and Imperato, 1988; Benwell and Balfour, 1997; Janhunen and Ahtee, 2004) and imaging (Marenco *et al*, 2004; Scott *et al*, 2007; Takahashi *et al*, 2008) studies, which demonstrate the importance of nicotine administration for DA release. As expected, the 8.4% increase in DA concentration found here with smoking a regular cigarette was less than the 20–29% increase found with cocaine (Volkow *et al*, 1999; Park and Park, 2000) or the 19–31% increase found with amphetamine (Carson *et al*, 1997; Hartvig *et al*, 1997; Drevets *et al*, 1999) administration in studies using similar methodology.

The finding of a reduction in craving with smoking a denicotinized cigarette also indicates that factors other than the rewarding effect of ventral striatal DA release are important for craving alleviation. Denicotinized cigarettes likely alleviate craving through either (1) the sensorimotor feel, taste, and smell of cigarettes (including some degree of airway sensory stimulation (Naqvi and Bechara, 2005)), (2) DA release in brain regions not studied here (such as the thalamus, hippocampus, and cerebral cortex (Christian *et al*, 2000; Slifstein *et al*, 2004)), (3) pharmacological effects of the thousands of constituents of tobacco smoke other than nicotine (Green and Rodgman, 1996), or (4) some combination of these factors. To clarify the issue of how denicotinized cigarettes exert their effect, studies of changes in brain activity (either blood flow or glucose metabolism) or other neurotransmitter systems in response to smoking these cigarettes would be informative. Though several groups have examined the smoking of denicotinized cigarettes as a control condition compared to smoking regular cigarettes during functional brain imaging (Rose *et al*, 2003, 2007; Zubieta *et al*, 2005), we are aware of only one study (cited below) that specifically examined the effects of smoking denicotinized cigarettes themselves on brain function.

Although (to our knowledge) no prior functional brain imaging studies have directly reported how denicotinized cigarettes alleviate craving, several functional brain imaging studies of cigarette craving itself (see Brody (2006) for review) point to brain circuitry that might mediate the diminished craving found in response to denicotinized cigarettes. When cigarette smokers are exposed to cigarette-related (compared to neutral) cues, craving is found to correlate with regional glucose metabolism or perfusion in the prefrontal cortex, orbitofrontal cortex, anterior cingulate cortex, insula, sensorimotor cortex, and hippocampus (Brody *et al*, 2002; Zubieta *et al*, 2005; Rose *et al*, 2007). Changes in activity in these regions, which mediate brain functions possibly related to craving (eg, working and

episodic memory, repetitive thoughts, arousal and sensorimotor function) could be responsible for the craving reduction seen with denicotinized cigarettes. The effects of smoking denicotinized cigarettes on craving may also be explained by their effect on neurotransmitter systems other than DA, as demonstrated in a recent study showing activation of mu-opioid receptor-mediated neurotransmission in the right anterior cingulate cortex from smoking denicotinized and regular nicotine cigarettes (Scott *et al*, 2007).

As for the other central finding of this study, the association between mood improvement and smoking-induced DA release is consistent with a large body of literature linking DA release with improved mood and pleasure. Most closely related to the present study are reports using ^{11}C-raclopride PET that have linked DA release in the striatum with hedonic responses to cigarette smoking (Barrett *et al*, 2004) and with happiness following nicotine nasal spray administration (Montgomery *et al*, 2007). Similar relationships between DA release and mood improvement or reward have been demonstrated in response to amphetamine administration (Drevets *et al*, 2001; Oswald *et al*, 2005), monetary reward (Pappata *et al*, 2002; Zald *et al*, 2004), and eating a meal (Small *et al*, 2003). In addition, DA concentration has been found to decrease in subjects who experience decreased happiness/euphoria in response to catecholamine depletion (Verhoeff *et al*, 2003). The present findings reinforce the previously demonstrated relationship between DA concentration and mood, and point to the consistency of these findings across rewarding stimuli.

The strengths of the present study included a relatively large sample size for this line of research; well-matched groups with regard to demographic variables, cigarette usage, and absence of comorbidity; and a statistical method that minimized type I error by using a single BP value. The primary limitations of this study were the ones inherent to PET scanning with ^{11}C-raclopride. Although this radiotracer is commonly used for the evaluation of DA release (Volkow *et al*, 1994; Carson *et al*, 1997; Schlaepfer *et al*, 1997; Ginovart *et al*, 2002; Brody *et al*, 2004, 2006; Marenco *et al*, 2004; Barrett *et al*, 2004; Scott *et al*, 2007) and generally demonstrates DA release when expected, the short half-life and variable pharmacokinetics of the radiotracer make its use logistically challenging for human studies, and can produce considerable variability within groups, as observed here (Figure 1). ^{11}C-raclopride is also not a reliable radiotracer for examining competitive displacement in regions with low specific binding outside the striatum. For measuring non-striatal DA receptor levels, a radiotracer with very high affinity (such as ^{18}F-fallypride) might be more appropriate (for reviews see Siessmeier *et al* (2005); Elsinga *et al* (2006)). Another study limitation was the unequal sample size between the two groups (done for practical reasons), which resulted in greater power to detect change in the larger regular cigarette group compared to the smaller denicotinized cigarette group. Also, due to the fact that de-identified cigarettes of the subjects' own brand were used in this study, it is possible that varying cigarette composition (eg, regular cigarettes smoked here contained 14.2 ± 0.5 mg tar, whereas Quest cigarettes contained 10 mg tar) or the fact that some subjects may have recognized their own brand of cigarettes could have affected study results. Despite these limitations, this study did demonstrate that smoking regular nicotine cigarettes resulted in VCD/NAc BP change in the hypothesized direction, and this fact does lend confidence to the findings here, as does the expected significant correlation for the total group between VCD/NAc BP reduction and mood improvement.

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CONFLICT OF INTEREST

The author(s) declare that, except for income received from my primary employer, no financial support or compensation has been received from any individual or corporate entity over the past 3 years for research or professional service, and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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ARTICLE

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Brain nicotinic acetylcholine receptor occupancy: effect of smoking a denicotinized cigarette

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Abstract

Our group recently reported that smoking a regular cigarette (1.2–1.4 mg nicotine) resulted in 88% occupancy of brain $\alpha_4\beta_2$* nicotinic acetylcholine receptors (nAChRs). However, this study did not determine whether nicotine inhalation or the many other pharmacological and behavioural factors that occur during smoking resulted in this receptor occupancy. If nicotine is solely responsible for $\alpha_4\beta_2$* nAChR occupancy from smoking, then (as estimated from our previous data) smoking a denicotinized (0.05 mg nicotine) or a low-nicotine (0.6 mg nicotine) cigarette (commonly used for research and clinical purposes) would result in substantial 23% and 78% $\alpha_4\beta_2$* nAChR occupancies, respectively, and a plasma nicotine concentration of 0.87 ng/ml would result in 50% $\alpha_4\beta_2$* nAChR occupancy (EC_{50}). Twenty-four positron emission tomography sessions were performed on tobacco-dependent smokers, using 2-[F-18]fluoro-A-85380 (2-FA), a radiotracer that binds to $\alpha_4\beta_2$* nAChRs. 2-FA displacement was determined from before to 3.1 hours after either: no smoking, smoking a denicotinized cigarette, or smoking a low-nicotine cigarette. Analysis of this PET data revealed that smoking a denicotinized and a low-nicotine cigarette resulted in 26% and 79% $\alpha_4\beta_2$* nAChR occupancies, respectively, across three regions of interest. The EC_{50} determined from this dataset was 0.75 ng/ml. Given the consistency of findings between our previous study with regular cigarettes and the present study, nicotine inhalation during smoking appears to be solely responsible for $\alpha_4\beta_2$* nAChR occupancy, with other factors (if present at all) having either short-lived or very minor effects. Furthermore, smoking a denicotinized cigarette resulted in substantial nAChR occupancy.

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Key words: Denicotinized cigarette, nicotine, nicotinic acetylcholine receptors, positron emission tomography, thalamus, tobacco.

Introduction

Nicotine is the constituent of cigarette smoke that is most closely linked to tobacco dependence (Henningfield and Fant, 1999). The binding of nicotine to nicotinic acetylcholine receptors (nAChRs) is the presumed pharmacological event mediating the feeling of reward (Leshner and Koob, 1999), reduced irritability and anxiety (West and Shiffman, 2001), heightened attentional performance (Newhouse et al., 2004), and improved reaction times (Ernst et al., 2001) that occur when a smoker smokes a cigarette. However, cigarette smoking is a behaviour that involves much more than the delivery of nicotine. Cigarette smoking includes the inhalation of thousands of constituents of tobacco smoke other than nicotine (Green and Rodgman, 1996) and results in a wide range of

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physical sensations (such as the taste, smell, and feel of a cigarette). Thus, there are many aspects of cigarette smoking other than nicotine inhalation that could affect nAChR occupancy either directly or through the release of endogenous acetylcholine. The central goal of the present study was to determine if factors associated with smoking other than nicotine inhalation result in nAChR occupancy.

Recently, denicotinized cigarettes (0.05–0.1 mg nicotine yield) have come into wider use for research and clinical purposes. For research, denicotinized cigarettes have been compared to regular cigarettes to differentiate the effects of nicotine inhalation from all other aspects of cigarette smoking. Smoking denicotinized cigarettes has been shown to relieve withdrawal to a similar degree as smoking regular cigarettes (Buchhalter et al., 2001; Dallery et al., 2003; Donny et al., 2007; Pickworth et al., 1999); however, denicotinized cigarettes provide less satisfaction (Butschky et al., 1995; Gross et al., 1997) and reward (Donny et al., 2007; Naqvi and Bechara, 2005) than regular cigarettes, and do not produce all of the physical manifestations of regular cigarettes (Buchhalter et al., 2001; Pickworth et al., 1999; Schuh et al., 2001). For clinical use, denicotinized cigarettes have been found to reduce craving in smokers attempting to quit (Rezaishiraz et al., 2007), and are currently being tested as an adjunct to standard smoking cessation treatments (Donny et al., 2007). Because of the expanding role of denicotinized cigarettes in both research studies and clinical treatment, it is important to understand the effects of these cigarettes on brain nAChR occupancy.

Our group recently examined the effects of smoking varying amounts of regular nicotine-containing cigarettes (1.2–1.4 mg nicotine) on brain $\alpha_4\beta_2$* nAChR occupancy. For that study, we used the radiotracer 2-[^{18}F]fluoro-3-(2(*S*)azetidinylmethoxy)pyridine (2-F-A-85380, abbreviated as 2-FA), which was developed recently for the in-vivo imaging of $\alpha_4\beta_2$* nAChRs with positron emission tomography (PET) (Chefer et al., 2003; Gallezot et al., 2005; Koren et al., 1998). 2-FA was injected and infused during PET scanning, and the extent of its displacement from smoking was determined. We found that the effective dose of a cigarette needed to occupy 50% of available $\alpha_4\beta_2$* nAChRs (ED_{50}) at 3.1 h after smoking was 13% and that the effective venous plasma nicotine concentration needed to occupy 50% of these nAChRs was 0.87 ng/ml [much less than the 10–50 ng/ml achieved during the day in typical daily smokers (Benowitz et al., 1990)]. Because subjects in this previous study smoked different amounts of regular nicotine-containing cigarettes, the study did not rule out the possibility that smoking-related factors other than nicotine might result in receptor occupancy. If such factors are responsible for nAChR occupancy, our previous study would have overestimated the effect of nicotine on nAChR occupancy and underestimated the value for EC_{50}.

To address this issue in the study presented here, we determined the percentage occupancy of $\alpha_4\beta_2$* nAChRs from smoking a denicotinized (0.05 mg nicotine yield) and a low-nicotine (0.6 mg yield) cigarette. Based on our previous determination of ED_{50} using regular cigarettes and the equation

$$\text{receptor occupancy} = \text{dose}/(\text{dose} + \text{ED}_{50}),$$

we predicted that smoking a denicotinized cigarette would result in a substantial 22–24% occupancy of $\alpha_4\beta_2$* nAChRs and smoking a low-nicotine cigarette would result in 77–79% occupancy of $\alpha_4\beta_2$* nAChRs, if nicotine inhalation from cigarette smoking is solely responsible for this occupancy. We also hypothesized that the EC_{50} would be the same for smoking low-nicotine cigarettes as it was for smoking regular cigarettes (i.e. 0.87 ng/ml), if nicotine inhalation alone is responsible for nAChR occupancy.

Method

General study design

Tobacco-dependent cigarette smokers underwent the following sequence of procedures (described in detail below): (1) screening over the telephone and in person, (2) two bolus + continuous infusion 2-FA PET sessions (at least 1 wk apart), with either no smoking, smoking of a denicotinized cigarette (0.05 mg nicotine), or smoking of a low-nicotine cigarette (0.6 mg nicotine) during a break in the middle of scanning, and (3) a structural magnetic resonance imaging (MRI) scan of the brain within 1 wk of PET scanning to aid in localization of regions on the PET scans.

Subject screening

Fifteen otherwise healthy tobacco-dependent smokers ($\geqslant$15 cigarettes/d) were recruited through newspaper advertisements and the internet. Initial screening consisted of an anonymous telephone interview in which smoking, medical, psychiatric, and substance use histories were obtained. Qualified subjects who wished to participate were then assessed in person using screening questions from the Structured Clinical Interview for DSM-IV (SCID; First et al., 1995) 2 d prior to PET scanning. The central inclusion criterion was the DSM-IV diagnosis of nicotine dependence,

while any history of an Axis I diagnosis other than nicotine dependence was exclusionary. Other exclusion criteria were pregnancy, the use of medications or history of a medical condition that might affect the central nervous system during scanning, and the use of more than the caffeine equivalent of two cups of coffee per day. Women of childbearing potential had a urine pregnancy test, and all subjects had a urine toxicology screen to verify subject reports. After a complete description of the study to subjects, written informed consent was obtained using forms approved by the local institutional review board.

During the initial visit, additional screening data were obtained, including the Smoker's Profile Form (which includes smoking history), the Fagerström Test for Nicotine Dependence (FTND; Fagerström, 1978; Heatherton et al., 1991), the Hamilton Depression Rating Scale (17-item HAMD; Hamilton, 1967), the Hamilton Anxiety Rating Scale (HAMA; Hamilton, 1969), and the Shiffman–Jarvik Withdrawal Scale (SJWS; Shiffman and Jarvik, 1976). An exhaled carbon monoxide (CO) level was obtained using the Micro-Smokerlyzer (Bedfont Scientific Ltd, Kent, UK) to verify smoking status [subjects were considered to be active smokers if a CO $\geqslant$8 parts per million (ppm) was obtained].

Because we were primarily interested in the effects of smoking a denicotinized cigarette on nAChR occupancy and because dosimetry concerns limited us to PET scanning subjects only twice, subjects were alternately assigned to receive either a no-smoking and a denicotinized cigarette PET session ($n=7$) *or* a low-nicotine and a denicotinized cigarette PET session ($n=8$). We did not assign subjects to the third possibility of having a no-smoking and a low-nicotine cigarette PET session, because our previous results with low dosages of regular cigarettes strongly indicated that smoking a low-nicotine cigarette (0.6 mg nicotine) would result in significant occupancy compared to no smoking. Due to the challenging two-night abstinence period and the $\sim$9 h PET scanning sessions (described below), six subjects did not complete both PET sessions to which they were assigned. Specifically, for subjects assigned to the no-smoking and denicotinized cigarette conditions ($n=7$), four completed both PET conditions, while three completed only the no-smoking condition. For subjects assigned to the low-nicotine and denicotinized cigarette conditions ($n=8$), five completed both PET sessions, while two completed only the low-nicotine and one completed only the denicotinized cigarette condition. Thus, a total of 24 PET sessions were used for this study ($n=7$ for the no-smoking condition, $n=10$ for the denicotinized cigarette condition, and $n=7$ for the low-nicotine cigarette condition).

Abstinence period and PET protocol

After the initial screenings, participants began smoking/nicotine abstinence at 18:00 hours two nights prior to PET scanning. They reported to our laboratory at 13:00 hours the day after initiating abstinence, and a brief clinical interview and an exhaled CO measurement were obtained. Participants were deemed to be compliant with the study protocol if they reported no smoking/nicotine since 18:00 hours the previous night and had an exhaled CO level of $\leqslant$8 ppm. Subjects were seen the following day for PET scanning, and were required to report continuous abstinence since two nights previously and have an exhaled CO level of $\leqslant$4 ppm in order to undergo PET scanning. Subjects were given financial incentives to maintain this abstinence.

PET scanning followed the same general protocol as in our previous report (Brody et al., 2006), except that either a denicotinized or low-nicotine cigarette was smoked (along with the no-smoking control condition) in this study. At 12:00 hours on the day of scanning, subjects arrived at the VA Greater Los Angeles Healthcare System PET Center, and abstinence was verified as described above. Each participant then had an intravenous line placed at 12:45 hours in a room adjacent to the scanner. At 13:00 hours, bolus+continuous infusion of 2-FA was initiated. The amount of 2-FA administered as a bolus was equal to the amount infused over 500 min ($K_{\text{bolus}}=500$ min) (Carson et al., 1993; Carson, 2000; Kimes et al., 2008). The K_{bolus} of 500 min was chosen based on a study which demonstrated this value as optimal for reaching steady state between blood and brain within 6–8 h (Kimes et al., 2008). Consistent with this paradigm, 147 MBq (3.98$\pm$0.06 mCi) of 2-FA was administered as an intravenous bolus. This same amount of 2-FA (147 MBq) was also diluted in 60 ml saline, and 58 ml (141 MBq) was infused over the next 480 min (7.3 ml/h). This bolus+continuous infusion method has been validated vs. the bolus-only method (Kimes et al., 2008). PET scans were obtained as series of 10-min frames.

After initiation of the 2-FA bolus+continuous infusion, subjects remained seated in a room adjacent to the PET scanner for the next 3 h to allow the radiotracer to reach a relatively steady state. At 16:00 hours, brain scanning commenced and continued for 60 min. At 17:00 hours, subjects had a 10-min break in scanning, during which they smoked either a low-nicotine cigarette (Quest 1 brand: 0.6 mg nicotine yield, $n=7$

PET sessions), a denicotinized cigarette (Quest 3 brand: 0.05 mg nicotine yield, $n=10$ PET sessions), or did not smoke ($n=7$ PET sessions). To maintain a blind for the smoking conditions, a low-nicotine or denicotinized cigarette was de-identified by a research assistant, who covered brand markings. This de-identified cigarette was given to a study investigator (A.L.B. or R.E.O.) who gave it to the participant to smoke during the break in scanning. Participants were observed throughout this break. Subjects were then scanned for 3 h 50 min more with three scheduled 10- or 15-min breaks. Scanning ended at 21:00 hours.

Blood samples (5 ml) for assay of venous plasma nicotine levels were drawn at six time-points: at the beginning of the first break in scanning (immediately before smoking for the scans with cigarette smoking), at the end of this break (immediately after smoking for the scans with cigarette smoking) and at 10, 30, 60, and 180 min after the first break in scanning. These samples were drawn from a separate catheter placed for this purpose. For four of the denicotinized cigarette scans, we were unable to obtain a sufficient number of samples for plasma nicotine levels due to poor venous access. Samples were centrifuged, and concentrations of venous plasma nicotine were determined in the laboratory of Dr P. Jacob (University of California, San Francisco) using a GC–MS/MS system (lower limit of detection 0.2 ng/ml).

Withdrawal symptoms were monitored immediately before and after smoking, and at each post-smoking break in scanning. Cigarette craving was monitored with the Urge to Smoke (UTS) Scale (Brody et al., 2002; Jarvik et al., 2000), an analogue scale (range 0–6) with 10 craving-related questions. Mood (from 'sad' to 'happy') and nervousness were measured with analogue rating scales (range 0–6) with single questions asking subjects to rate their levels of these two states.

PET scans were obtained on a General Electric Advance NXi scanner (General Electric Medical Systems, Milwaukee, WI, USA) with 35 slices in 3D mode, transaxial resolution FWHM 5.2–7.7 mm (Dhawan et al., 1998). Attenuation correction scanning was performed with the germanium rotating rod source built into the scanner for 5 min at the end of the scanning session, and this attenuation correction was applied to all scans. 2-FA was prepared using a previously published method (Dolle et al., 1998).

MRI scanning

An MRI scan of the brain was obtained for each subject within a week of their first PET scanning session, with the following specifications: three-dimensional Fourier-transform (3D FT) spoiled-gradient-recalled acquisition with TR = 30 ms, TE = 7 ms, 30° flip angle, two acquisitions, 256 × 192 view matrix. The acquired volume was reconstructed as roughly 90 contiguous 1.5-mm-thick transaxial slices.

Region of interest (ROI) placement

PET-to-MRI co-registration was performed using an automated image registration method (Woods et al., 1993) within MEDx 3.3 (Sensor Systems Inc., Sterling, VA, USA). Each summed PET scan (lasting 30–60 min) was co-registered to the MRI separately, and the individual 10-min time-frames within each scan were co-registered to MRI scans using the transformation matrix parameters from the co-registration of the summed images.

ROIs were drawn on MRI within MEDx and transferred to the co-registered PET scans. ROIs included the thalamus, brainstem, and cerebellum, which were drawn as whole structures. These regions were chosen based on previous reports indicating a range of nAChR densities in these regions (Fujita et al., 2003; London et al., 1985; Valette et al., 1999). ROI placement on each PET scan frame was visually inspected to minimize the effects of co-registration errors and movement issues, with the procedure being repeated if there was a noticeable problem. Mean volumes for the thalamus, brainstem, and cerebellum were 8 ± 1, 15 ± 3, and 92 ± 18 cm^3, respectively.

Demographic and rating-scale data analysis

Means ± standard errors of the mean (S.E.M.) were determined for demographic and rating-scale data. For withdrawal-symptom rating-scale data, changes from before to after the smoking break in scanning were evaluated using paired Student's t tests (one-tailed, given the unidirectional hypotheses that withdrawal symptoms would be alleviated with smoking).

Determination of nAChR occupancy from smoking a denicotinized and a low-nicotine cigarette

For the determination of $\alpha_4\beta_2$* nAChR occupancies from smoking a denicotinized cigarette and a low-nicotine cigarette, the general method was that the fraction of total radioactivity displaced from smoking (corrected for the imperfect steady state determined from the no-smoking scans) to the maximum fractional displacement of radioactivity (from our previous study, Brody et al., 2006) was determined. This ratio is equal to the fraction of specifically bound

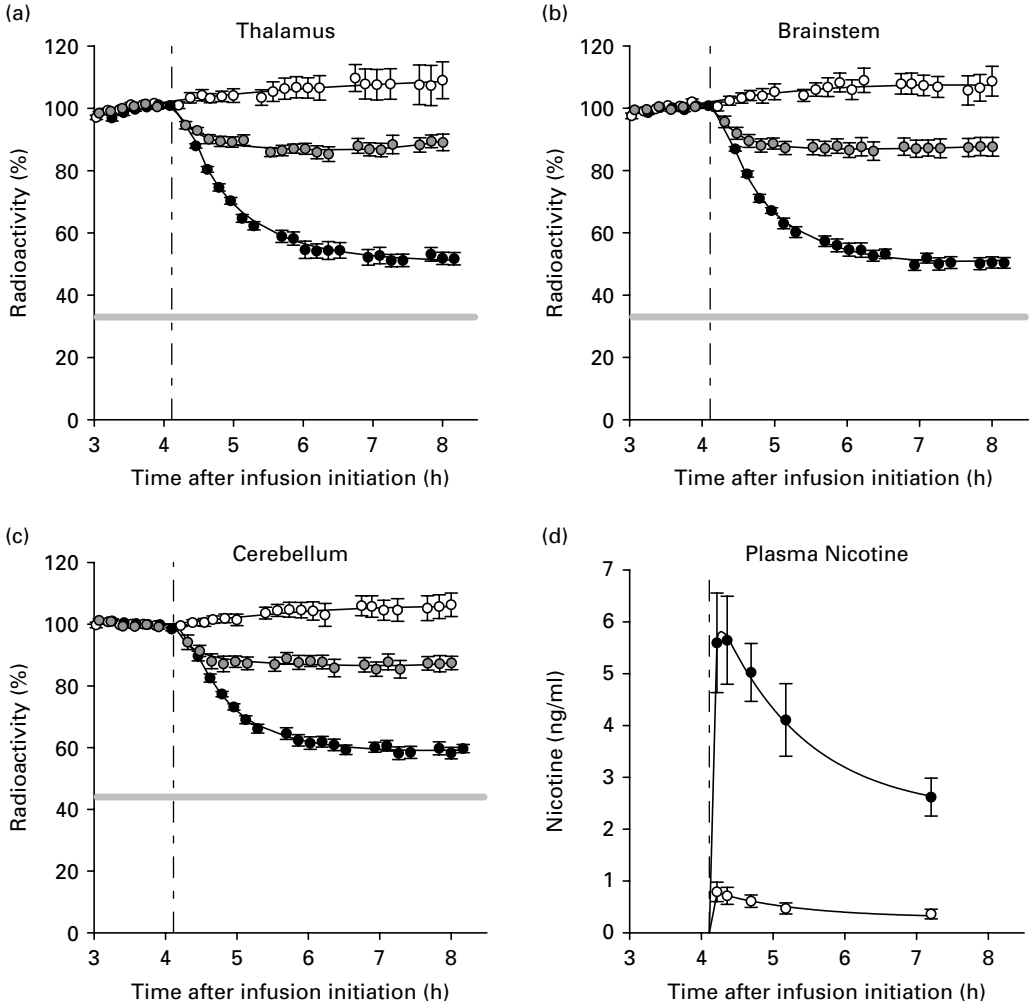


Figure 1. (a)–(c) Time–activity curves demonstrating changes in percent radioactivity for the three regions of interest (thalamus, brainstem, and cerebellum) over time following the initiation of bolus + infusion of 2-FA. The white, grey, and black circles represent the mean values (±S.E.M.) for the scans with no smoking ($n=7$), smoking of a denicotinized cigarette (0.05 mg nicotine yield) ($n=10$), and smoking of a low-nicotine cigarette (0.6 mg nicotine yield) ($n=7$), respectively. The horizontal grey lines in panels (a)–(c) represent the percent of maximum fractional displaceable radioactivity for each region, which were obtained from the asymptotic portions of the curves in figure 4 of our previous study in which subjects smoked regular cigarettes to satiety (Brody et al., 2006). (d) Changes in mean plasma nicotine levels (ng/ml) with time in subjects who smoked a low-nicotine (●) or denicotinized (○) cigarette.

radioactivity that was displaced from smoking, and is equal to the fractional occupancy of $\alpha_4\beta_2$* nAChRs from smoking.

As for the details of this analysis, the mean radioactivity for each 10-min PET frame for each condition was converted to a percent of total mean radioactivity at baseline for each ROI and plotted vs. time (Figure 1). The total concentration of tissue radioactivity before (C_T) and after (C_T') the smoking break in scanning was determined by averaging data from the six 10-min frames (1 h) prior to the break in scanning [180–240 min (mean 210 min) after bolus injection] and the last seven 10-min frames of the PET scanning session [395–480 min including a 15-min break (mean 438 min) after bolus injection] for each ROI. The displacement of total radioactivity ($C_T - C'_T$ for each cigarette-smoking condition (no smoking, denicotinized cigarette, and low-nicotine cigarette) was then calculated as a percent of total concentration of tissue radioactivity (C_T). For each ROI, the percent total radioactivity displacement from the two smoking conditions was corrected for the imperfect steady state (+2.5% per h) determined from the no-smoking condition scans. This corrected percent radioactivity

Table 1. Demographic and rating-scale variable

Variable	Total group ($n=15$)	No smoking ($n=7$)	Denicotinized cigarette ($n=10$)	Low-nicotine cigarette ($n=7$)
Age (yr)	35.7±3.1	35.3±5.0	33.5±3.2	38.6±3.6
Female/male	6/9	2/5	5/5	3/4
Cigarettes/d	18.7±0.5	18.8±0.9	17.8±0.6	18.7±0.7
Years smoking	13.5±2.6	15.1±3.7	12.2±2.9	13.1±4.2
Education (yr)	13.5±0.3	13.9±0.4	13.8±0.4	13.3±0.6
FTND	5.7±0.3	5.7±0.5	5.9±0.4	5.9±0.6
HAMD	2.2±0.4	2.7±0.5	1.7±0.5	2.0±0.6
HAMA	2.3±0.4	3.0±0.6	2.3±0.7	2.0±0.6
SJWS per item	3.9±0.1	3.8±0.3	3.7±0.2	3.9±0.2

FTND, Fagerström Test for Nicotine Dependence; HAMD, Hamilton Depression Rating Scale; HAMA, Hamilton Anxiety Rating Scale; SJWS, Shiffman–Jarvik Withdrawal Scale.

Values are stated as mean (±standard error of the mean); p values (Student's t tests) for demographic and rating scale variables (and a χ^2 test for female/male ratio) between the subjects assigned to the three conditions (no smoking, denicotinized cigarette, and low-nicotine cigarette) were all non-significant (range −0.3 to 1.0).

displacement was then divided by the percent of maximum displaceable radioactivity for each ROI, as determined from the asymptotic portions of the saturation curves in our previous study with smoking various amounts of regular cigarettes (see table 1 and figure 4a in Brody et al., 2006). The percentages of maximum displaceable radioactivity in the previous study for thalamus, brainstem, and cerebellum were 67%, 67% and 56%, respectively. These percent displaceable radioactivities are equal to 100% minus the non-displaceable radioactivity (thereby accounting for both non-specifically bound and free radiotracer). The ratio of percent total radioactivity displaced from smoking to percent of maximum displaceable radioactivity is equal to the percent of specifically bound radiotracer that is displaced, which is the percent $\alpha_4\beta_2$* nAChR occupancy. The percent $\alpha_4\beta_2$* nAChR occupancies for both the denicotinized and low-nicotine cigarette conditions were then compared to the predicted values of 22–24% and 77–79%, respectively, which were calculated from our previous data with regular cigarettes as described above.

Comparison of $\alpha_4\beta_2$ nAChR occupancy between the no-smoking, denicotinized cigarette, and low-nicotine cigarette conditions*

To compare $\alpha_4\beta_2$* nAChR occupancy between the three conditions, separate analyses were performed for the three potential comparisons (no smoking vs. low-nicotine cigarette, no smoking vs. denicotinized cigarette, and denicotinized vs. low-nicotine cigarette). Separate analyses were used because the no smoking vs. low-nicotine cigarette comparison had no subject overlap, while the other two comparisons had partial subject overlap (due to some subjects not completing the study).

For comparison of the no-smoking vs. low-nicotine cigarette conditions (no subject overlap), unpaired t tests were performed for the ROI data between conditions. Bonferroni correction was applied to account for the fact that three brain ROIs were tested (thalamus, brainstem, cerebellum).

For the comparisons of both the no-smoking vs. denicotinized cigarette conditions and the denicotinized cigarette vs. low-nicotine cigarette conditions, two different (but overlapping) analyses were performed, due to the unusual circumstance of partial overlap in the subject pool for the conditions being compared. First, an unpaired t test was performed for both comparisons using subjects from each condition who did not complete both conditions. And second, two independent sequential tests were performed, namely a paired t test between each pair of conditions for the subjects who underwent both conditions, followed by an unpaired t test for the remaining subjects who received only one scanning session. For this second analysis, the overall p value is given by $p1p2[1-\ln(p1p2)]$ where $p1$ and $p2$ are the p values for the two sequential tests. For the independent samples in these sequential tests, multiplying the p values by each other and then by a correction factor was felt to be

a conservative approach (see Eadie et al., 1971). Bonferroni correction was applied for both analyses to account for the fact that three ROIs were tested.

Determination of EC_{50}

To determine the venous plasma nicotine level sufficient to result in 50% occupancy of $\alpha_4\beta_2$* nAChRs (EC_{50}), we used a similar method to that in our previous report (Brody et al., 2006). Percent $\alpha_4\beta_2$* nAChR occupancy for each ROI was plotted against plasma nicotine concentration at the break in scanning corresponding to the middle of the post-smoking radioactivity measurements used for calculations. The plotted data were fitted using nonlinear regression with SigmaStat software (Systat Software Inc.) to determine the EC_{50}.

Correlations between $\alpha_4\beta_2$ nAChR occupancy and cigarette withdrawal symptoms*

To determine associations between $\alpha_4\beta_2$* nAChR occupancy and alleviation of cigarette withdrawal, correlation coefficients for the entire study group (corrected for condition) were determined between percent $\alpha_4\beta_2$* nAChR occupancy and change in the three primary withdrawal rating scales (craving/UTS, mood, nervousness) from before to 3.1 h after smoking.

Results

Clinical results

The subject population consisted of adults (mean age $\pm$ S.E.M., 35.7 ± 3.1 yr), was mostly male (nine males, six females), and smoked moderately (18.7 ± 0.5 cigarettes/d) for many years (13.5 ± 2.6 yr). Subjects were moderately nicotine-dependent (mean total FTND scores: 5.7 ± 0.3), had low levels of depression and anxiety (HAMD and HAMA scores: 2.2 ± 0.4 and 2.3 ± 0.4, respectively), and experienced moderate withdrawal during the initial visit (mean per item SJWS score $= 3.9 \pm 0.1$). All subjects reported compliance with the abstinence protocol, and exhaled CO levels at the time of scanning (2.8 ± 0.4 ppm) were consistent with these subject reports. The groups that underwent the three scanning conditions did not significantly differ in demographic or rating scale variables (Table 1).

From before to after the smoke break, subjects who smoked a cigarette had significant reductions in craving (UTS scores: 4.8 ± 0.6 to 2.4 ± 0.4 and 5.0 ± 0.5 to 3.6 ± 0.6 for the low-nicotine and denicotinized cigarette conditions, respectively, paired Student's t tests, $p = 0.03$ and $p = 0.04$, respectively). Although the reduction in mean UTS score was more pronounced in the low-nicotine cigarette group than the denicotinized cigarette group (UTS score change: -2.4 vs. -1.4, respectively), this difference did not reach statistical significance. The no-smoking group had no change in mean UTS score from before to after the break in scanning (UTS scores: 2.5 ± 0.5 to 2.5 ± 0.5, paired Student's t test, n.s). For the last break in scanning (which corresponded to the middle of the post-smoking PET scanning frames used for data analysis, 3.1 h after smoking), mean UTS scores returned towards baseline for both the low-nicotine and the denicotinized cigarette groups (3.8 ± 0.7 and 4.2 ± 0.7, respectively), and were no longer significantly different from baseline (paired Student's t tests, n.s.).

Nervousness and mood scores did not change significantly from the beginning to end of the smoke break for the three conditions (nervousness: 4.4 ± 0.8 to 3.7 ± 0.7, 3.0 ± 0.6 to 3.4 ± 0.5, and 1.7 ± 0.3 to 1.4 ± 0.3 for the low-nicotine cigarette, denicotinized cigarette, and no-smoking conditions, respectively; paired Student's t tests, n.s.; mood: 3.9 ± 0.7 to 3.9 ± 0.4, 4.7 ± 0.4 to 3.7 ± 0.5, and 4.3 ± 0.7 to 4.6 ± 0.6 for the three groups, respectively; paired Student's t tests, n.s.). [As an aside, subjects undergoing the no-smoking condition had significantly lower UTS and nervousness scores prior to the break in scanning than those undergoing the two smoking conditions (Student's t tests, $p = 0.002$ for both comparisons for UTS scores and $p < 0.05$ for both comparisons for nervousness scores).]

Determination of nAChR occupancy from smoking a denicotinized and a low-nicotine cigarette

Time–activity curves for the three ROIs (Figure 1a–c) demonstrated that displacement of 2-FA was consistent with the amount of nicotine in the cigarette smoked. Compared to the no-smoking control condition (which had a mean 2.5% per h increase in radioactivity), smoking a denicotinized cigarette resulted in $17 \pm 1\%$, $16 \pm 2\%$, and $16 \pm 2\%$ displacement of total radioactivity in the thalamus, brainstem, and cerebellum, respectively. Smoking a low-nicotine cigarette resulted in $52 \pm 2\%$, $53 \pm 2\%$, and $43 \pm 2\%$ displacement of total radioactivity in these three brain regions, respectively.

Receptor occupancy from smoking a denicotinized cigarette (displacement of specifically bound radiotracer) was $25 \pm 2\%$, $25 \pm 3\%$, and $28 \pm 4\%$ (mean 26%) for the thalamus, brainstem, and cerebellum,

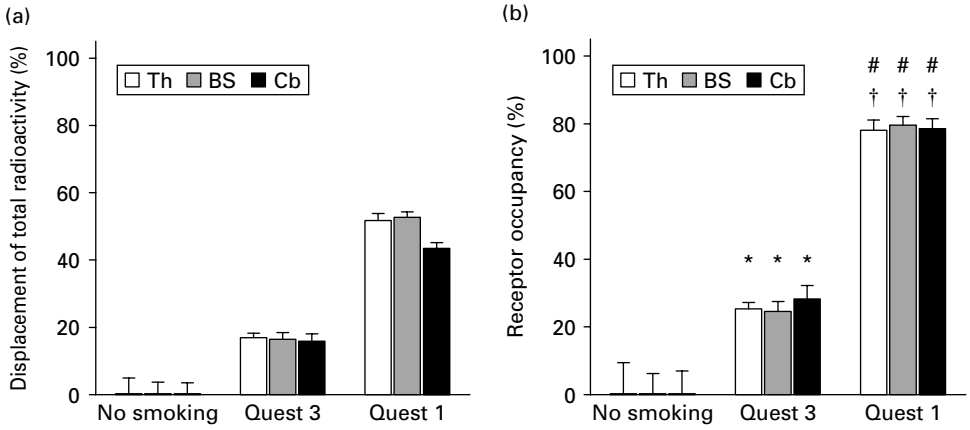


Figure 2. (a) The mean (±S.E.M.) percent displacements of total radioactivity for the three smoking conditions [no smoking, Quest 3 (denicotinized cigarette), Quest 1 (low-nicotine cigarette)] for the three regions of interest (Th, thalamus, BS, brainstem, Cb, cerebellum) from before to 3.1 h after smoking. (b) The mean (±S.E.M.) receptor occupancies for the three smoking conditions for the three regions of interest (ROIs). The * symbol indicates a significance level of $p<0.05$ for Student's t test comparisons (following Bonferroni correction for the three ROIs) between the denicotinized cigarette and the no-smoking conditions. The # and † symbols indicate significance levels of $p<0.0001$ for Student's t test comparisons (following Bonferroni correction for the three ROIs) between the low-nicotine cigarette condition and the no-smoking and denicotinized cigarette conditions.

respectively, while receptor occupancy from smoking a low-nicotine cigarette was 78±3%, 80±3%, and 79±3% (mean 79%) for the three brain regions, respectively. These receptor occupancies were similar to the predicted values of 22–24% and 77–79%, respectively, and indicate that nicotine inhalation is primarily responsible for $\alpha_4\beta_2$* nAChR occupancy from smoking. Plasma nicotine levels were also consistent with the amount of nicotine in the cigarette smoked (Figure 1d).

Comparison of $\alpha_4\beta_2$ nAChR occupancy between the no-smoking, denicotinized cigarette, and low-nicotine cigarette conditions*

For the comparison of $\alpha_4\beta_2$* nAChR occupancy between the no-smoking and low-nicotine cigarette conditions, all three brain regions had significantly different occupancies (unpaired Student's t test, two-tailed, $p<0.0001$, for the three regions, following Bonferroni correction) (Figures 2 and 3). These results indicate that smoking a low-nicotine cigarette results in significant receptor occupancy compared to no smoking.

For the comparison of $\alpha_4\beta_2$* nAChR occupancy between the no-smoking and denicotinized cigarette conditions, all three brain regions were significantly different with both statistical methods applied (for the first analysis, unpaired Student's t tests, two-tailed, $p<0.01$ for the three regions; for the second analysis using sequential tests, $p=0.01$, 0.03, and 0.04 for the thalamus, brainstem, and cerebellum, respectively; all results are Bonferroni-corrected) (Figures 2 and 3). These results indicate that smoking a denicotinized cigarette results in significant receptor occupancy compared to no smoking.

For the comparison of $\alpha_4\beta_2$* nAChR occupancy between the low-nicotine and denicotinized cigarette conditions, all three brain regions were significantly different with both statistical analysis methods (for the first analysis, unpaired Student's t tests, two-tailed, $p<0.0001$, for the three regions; for the second analysis using sequential tests, $p<0.0001$ for the three regions; all results are Bonferroni-corrected) (Figures 2 and 3). These results indicate that smoking a low-nicotine cigarette results in significantly greater receptor occupancy than smoking a denicotinized cigarette.

Determination of EC_{50}

The effective venous plasma concentration of nicotine needed to occupy 50% of $\alpha_4\beta_2$* nAChRs (EC_{50}) was 0.79±0.08, 0.77±0.10, and 0.68±0.10 ng/ml for the thalamus, brainstem, and cerebellum, respectively (Figure 4a–c), with the mean EC_{50} being 0.75±0.08 ng/ml (Figure 4d).

Correlations between $\alpha_4\beta_2$ nAChR occupancy and cigarette withdrawal symptoms*

There were no significant correlations between receptor occupancy and change in withdrawal symptoms

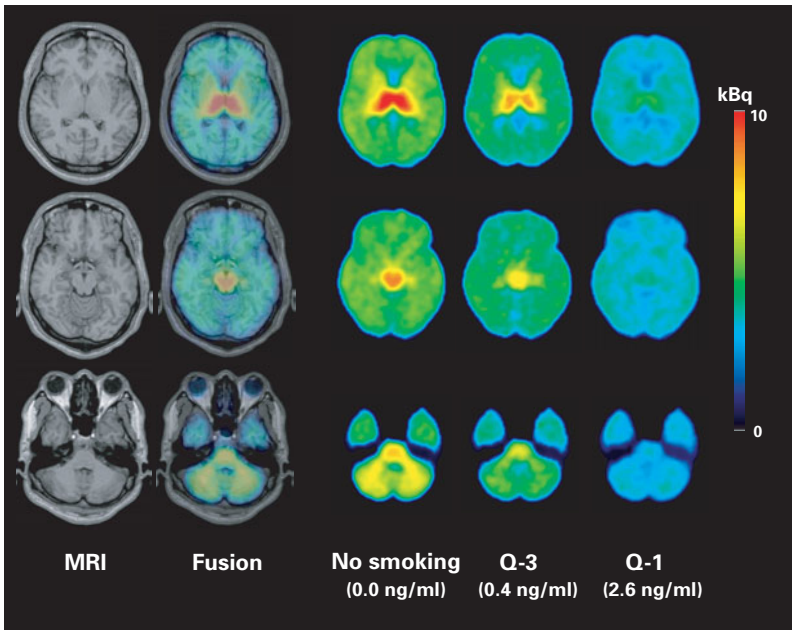


Figure 3. Radioactivity levels on mean positron emission tomography (PET) images 3.1 h after the smoke break in scanning. Column 1 shows a magnetic resonance imaging (MRI) scan with representative slices from three levels of interest for this study (row 1 at the level of the thalamus, row 2 at the level of the brainstem, and row 3 at the level of the cerebellum). Column 2 demonstrates a PET/MRI fusion used for transfer of drawn regions of interest from MRI to PET. Columns 3–5 show mean PET images from the three study groups [no smoking, Q-3 (denicotinized cigarette), Q-1 (low-nicotine cigarette)] 3.1 h after the smoking break in scanning, demonstrating radiotracer displacement for the two active smoking conditions, but not for the no-smoking condition. Mean plasma nicotine levels at the 3.1 h time-point are shown below columns 3–5.

(correlation values -0.27 to -0.13, d.f. $= 13$, p values > 0.05), indicating that factors other than nicotine binding to $\alpha_4\beta_2$* nAChRs are at least partly responsible for the improvement in withdrawal symptoms seen with smoking either low-nicotine or denicotinized cigarettes, or (alternatively) that the sample size here was too small to detect such correlations.

Discussion

The present findings indicate that inhalation of nicotine during cigarette smoking is solely responsible for occupancy of brain $\alpha_4\beta_2$* nAChRs. The results which support this conclusion are that: (1) the receptor occupancy after smoking an entire denicotinized cigarette (26%) was very similar to the predicted values (22–24%) calculated from our previous study using regular cigarettes (Brody et al., 2006), and (2) the EC_{50} found here (0.75 ng/ml) was compatible with the previously determined EC_{50} (0.87 ng/ml). The slight discrepancies between results of the present study and the predictions based on our previous study are probably due to small errors in measurement. Although it is possible that the slightly higher receptor occupancy (2–4%) found here with smoking a denicotinized cigarette is real (perhaps due to intake of the other constituents of tobacco smoke or behavioural factors affecting endogenous acetylcholine levels), these effects would be very minor (if present at all) compared to the effects of nicotine. In our previous study, smoking a full regular cigarette and smoking to satiety resulted in 88% and 95% receptor occupancy, respectively, so that any small effect of factors other than nicotine would be insignificant in daily cigarette smokers compared to the effects of their regular smoking.

The present study also demonstrated significant $\alpha_4\beta_2$* nAChR occupancy from smoking a denicotinized cigarette, which has implications for the basic assumption both in research and clinical practice that denicotinized cigarettes provide little pharmacological effect of nicotine. While the present study demonstrated a mean 26% $\alpha_4\beta_2$* nAChR occupancy 3.1 h after smoking a denicotinized cigarette, we can infer that the percent $\alpha_4\beta_2$* nAChR occupancy would be higher sooner after smoking, since nicotine plasma levels were twice as large within the first hour after smoking (Figure 1d). Therefore, researchers, clinicians, and smokers themselves should consider the fact that smoking denicotinized cigarettes results in substantial

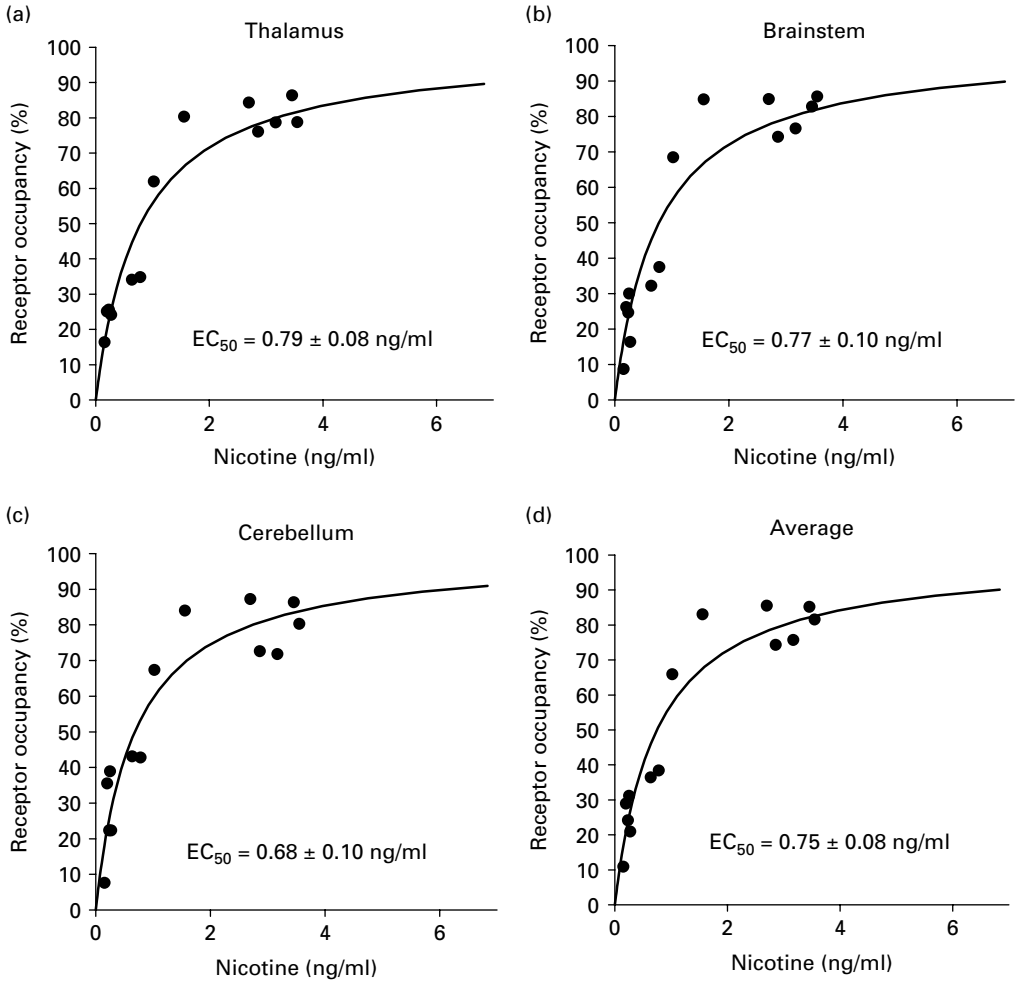


Figure 4. (a)–(c) Graphs showing percent $\alpha_4\beta_2$* nicotinic acetylcholine receptor occupancy as a function of plasma nicotine concentration (ng/ml) for the three regions of interest and (d) for the mean of these three regions. From these graphs, the effective plasma concentration of nicotine needed to occupy 50% of available receptors (EC_{50}) was determined for the three regions separately and for the mean of the regions.

$\alpha_4\beta_2$* nAChR occupancy, even if this effect is much smaller than that of smoking a regular cigarette.

This study also complements other lines of research indicating that factors other than nicotine binding to $\alpha_4\beta_2$* nAChRs are responsible for withdrawal alleviation from smoking. In the present study, there were no significant correlations between changes in withdrawal symptoms and $\alpha_4\beta_2$* nAChR occupancy, and craving was returning to baseline levels during the post-smoking PET time-points of interest despite PET scanning evidence of continued $\alpha_4\beta_2$* nAChR occupancy. While the absence of correlations with withdrawal symptoms and the return of craving with continued $\alpha_4\beta_2$* nAChR occupancy do not prove that there is no relationship between $\alpha_4\beta_2$* nAChR occupancy and withdrawal, these findings are consistent with the wealth of literature demonstrating that factors other than nicotine intake are important for withdrawal alleviation. This literature includes studies demonstrating that denicotinized cigarettes alleviate cigarette craving, often to a comparable degree as smoking regular cigarettes (Buchhalter et al., 2001; Dallery et al., 2003; Donny et al., 2007; Pickworth et al., 1999), and studies of nicotine replacement therapies (Gross and Stitzer, 1989; Muramoto et al., 2003; Rose et al., 1990; West and Shiffman, 2001), which demonstrate incomplete withdrawal alleviation with these medications. Thus, our study results support the theory that factors other than nicotine binding to brain $\alpha_4\beta_2$* nAChRs are at least partly responsible for relief of craving (and other withdrawal symptoms) from smoking.

A limitation of this study is the slow brain kinetics of 2-FA, requiring assessment of receptor occupancy >3 h after smoking. If smoking-induced receptor occupancy through non-nicotine mechanisms is short-lasting and disappears in the first minutes after smoking, it would not be detected with our method. New radioligands with faster kinetics will be needed to determine such effects. Nonetheless, even if there are short-lasting effects on nAChR occupancy from non-nicotine aspects of smoking, the low values for plasma EC_{50} found in both of our studies suggest that brain $\alpha_4\beta_2$* nAChRs in smokers are almost completely occupied by nicotine and are probably in the inactive (desensitized) state (Quick and Lester, 2002) throughout the day.

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Statement of Interest

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ORIGINAL INVESTIGATION

Alcohol-induced increases in smoking behavior for nicotinized and denicotinized cigarettes in men and women

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Abstract
Introduction Alcohol has been shown to increase smoking urges and smoking behavior. However, alcohol's effects on specific components of smoking behavior for nicotine versus non-nicotine factors and potential sex differences in this response have not been investigated.
Methods Forty-two young male and female non-dependent, heavy social drinking smokers participated in two double-blind laboratory sessions. They were randomized to either an alcohol (0.8 g/kg; n = 29) or placebo (n = 13) beverage pre-administration group. After beverage consumption, they were assessed for smoking urges and then given the opportunity to smoke cigarettes which were either all nicotinized (0.6 mg/cigarette) or denicotinized (≤0.05 mg/cigarette) over a 3-h period; smoking behavior was quantified by a smoking topography device. Subjects took standardized puffs of the session's cigarette both before and after beverage administration to provide a reference when making future smoking choices.
Results Alcohol, compared with placebo beverage, increased both men's and women's smoking urge, as well as subjective ratings of smoking reference puffs for either nicotinized or denicotinized cigarettes. In terms of smoking choice behavior, regardless of cigarette type, alcohol (>placebo) increased men's smoking behavior, including puff count, volume, and duration. In contrast, for women, smoking topography measures did not differ between alcohol and placebo conditions.
Discussion In summary regardless of nicotine content, in men, alcohol increased smoking urge and behavior, whereas in women, alcohol increased smoking urge but did not increase smoking behavior. These results indicate that the mechanisms underlying co-use of alcohol and tobacco in women may be more complex than in men.

Keywords Alcohol · Nicotinized and denicotinized cigarettes · Smoking topography · Sex differences · Non-daily smoker

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Introduction

Epidemiological and clinical data demonstrate a strong positive relationship between alcohol drinking and cigarette smoking (Ait-Daoud et al. 2005; Bien and Burge 1990; Falk et al. 2006; Istvan and Matarazzo 1984). Heavier drinkers, are more likely to smoke than their moderate or light drinking counterparts (Falk et al. 2006). This positive relationship has been supported by data from controlled laboratory studies showing that acute alcohol, compared with placebo beverage, increases cigarette smoking behavior (Griffiths et al. 1976; Henningfield et al. 1983, 1984).

Alcohol also increases urge to smoke, either in the presence of salient smoking cues (Burton and Tiffany 1997; Glautier et al. 1996; Mintz et al. 1985; Sayette et al. 2005) or in the absence of salient cues (Epstein et al. 2007; King and Epstein 2005). In terms of mechanisms, co-use of these substances may relate to positive or negative reinforcement. Recent studies have supported the former, as one study showed that the stimulating and not sedating effects of alcohol partially mediated alcohol's increase of smoking urges (Epstein et al. 2007), and another study indicated that alcohol may also enhance the rewarding effects of nicotine, including smoking satisfaction and relief of cigarette craving (Rose et al. 2004).

There is a growing body of literature demonstrating sex differences in nicotine responses and drinking–smoking interactions (Drobes 2006; Field and Duka 2004; McClernon et al. 2008; Perkins 1995a; Perkins et al. 1997, 2006). It has been shown that women smokers are less successful and less confident in nicotine discrimination (Perkins 1995b; Perkins et al. 1997), have greater sensitivity to instructional set on ratings of nicotine reward and reinforcement (Perkins et al. 2006), and may have less success in smoking cessation using standardized treatments such as nicotine replacement than men (Bjornson et al. 1995; Perkins and Scott 2008; Royce et al. 1997; Senore et al. 1998; Wetter et al. 1999). Moreover, women may be less sensitive to smoking's effects on alcohol behaviors, as evidenced by lower alcohol consumption after nicotine administration (Acheson et al. 2006) and less responding for alcohol after ad libitum smoking (Perkins et al. 2000) compared with their male counterparts. Finally, while for both sexes, heavier co-use of cigarettes is associated with heavier alcohol drinking, only in men is there an association between alcohol-related increases in smoking behavior and urge (King et al. 2008).

While alcohol's effects on smoking urge and behavior have been demonstrated, as well as potential sex differences in their interaction, the mechanisms underlying this relationship remain unknown. More specifically, it is unclear whether alcohol increases smoking urge and behavior for the reinforcing effects of nicotine specifically, or for the associated features of smoking (handling, smoke, airway sensations, etc.). In addition, since the majority of prior studies on alcohol-nicotine interactions have been conducted in regular heavy smokers, it is unclear if results may extend to light/non-daily smokers, who do not show tobacco withdrawal symptoms that may confound other subjective assessments. Non-daily smokers are of interest in examining alcohol-smoking interactions because they may be particularly sensitive to alcohol's effects on subsequent smoking urge and behavior, smoke proportionately more of their cigarettes in the context of alcohol than their nicotine-dependent counterparts (Shiffman and Paty 2006), and do not experience the confound of withdrawal effects from either substance in a controlled laboratory setting (Shiffman 1989; Shiffman et al. 1995).

Therefore, the goal of the current study was to further elucidate alcohol-smoking interactions by examining alcohol's effects on heavy social drinkers' smoking of nicotinized versus denicotinized cigarettes and potential sex differences in these responses. Subjective and objective measures of smoking urge and behavior were obtained during an alcohol challenge session, including quantifiable aspects of smoking behavior assessed via a smoking topography device during the 3-h post-drink period. It was predicted that alcohol, relative to placebo, would increase smoking behavior and that this increase would be preferential to nicotinized cigarettes. Further, based on the theory (Perkins 2001) that women's smoking (versus men's) may relate more strongly to exteroceptive factors, it was predicted that women would show less alcohol elicitation of smoking behavior than men.

Methods

Recruitment and screening

Subjects were 42 young adults with concomitant non-dependent, light smoking and non-dependent, heavy social drinking patterns. This group was chosen because of the strong association between alcohol drinking and cigarette smoking evident in heavy drinkers. They were recruited through newspaper and online advertisements in local media and by word-of-mouth referrals. Interested candidates completed both a brief phone interview and more extensive in-person screening to determine study eligibility.

At screening, candidates provided written informed consent and met the following inclusion criteria: age between 21 and 35; body mass index (BMI) between 19 and 30; and non-dependent, lifetime patterns of cigarette smoking and alcohol drinking. Self-reported alcohol and cigarette use patterns were confirmed by daily estimates of each substance as part of a 1-month timeline follow-back interview (TLFB; Sobell et al. 1979; Sobell and Sobell 1995). Smoking criteria included smoking between 1 and 50 total cigarettes per week, with typical daily use of no more than seven cigarettes, while allowing up to 15 cigarettes on heavy drinking days. Alcohol drinking criteria included a predominant adult drinking pattern of ten or more alcoholic drinks weekly with regular "binge" drinking episodes one to five times per week. As in other studies, a "binge" drinking episode was defined as consuming five or more drinks per occasion for men and four or more for women (SAMHSA 2005). Smoking and drinking criteria for inclusion were chosen to be consistent with prior studies in light smokers or tobacco chippers (Epstein et al. 2007;

King et al. 2002; King and Epstein 2005; Ray et al. 2007; Sayette et al. 2005).

All candidates underwent a medical examination by a resident physician, completed a diagnostic interview with a trained assessor, provided blood and urine samples, and completed several psychosocial and health history questionnaires. Participants were excluded if they were taking any psychotropic medications, had a major medical or psychiatric condition (including past or current alcohol, nicotine, or other substance dependence), or had a positive urine toxicology screen (morphine, cocaine, methamphetamine, barbiturates, or benzodiazepines). Persons with alcohol and/or nicotine dependence, as determined by structured clinical interview for DSM-IV interview (SCID; First et al. 1995), were excluded to avoid potential withdrawal during the sessions. Finally, women were excluded if they were pregnant or breastfeeding.

Procedures

The study employed a two session, double-blind, double-dummy design that included a 15-min drinking period followed by 3 h of post-drink assessment of subjective effects and smoking behavior. The within-subjects factor was cigarette type (nicotinized versus denicotinized cigarettes), and the between-subjects factor was beverage type (alcohol versus placebo), with randomization to the alcohol or the placebo group at a 2:1 ratio. There were 29 subjects (14 females) in the alcohol administration group and 13 subjects (six females) in the placebo administration group. The rationale for the unequal grouping randomization derived from our prior data indicating that young adult heavy drinking chippers smoke infrequently on non-alcohol drinking days (King et al. 2008) and exhibit little change in subjective smoking urges after consuming a placebo beverage (Epstein et al. 2007; King and Epstein 2005). Therefore, it was assumed that they would not engage in significant choice smoking behavior to placebo relative to an intoxicating alcoholic beverage.

Test sessions began between 2:00 and 5:00 p.m. and were held at the Clinical Addictions Research Laboratory at the University of Chicago. Each session lasted 4 h and were separated by at least 48 h; the average interval between sessions was 6.6 ± 1.0 SEM days. Prior to each session, participants were instructed to abstain from alcohol and medications for 48 h and from caffeine, food, and cigarettes for 3 h. Upon arrival, the subject verified abstinence by providing an expired air carbon monoxide breath test (Smokerlyzer®, Bedfont Scientific, Medford, NJ, USA) of less than 10 ppm and an alcohol breathalyzer reading (Alco-Sensor III, Intoximeter, St. Louis, MO, USA) of less than 0.003 mg%. Subjects also provided a urine sample prior to each session for drug toxicology testing and a pregnancy test (for females). This was followed by consumption of a small snack (20% daily calories) to reduce potential negative effects of hunger on mood and/or nausea while drinking alcohol.

In Fig. 1, the study timetable, procedures, and measures are presented. Thirty minutes prior to beverage consumption, participants completed baseline measures and took their first set of two standardized reference puffs from that session's randomized cigarette type. The puffs were standardized through an automated computer display prompting the initiation and discontinuation of each puff. They were included to provide a reference by which to make future smoking choices during that session (e.g., see Rukstalis et al. 2005). Following the completion of these procedures, the participant ingested a placebo gel capsule followed by the allocated study beverage (alcohol or placebo) over a 15-min time period (two 5-min periods of drinking separated by 5 min of rest to standardize alcohol intake). Fifteen minutes later, the subject took his/her second set of two standardized reference puffs. This was conducted to examine the effects of the beverage (alcohol or placebo) on subjective responses to the standard reference puffs. Starting at 60-min post-initiation of beverage consumption and at 0.5-h intervals for the next 3 h, the subject was given the option of smoking a cigarette via a smoking topography device (details below). This interval was chosen because smoking urge has been shown to peak approximately 1 h after alcohol consumption, i.e., when blood alcohol contents (BACs) are peaking, and remain elevated for several hours thereafter (Epstein et al. 2007; King and Epstein 2005). Each cigarette was presented to the subject with the following instructions: "*At this point, you have the option of having a cigarette. You can smoke as much or as little as you'd like. You'll have 5 min to decide if you'd like to smoke, and I'll be back after that time*". After 5 min, the experimenter returned to the room and removed the cigarette if it was not chosen. If the cigarette was chosen, once completed, the experimenter removed the cigarette butt and lighter.

Between time points, participants were allowed to relax in the living room-like subject rooms, watch television or movies, or read. At the end of each session, subjects were transported home by a livery service. Each participant was debriefed and compensated $150 after completing the second study visit. The study was fully approved by the University of Chicago Institutional Review Board.

Beverages

During each of the two sessions, subjects in the alcohol group consumed a beverage containing a 0.8 g/kg dose of alcohol (equivalent to four to five standard alcoholic drinks), and subjects in the placebo group consumed an identical placebo beverage (1% alcohol by volume added as a taste mask). Beverages were parsed into two equal

Time (min):	-30 pre	-30 post	0	+30 pre	+30 post	+60	+90	+120	+150	+180
Reference Puffs		X			X					
Visual Analog (VAS)	X	X		X	X					
Cigarette Effects (CES)		X			X					
Beverage[a]			X							
Smoking Urge (BQSU)		X			X	X				
BAC		X			X	X		X		X
Optional Cigarettes[b]						(X)	(X)	(X)	(X)	(X)
Smoking Topography						X	X	X	X	X

Fig. 1 Session timeline of events and study measures. Time measured in relation to initiation of beverage consumption. *Superscript a*: participants were assigned to either alcohol or placebo beverage administration group for both sessions (between-subjects factors). *Superscript b*: subjects were given the option of smoking a cigarette via a smoking topography device at 30-min intervals following beverage consumption. Sessions were identical aside from cigarette type (within-subjects factor)

portions, served in clear, plastic, lidded cups, and consumed through a straw. To reduce alcohol expectancies, two strategies were employed: (a) subjects ingested a small gel capsule containing only dextrose prior to consuming the beverage; and (b) subjects were informed that the session beverage may contain alcohol, a stimulant, a sedative, a combination of these substances, or a placebo. Previous studies by our group have used similar blinding procedures (Conrad et al. 2009), and the gel capsule served to further reduce alcohol expectancies. The validity of this procedure was supported as subjects' ratings of beverage content revealed that in 59% of alcohol sessions, subjects thought they had received a substance other than alcohol, and in 46% of placebo beverage sessions, subjects thought they had received an active substance. Session beverages were comprised of Kool-Aid (Kraft Foods, Inc, Northfield, IL, USA), Splenda® (McNeil Nutrionals, LLC, Fort Washington, PA, USA), water, and the appropriate dose of 190-proof ethanol based on body weight. Due to body water differences between the sexes, women received an approximate 85% dose as compared with men (Frezza et al. 1990; Sutker et al. 1983). Measures of BAC were obtained at baseline and during two points on both the ascending (+30, +60) and descending (+120, +180) blood alcohol curve (Fig. 1), utilizing an Alco-Sensor IV breathalyzer (Intoximeter, St. Louis, MO, USA).

Subjective measures

Smoking urges were assessed at baseline and at two intervals during the first 1 h following beverage consumption by the ten-item brief questionnaire on smoking urges (BQSU; Cox et al. 2001; see study timetable, Fig. 1). Responses to the standard reference puffs were measured via several visual analog scale (VAS) items and a modified cigarette evaluation scale (CES; Rose et al. 2000). The VAS items have been shown to be sensitive to acute smoking (Mendelson et al. 2008; Perkins et al. 1993, 1994). They were rated from 0 ("none") to 100 ("most") and included *feeling stimulated*, *desire to smoke*, *dizzy*, *relaxed*, *head rush*, and *pleasure from cigarette*. Seven CES items were each rated on a Likert scale from 1 ("not at all") to 7 ("extremely") and included *satisfaction, reduced craving, strength and enjoyment of cigarette sensations in the throat and chest, perceived nicotine content, similarity to the subject's preferred brand, and enjoyment of cigarette taste*. Subjective measures were not included after the optional cigarette choice interval was initiated (i.e., after the 60-min post-drinking time point), due to the potential confound of choice behavior on subjective ratings.

Cigarettes

During each session, the participant received cigarettes (Quest® brand; Vector Tobacco Inc., Morrisville, NC, USA) that were either all nicotinized (0.6 mg nicotine yield per cigarette) or all denicotinized (≤0.05 mg nicotine yield per cigarette). The cigarettes were blinded by a physical ink marking over the brand recognition on the filter.

Objective smoking-related measures

All cigarettes were smoked through a device designed to measure the various smoking topography indices of each individual cigarette puff (Clinical Research Support System Smoking Topography Machine, Plowshare, Baltimore, MD, USA). The device was a small (2.5 × 2.2 × 1.2″), light-weight (3.1 oz), handheld black box with an entry port in which to place a cigarette and a small tube through which to inhale the smoke. Data from each cigarette puff was recorded on the device and later downloaded following each session. The primary measures obtained were puff count (the total number of puffs for each cigarette), puff volume (capacity of each puff in milliliters), and puff duration (length of each puff in milliseconds). The sum of each smoking topography variable across all five intervals at each

session was used for analysis in order to examine the cumulative effect of alcohol on smoking behavior.

Statistical analyses

Background and demographic characteristics were compared by sex (men and women) and drinking group (alcohol and placebo) using Student's *t* tests and Chi-square tests, where appropriate. Because men were older and had more years of education than women, these variables were used as covariates in analyses of subjective and objective outcomes. The subjective effects of the standardized reference cigarette puffs (i.e., nicotinized versus denicotinized) were analyzed by analyses of variance (ANOVA) with beverage (alcohol and placebo) and sex as the between-subjects factors and cigarette type (nicotinized and denicotinized) as the within-subjects factor. Similar ANOVAs were conducted to examine smoking topography measures. Simple effects tests were used for post hoc examinations of significant main effects and interactions. Pearson product moment partial correlations were also used to examine the relationship between changes in subjective response and subsequent smoking behavior, controlling age, education, sex, and beverage type with behavior as the outcome and changes in subjective response as the independent variable. Three subjects (one male and two females) made no cigarette choices throughout the entire study and therefore were not included in the smoking topography analyses.

Results

Participant characteristics

Background and demographic characteristics were similar between the sexes (see Table 1) with the exception of men being slightly older and having more years of education than women ($ps \leq 0.05$). These characteristics were also similar between beverage condition groups, though BMI was slightly higher in the alcohol pre-administration group ($p < 0.05$). No sex differences were observed in self-reported alcohol drinking and cigarette smoking behaviors or in estimated nicotine content of preferred cigarette brand across sex or beverage group (Table 1). Both men and women's smoking behaviors increased as a function of drinking day type. There was infrequent use of other substances, with marijuana as the most frequently used (see Table 1).

Subjective measures: reference puffs

The first set of reference puffs had no effect on smoking urge, subjective effects, or evaluation of cigarettes ($p \geq 0.17$). In contrast, the second set of reference puffs, i.e., conducted 15 min after beverage completion, did produce several differential effects by sex and cigarette type. First, after either alcohol or placebo beverage, the nicotinized puffs increased women's but not men's ratings of "feeling stimulated" (cigarette type×sex; $F(1,35) = 6.69$, $p = 0.01$; *women*: nic>denic, $p < 0.001$). Second, for both sexes, after consumption of either alcohol or placebo, the nicotinized puffs increased ratings of desire to smoke, satisfaction, enjoyable sensation, and enjoyable taste (cigarette type; $F(1,35) = 5.91$, $p < 0.05$). Finally, alcohol (versus placebo) increased ratings of head rush, nicotine content, similarity to own brand, enjoyable cigarette taste, and reduced cigarette craving (beverage group; $F(1,35) = 4.04$, $p \leq 0.05$), regardless of the nicotine content of the puff (see Fig. 2).

Subjective measures: smoking urge

For both sexes, alcohol, compared with placebo beverage, significantly increased smoking urge (beverage group×time; $F(4,35) = 5.16$, $p < 0.01$). The pre-drink baseline BQSU scores were comparable between beverage conditions and directionally but non-significantly higher in women. Within 30 min of alcohol consumption, BQSU scores increased for both sexes (men: baseline 20.0 ± 3.2 versus post-beverage 32.4 ± 3.7; women: baseline 26.3 ± 3.0 versus post-beverage 37.2 ± 3.9) (see Fig. 3) and remained elevated at 60 min.

Objective measures

As expected, alcohol increased BAC levels steadily in the first hour (30 min post-drink: 0.084 ± 0.004; 45 min post-drink: 0.097 ± 0.003; 60 min post-drink: 0.096 ± 0.002) which declined during the elimination phase (120 min post-drink: 0.075 ± 0.002; 180 min post-drink: 0.057 ± 0.002). The BAC levels did not differ between men and women ($p = 0.24$).

There were no differences in cigarette choice for the nicotinized versus denicotinized cigarettes (cigarette type: $F(1,77) = 1.84$, p=ns); therefore, all subsequent analyses examining beverage and sex effects were conducted collapsing on cigarette type. Examination of smoking choice behavior over the entire smoking choice interval, however, revealed several sex differences as a function of beverage type. These sex differences were corroborated by measures of smoking topography, including puff count, volume, and duration. Figure 4 depicts smoking choice behavior as well as smoking topography indices by beverage type and sex. In men, but not in women, alcohol (versus placebo) significantly increased smoking choice (beverage type×sex: $F(1, 35) = 6.58$, $p < 0.05$; simple effects *men*: alcohol>placebo, $p < 0.01$) and all three smoking topography measures, including puff count (beverage×sex; $F(1, 35) = 7.46$, $p = 0.01$; simple effects,

Table 1 Background characteristics and substance use in men and women

	Alcohol		Placebo	
	Men (n=15)	Women (n=14)	Men (n=7)	Women (n=6)
General characteristics				
Age (years)	26.1 (0.9)*	23.7 (0.6)	24.7 (1.5)*	22.5 (0.7)
Race (Caucasian)	11 (73%)	9 (64%)	5 (71%)	4 (67%)
BMI (kg/m^2)	25.2 (0.7)*	24.3 (0.9)*	23.5 (0.7)	22.5 (0.7)
Education (years)	16.1 (0.5)*	14.8 (0.4)	15.8 (0.3)*	15.0 (0.5)
Smoking behavior				
Frequency (days/month)	13.9 (1.9)	15.8 (1.9)	12.3 (1.8)	14.7 (2.1)
Quantity (number of cigarettes/smoking day)	4.3 (0.6)	4.2 (0.4)	3.0 (0.5)	3.9 (0.8)
Estimated nicotine content of preferred brand of cigarettes (mg)	0.91 (0.1)	0.87 (0.0)	0.97 (0.1)	0.95 (0.1)
Drinking behavior				
Frequency (days/month)	12.5 (1.5)	12.1 (1.2)	11.4 (1.6)	11.3 (1.9)
Quantity (number of drinks/drinking day)	6.6 (0.7)	5.1 (0.4)	5.8 (0.7)	4.9 (0.8)
Heavy drinking frequency (number of heavy drinking days/month)[a]	8.2 (1.0)	8.1 (0.9)	6.3 (0.7)	8.2 (2.1)
Lifetime alcohol abuse	12 (80%)	11 (78%)	7 (100%)	4 (67%)
Smoking and drinking behavior				
Average number of cigarettes smoked on:				
Non-drinking days	1.1 (0.4)	1.5 (0.3)	1.4 (0.3)	1.4 (0.8)
Light drinking days[b]	1.6 (0.4)	2.5 (0.5)	2.0 (1.0)	2.0 (1.1)
Heavy drinking days[c]	5.0 (0.9)	5.8 (0.8)	5.0 (1.3)	4.8 (0.9)
Other substance use				
Marijuana (% using once/week or more)	4 (27)%	2 (14%)	4 (57%)	2 (33%)

Data are mean (±SEM) or n (%)

Smoking and drinking data presented are obtained from a timeline follow-back interview

*$p \leq 0.05$ (age and education: men>women; body mass index: alcohol>placebo)

[a] Heavy drinking=5+ drinks/occasion for men, 4+ for women

[b] light drinking days=days in which one to four drinks reported for men, one to three for women;

[c] heavy drinking days=days in which 5+ drinks reported for men, 4+ for women

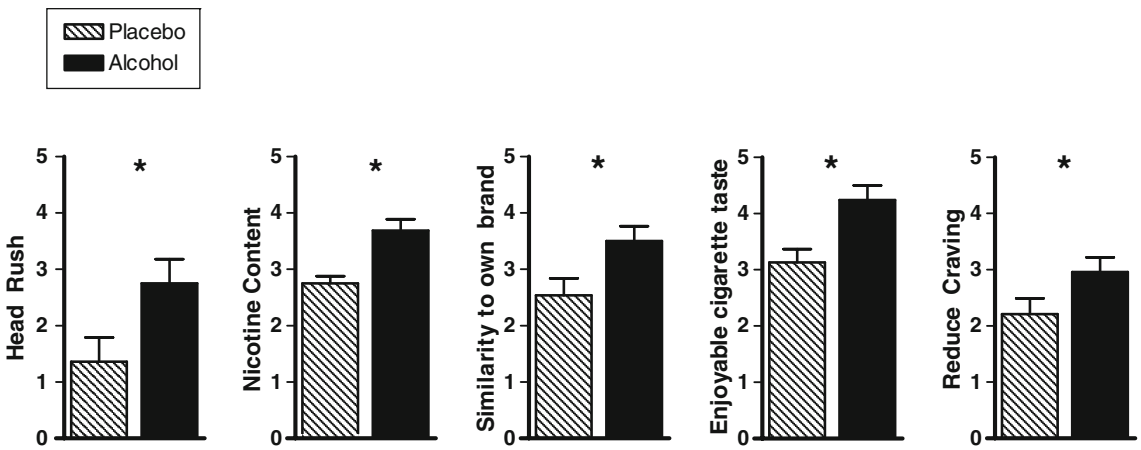


Fig. 2 Subjective responses (mean, SEM) to the second set of reference puffs (collapsed on cigarette type) in the alcohol and placebo conditions. Head rush measured on a visual analog scale (1–10 cm). Nicotine content, similarity to own brand, enjoyable cigarette taste, and reduce craving measured on a Likert scale (1–7). *$p < 0.05$

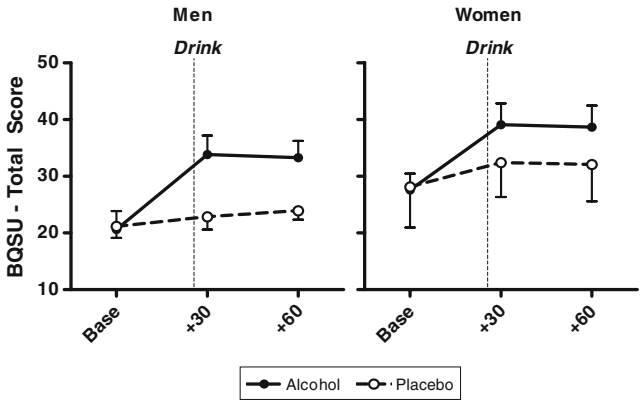


Fig. 3 Smoking urge (BQSU total mean, SEM) in men and women. Time measured in relation to initiation of beverage consumption. $p < 0.001$, smoking urge was higher at 30 min post-beverage consumption as compared with baseline ratings in both men and women

men: alcohol>placebo, $p < 0.01$), puff volume (beverage× sex; $F(1, 35) = 4.99$, $p < 0.05$; simple effects, *men*: alcohol>placebo, $p = 0.01$), and puff duration (beverage× sex; $F(1, 35) = 4.36$, $p < 0.05$; simple effects, *men*: alcohol>placebo, $p = 0.01$). Calculation of partial Eta-squared revealed a medium effect size of the beverage type×sex interaction (cigarettes chosen: $h_p^2 = 0.14$; puff count: $h_p^2 = 0.18$; puff volume: $h_p^2 = 0.13$; puff duration: $h_p^2 = 0.12$; Cohen 1973). For women, there were no differences in smoking choice or topography measures between the alcohol and placebo conditions; in either beverage session, cigarettes chosen, puff count, volume, and duration for women were similar to those observed in men in the alcohol condition (see Fig. 4). In other words, alcohol selectively increased men's smoking behavior but women's smoking behavior did not differ in the alcohol and placebo conditions.

Relationship between subjective and objective measures

Smoking urge during the 60 min after beverage consumption was significantly correlated with subsequent smoking behavior, including both puff volume ($r(38)=+0.36$, $p < 0.05$) and puff duration ($r(38)=+0.38$, $p < 0.05$). These effects were apparent even after controlling for sex and beverage type, as well as age and education, as increases in smoking urge showed moderate relationships to smoking uptake measures, including puff duration ($p < .05$), and puff volume and count ($p \leq 0.08$). However, other subjective measures taken during the initial post-beverage interval, such as feeling stimulated and cigarette pleasure, were not associated with subsequent smoking topography measures.

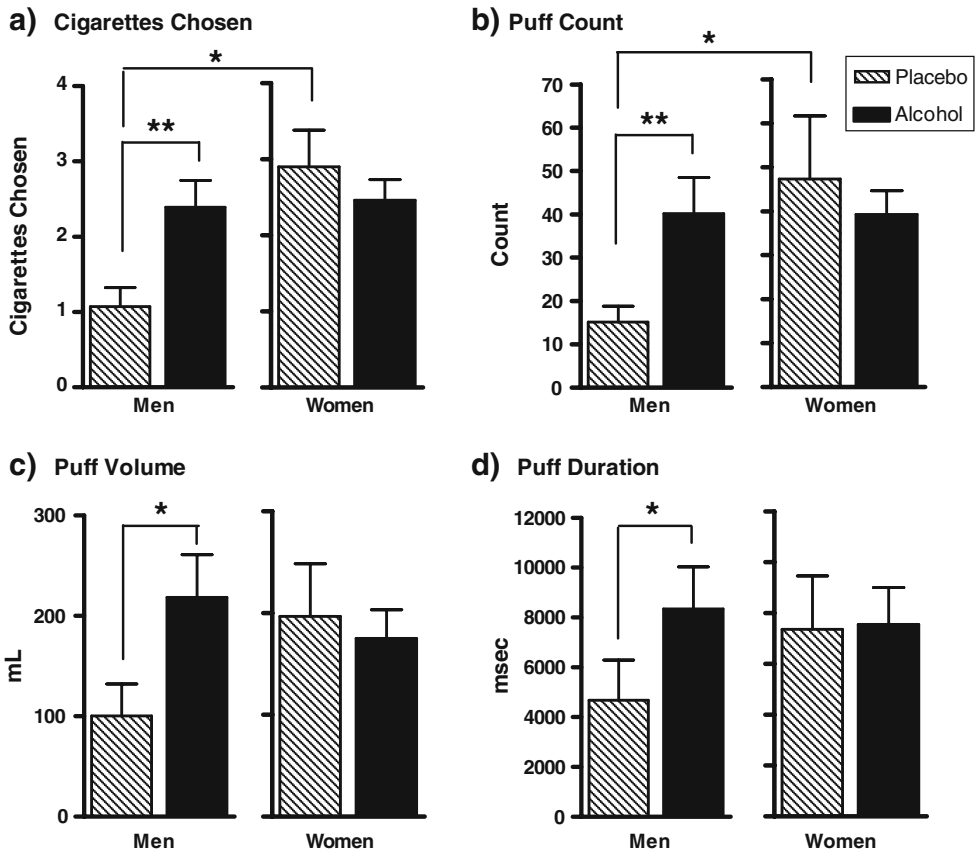


Fig. 4 Smoking choice and topography measures (mean, SEM) for optional cigarettes smoked by men and women. Data collapsed on cigarette type as there were no significant differences between nicotinized and denicotinized cigarettes for choice or topography indices. Cigarettes chosen were averaged across nicotinized and denicotinized sessions. **a** $*p < 0.05$, women showed more cigarettes chosen than men; women showed more cigarettes chosen in the placebo condition than men. **b** $**p \leq 0.01$, alcohol increased puff count in men; $*p < 0.05$, women showed higher puff count in the placebo condition than men. **c** $*p < 0.05$, alcohol increased puff volume in men. **d** $*p < 0.05$, alcohol increased puff duration in men

Discussion

In this study, we have further elucidated differences in alcohol-smoking interactions between men and women non-dependent drinker-smokers. First, we replicated prior findings by our group and others that under well-controlled laboratory conditions, an intoxicating dose of alcohol significantly increases smoking urge (Epstein et al. 2007; King and Epstein 2005; Ray et al. 2007; Sayette et al. 2005), and both men and women are sensitive to this effect (King et al. 2008). Second, we extended this finding to show that both men and women are sensitive to alcohol's effects on numerous subjective ratings (i.e., head rush, enjoyable cigarette taste) after two brief cigarette puffs. Third, we found that in men, but not in women, alcohol significantly increased smoking behavior, as measured by cigarette choice and corroborated by puff count, volume, and duration. So, while women self-reported greater smoking urges after drinking alcohol compared with placebo, their smoking choice behavior and topography did not differ after consuming either beverage, and this smoking level was comparable to men's smoking behavior observed only after alcohol drinking. Fourth, as mentioned earlier, while subjective responses to the nicotinized (0.6 mg yield) reference puffs indicated greater satisfaction and enjoyment compared with the denicotinized (≤0.05 mg yield) reference puffs, subsequent smoking choice in either beverage condition was not preferential to nicotinized versus denicotinized cigarettes.

Taking all the results together, it appears that smoking-drinking interactions are more complex in women than in men. Our results may extend Perkins' (2001) theory that women, compared with men, smoke less to interoceptive factors and more to associated stimuli and psychophysical sensations of smoking (Benowitz and Hatsukami 1998; Perkins et al. 1999). It is unclear why women smoked in the laboratory at similar rates between the alcohol and placebo conditions. The moderate overall relationship observed in this study between smoking urge and behavior, which is similar to other studies, supports the notion that substance use behavior may be influenced by a number of factors beyond craving (For review, see Sayette et al. 2000). For instance, women may be more sensitive than men to the contextual and social factors involved in frequent cigarette choice offerings by the research assistant during the 2-h choice interval, regardless of beverage content. Compared with men, women have been shown to be more sensitive to instructional set stating accurate nicotine content of cigarettes on subsequent smoking reinforcement (Perkins et al. 2006) and to show a lack of association of alcohol-induced smoking urge in the laboratory with real-world co-use of alcohol and cigarettes (King et al. 2008). However, while smoking choice did not differ by beverage group in women, the similarity of topography between groups suggests that this explanation may not fully account for smoking behavior. Another possibility is that women were less sensitive than men to alcohol's effects on respiration and bronchodilation (Sisson 2007), which may affect smoking behavior. Future studies comparing prompted smoking choice versus ad libitum smoking, and also discerning physiological mechanisms, after alcohol or placebo consumption may help determine the contribution of these factors to alcohol-smoking interactions between the sexes.

Another interesting and somewhat surprising finding in the present study is the lack of clear preference, in either alcohol or placebo conditions, for choosing to smoke nicotinized compared with denicotinized cigarettes. While both men and women demonstrated sensitivity to the post-beverage reference puffs for nicotinized cigarettes, including higher smoking desire, satisfaction, and enjoyment, subsequent smoking topography was similar between cigarette types. This finding is in contrast to a prior study showing that lower nicotine dependence may relate to greater reward from nicotine versus non-nicotine cigarettes (Brauer et al. 2001). However, a more recent study demonstrated that the converse relationship, i.e., smoking's impact on subsequent alcohol self-administration, was observed after subjects smoked nicotinized versus denicotinized cigarettes (Barrett et al. 2006). It is has been posited that smoking reinforcement relates to both the sensory aspects of smoking as well as the direct central nervous system effects of nicotine (Rose et al. 2000). In this study, we utilized the Quest® brand of cigarettes because of the similarity in taste and appearance of the nicotinized and denicotinized cigarettes. However, the highest level of nicotine yield from this brand is 0.6 mg, which is lower than the yield of most marketed brands of cigarettes (FTC 2007); therefore, it cannot be ruled out that this lower level of nicotine may have impacted smoking uptake behavior due to inability to reach the threshold of nicotine necessary to activate brain reward pathways and motivational salience (Kalivas and Volkow 2005). While beyond the scope of the present study, future research is needed to discern the potential interaction of taste discrimination, sensory aspects of smoking, and the neurobiological effects of varying levels of nicotine content on social smoking-drinking behaviors.

Although there are many strengths in the present study, including a well-controlled laboratory paradigm with a placebo control, a priori investigation of sex differences, and smoking uptake measurement using a smoking topography device, there are several limitations worth noting. First, the sample size of the placebo group was relatively small. This was chosen because prior studies have shown little to no smoking urge changes after placebo consumption (Epstein et

al. 2007; King and Epstein 2005) and infrequent cigarette smoking on non-drinking days in similar samples of non-dependent drinker-smokers (King et al. 2008). Given the relatively small sample size in the placebo group, caution should be taken to avoid over-interpreting results and replication in larger sample sizes is warranted. Because women's smoking pattern may reflect a more complex interplay between alcohol and smoking behaviors, it would be important for future studies to fully examine factors potentially underlying sex differences, such as social influences and instructional set (Perkins et al. 2006; Rose et al. 1996; Wilson et al. 1995), gonadal hormones, and menstrual cycle effects (Allen et al. 1999; Hamilton and Yonkers 1996), and laboratory versus natural environment assessment of co-use via ecological momentary assessment and other advanced technological tools. Second, we studied only heavy drinking, non-daily smokers, and it is unclear if results may generalize to other drinking and smoking subgroups. Heavy drinkers were of interest in the current study as epidemiological data shows that most non-daily smoking occurs in the context of alcohol use, especially in young adults (Harrison et al. 2008). Other survey-based research has also demonstrated this association, as experimenting and non-daily smokers will delay smoking until after consuming larger quantities of alcohol as compared with their regular smoker counterparts (Harrison et al. 2009). Preliminary data by our group indicates that light/moderate social drinkers may also be sensitive to alcohol's elicitation of smoking urge, although not to the extent of heavier social drinkers (King et al. 2009). Larger studies of alcohol-related smoking urge and behavior within a wider range of drinkers would enable us to understand if the current study findings are specific to heavier drinkers or if they generalize to both lighter drinkers and/or to alcohol-dependent drinkers. Finally, this study measured smoking behavior at maximum BAC levels, when smoking urge has been shown to peak; ascending BAC limb effects of alcohol on smoking behavior were not assessed. Studies of alcohol's effects on smoking behavior throughout the BAC curve would provide more information on the temporal effects of alcohol on smoking choice.

In summary, the present study showed that alcohol and cigarette co-use may involve more complex factors in women compared with men. For men, consuming an intoxicating dose of alcohol, versus a placebo beverage, produced increases in smoking urge and subsequent measures of smoking behavior. In women, while alcohol also increased smoking urge, subsequent smoking behaviors were similar regardless of whether the beverage contained an intoxicating dose of alcohol or a placebo. Interestingly, in both sexes, smoking behavior in the laboratory was not specific to nicotine-containing cigarettes. Future studies with larger sample sizes and more detailed assessment of the complex interplay and development of comorbid alcohol drinking and cigarette smoking will provide valuable information to guide prevention and treatment efforts for the sexes.

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