GENE AND GENE BY SEX ASSOCIATIONS WITH INITIAL SENSITIVITY TO NICOTINE IN NONSMOKERS

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Abstract

Genetic variation may influence initial sensitivity to nicotine (i.e. during early tobacco exposure), perhaps helping to explain differential vulnerability to nicotine dependence. This study explored associations of functional candidate gene polymorphisms with initial sensitivity to nicotine in 101 young adult nonsmokers of European ancestry. Nicotine (0, 5, 10 μg/kg) was administered via nasal spray followed by mood, nicotine reward (e.g. "liking") and perception (e.g. "feel effects") measures, physiological responses, sensory processing (pre-pulse inhibition of startle), and performance tasks. Nicotine reinforcement was assessed in a separate session using a nicotine vs. placebo spray choice procedure. For the dopamine D4 receptor (DRD4 VNTR), presence of the 7 repeat allele was associated with greater aversive responses to nicotine (decreases in "vigor", positive affect, and rapid information processing; increased cortisol) and reduced nicotine choice. Individuals with at least one DRD4 7-repeat allele also reported increased "feel effects" and greater startle response, but in men only. Also observed in men but not women were other genetic associations, such as greater "feel effects" and anger, and reduced fatigue, in the dopamine D2 receptor (DRD2 C957T SNP) TT versus CT or CC genotypes. Very few or no significant associations were seen for the DRD2/ANKK1 TaqIA polymorphism, the serotonin transporter promoter VNTR or 5HTTLPR (SLC6A4), the dopamine transporter 3' VNTR (SLC6A3), and the mu opioid receptor A118G SNP (OPRM1). Although these results are preliminary, this study is the first to suggest that genetic polymorphisms related to function

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in the dopamine D4, and perhaps D2, receptor may modulate initial sensitivity to nicotine prior to the onset of dependence and may do so differentially between men and women.

Keywords

nicotine; sensitivity; genetics; dopamine; reward; reinforcement

INTRODUCTION

Although nearly three-quarters of U.S. adults have experimented with cigarettes, less than half of those ever exposed go on to become nicotine dependent (Anthony *et al*., 1994). Understanding this differential vulnerability to dependence is a major emphasis of research on smoking initiation and prevention (e.g., Audrain-McGovern *et al*., in press). Substantial research supports the heritability of smoking onset, intensity, and persistence (Vink *et al*. 2005), but the specific genes that are responsible remain uncertain.

Prior research suggests that genetic factors potentially related to neurotransmitter function may influence the onset and progression of smoking in adolescents. The A1 allele of the DRD2/ANKK1 TaqIA polymorphism may interact with other vulnerability characteristics, such as depression, to accelerate smoking progression (Audrain-McGovern *et al*. 2004), and with protective factors, such as sports participation, to deter smoking progression (Audrain-McGovern *et al*. 2006). The short allele of the promoter variant in the serotonin transporter gene (SLC6A4 5HTTLPR VNTR) is associated with onset of smoking in teens (Gerra *et al*., 2005). Other studies suggest that the dopamine transporter (SLC6A3) gene (rs27072) A allele is associated with increased risk of early smoking onset (Ling *et al*. 2004). It should be noted, however, that these other findings often have not been replicated (see Munafo et al., 2004 for a meta-analysis).

There is also evidence for associations of these and other specific genetic variants with smoking behavior or clinical outcome in smoking cessation trials with medications in adults (Comings *et al*. 1996; Shields *et al* 1998; Bierut *et al*. 2000; Lerman *et al*. 2003; 2004; 2006; Munafo *et al.,* 2004; 2006; Swan et al. 2007; David *et al*. in press; Yudkin *et al*. 2004), although lack of replication is a concern in this literature as well (e.g., Munafo *et al*. 2004; 2006). Also, the mu opioid receptor polymorphism (OPRM1) has been related to acute preference for nicotine via smoking in women but not men (Ray *et al*. 2006), suggesting some genetic influences may be moderated by subject sex.

The mechanisms by which specific genes may increase risk of onset of nicotine dependence are not clear. Because risk of dependence may be enhanced in those with greater initial sensitivity to nicotine, i.e. upon early exposure, genes may influence risk by enhancing that initial sensitivity. Pomerleau (1995) has proposed a "sensitivity" model, in which teens at greater risk for nicotine dependence experience greater positive, and possibly aversive, responses to nicotine upon first exposure to smoking, compared to those at lower risk. The rationale is that greater responses to early exposure may increase the likelihood of repeated smoking exposures, fostering smoking escalation and dependence. Empirical evidence is limited, but adults who currently smoke retrospectively report having had greater pleasant sensations the first time they ever smoked, compared to adults who never smoked regularly but had some exposure (Pomerleau *et al*., 2004; O'Connor *et al.*, 2005; Hu, *et al.*, 2006). Similarly, among teens who currently smoke, retrospective reports of greater feelings of "relaxed", "high", and "dizziness" from their first cigarette are associated with a more rapid escalation of smoking (Hirschman, *et al*., 1984; Blitstein, *et al.*, 2003). This model follows from animal research showing genetic or other individual differences in initial sensitivity to

nicotine (Marks *et al*., 1991; Schechter *et al*. 1995). Some rat strains that are more sensitive than others to nicotine upon initial exposure may show greater subsequent acquisition of nicotine reinforcement (Shoaib *et al*. 1997; Le *et al*., 2006). (Note that the "exposure" model, an alternative view based on observations in the alcohol literature, makes the opposite prediction, that attenuated initial sensitivity to drugs increases vulnerability to dependence; Schuckit and Smith, 1996.)

Thus, although many other mechanisms for heritability of nicotine dependence are possible, genetic factors may alter risk of nicotine dependence by influencing initial sensitivity to the acute rewarding and reinforcing effects of nicotine. In this study, we examined the association of specific genetic polymorphisms with initial sensitivity to acute nicotine administration in young adult nonsmokers. Because the specific effects of nicotine that promote reinforcement in humans are not known, we examined a wide variety of acute nicotine responses. Candidate genes and SNPs were selected based on the following criteria: (1) genes in pathways implicated in the neurobiology of nicotine, including the dopamine, serotonin and endogenous opioid pathway; (2) within those pathways, genes that have been linked to smoking initiation or nicotine dependence phenotypes in prior research (Lerman *et al*., 2007); and (3) within these genes, polymorphisms with documented functional effects (in vitro or in vivo) and minor allele frequencies >0.10). Based on these criteria, we selected the following polymorphisms: dopamine D4 receptor (DRD4 VNTR), dopamine D2 receptor (DRD2 C957T SNP and DRD2/ ANKK1 TaqIA SNP), the dopamine transporter (SLC6A3 VNTR), the mu opioid receptor exon 1 SNP (OPRM1 A118G), and the serotonin transporter promoter variant (SLC6A4 5HTTLPR VNTR).

METHODS

Participants

Participants were 101 young adult nonsmokers (36 m, 65 f) of European ancestry aged 21–39, with ≤ 10 lifetime tobacco exposures and no use in the prior 3 years. Lifetime tobacco use was assessed twice (for reliability), during an initial telephone screening and at a subsequent in-person interview. Current problem alcohol use was an exclusion criterion, determined by self-report of more than 24 drinks per week. Mean ± SEM age was 25.0±0.4 yrs, 58.4% were college graduates, and 80.2% were single. About half (n=52) had prior tobacco experience (mean of 2.5 lifetime exposures), with the first exposure occurring at 15.7±0.4 years of age, but they had had no tobacco exposure in the past 8.2±0.6 years (range = 3–20 yrs).

Dependent measures

The dependent measures in this study were self-reported reward-related and mood items, physiological responses, sensory processing and attention (startle response, and pre-pulse inhibition of the startle response), task performance, and a nicotine choice reinforcement measure.

Self-report

Spray ratings of nicotine reward, incentive salience, and perception were assessed using visual-analog scale (VAS) items rated 0–100 (anchored by "not at all" and "extremely"). Items tapping nicotine "reward", or its hedonic value (Everitt and Robbins, 2005), were "liking" and "satisfying." Incentive salience was assessed by the item of "want more", adapted from a similar item shown to be sensitive in smokers to duration of smoking abstinence and to individual differences (e.g., Evans *et al*. 1999). Perception of the nicotine content in sprays was assessed by "feel the effects" and "how much nicotine". These items have been shown in other research to be sensitive to nicotine administration and to individual differences in sensitivity to nicotine intake via smoking (e.g., Perkins *et al.* 2006). In addition, a VAS item

assessing nasal "irritation" determined the degree of sensory irritation due to the spray, for use as a covariate.

Mood measures included a) the Positive And Negative Affect Scale (PANAS; Watson *et al.* 1988), with positive and negative affect subscales; b) the Profile of Mood States (POMS; McNair *et al.* 1971), with subscales labeled tension, anger, fatigue, vigor, depression; and c) a series of 12 VAS items (rated 0–100): comfortable, satisfied, pleasant, relaxed, buzzed, jittery, anxious, tired, sedated, alert, stimulated, and nausea. These and similar measures have been used in many prior studies of acute nicotine effects in smokers and nonsmokers (e.g., Perkins *et al.* 2001a; 2001b; Kalman and Smith 2005). POMS scale scores were converted to 0–100 scores to simplify comparison with VAS items.

Physiological responses

Heart rate (HR, in beats per minute), systolic and diastolic blood pressure (BP, in mmHg), were obtained by Dinamap blood pressure recorder (Critikon Inc., Tampa FL). Cortisol was obtained by saliva sample using a Sarstedt salivette (dental swab) that was analyzed by Salimetrics, LLC (State College, PA, www.salimetrics.com).

Sensory processing

Greater magnitude of eyeblink response to a loud acoustic stimulus (i.e. startle) is associated with negative affect (Filion *et al.*, 1998). The degree to which this response is attenuated by a mild acoustic stimulus immediately preceding the startle probe (pre-pulse inhibition, PPI) provides an index of attention to sensory stimuli (Swerdlow *et al.,* 1992). Cigarette smoking has been shown to acutely enhance PPI (Duncan *et al.* 2001), suggesting that nicotine can improve attention and sensory gating, but other studies indicate that smoking may impair PPI (Hutchison *et al.* 2000). Effects of smoking on startle response are unclear (Duncan *et al.* 2001; Hutchison *et al.* 2000). Startle and PPI were assessed by presenting short (50 msec) bursts of rapid onset loud (106 dB) acoustic stimuli either alone (to measure startle) or preceded 120 msec by milder (84 dB) 20-msec bursts (to assess PPI), with background white noise of 75 dB. During each of the 3 dose trials per session, we presented 6 startle and 6 PPI probes in random order, with an inter-trial interval ranging from 9–23 sec (mean of 15 sec). The raw EMG signal was amplified and then filtered using Biopac AcqKnowledge software (Biopac, Goleta CA) to produce a characteristic eye-blink maximum ("max") amplitude and area under the curve ("AUC") for each measure (startle, PPI) and each dose trial. PPI values were expressed as a percent of the startle response obtained during the same trial; smaller values indicate greater inhibition of startle, or greater sensory gating (Swerdlow *et al.,* 1992).

Performance tasks

Performance tasks assessed after each dose administration included finger-tapping speed, handsteadiness, Sternberg rapid information-processing, and memory recall. Nicotine dose effects on finger-tapping speed (increasing), handsteadiness (worsening), and memory recall (curvilinear) have been shown in nonsmokers as well as smokers (Perkins *et al.* 1994a; 2001a). Most of these tasks are described elsewhere in more detail (Perkins *et al.* 2001a). For the Sternberg rapid information processing task, subjects were given one or five "target" letters to retain in short-term memory. They then were to respond as quickly as possible to a series of letter pairs, indicating whether the given letter pair did ("hits") or did not ("correct rejections") contain a target letter. The difference in reaction time in msec between the one- and five-letter trials ("D-prime") on items requiring correct rejection (involving processing of all target letters) was the primary measure of memory scanning speed (information processing; Schneider and Shiffrin, 1977). Responding has been shown to be improved (i.e. faster) by nicotine in smokers under distracting conditions involving auditory presentation of non-target letters while attempting to process target letters presented visually on a computer monitor

(Grobe *et al*. 1998). This task was presented in the current study under both distracting and non-distracting conditions, in random order, following each dose administration.

Nicotine Reinforcement

The relative reinforcing effects of nicotine were determined by the number of nicotine (1.25 µg/kg/spray) versus placebo sprays selected in a choice procedure, described below. Greater nicotine choice via this procedure has been related to greater pleasurable responses to nicotine in nonsmokers, as well as smokers (Perkins *et al.* 2001b). Nicotine choice also increases in smokers with overnight abstinence (Perkins *et al*. 1996), predicts greater withdrawal severity and faster relapse in smokers trying to quit (Perkins *et al*. 2002), and is sensitive to individual differences, including obesity status (Blendy *et al.* 2005) and genetic variation (Ray *et al*. 2006).

Nicotine dosing

Nicotine was administered via a nasal spray procedure developed by us and used in many prior studies (e.g. Perkins *et al*. 1986; 1994a; 2001a). Each participant received 0, 5, and 10 µg/kg doses, with dose administration spread over 8 sprays (2 @ 30 secs). The relatively low 5 and 10 µg/kg doses, which produce plasma nicotine levels comparable to about 1/4 and 1/2 typical cigarette, respectively (Perkins *et al*. 1994a; 2001a), were selected because these are typical of amounts naive individuals are likely to absorb in initial experimentation with smoking (Eissenberg and Balster, 2000), which we were trying to simulate in these assessments. A blood sample was obtained by venipuncture at the end of each session and analyzed for plasma nicotine concentration by gas chromatography with nitrogen-phosphorus detection using 5-methylnicotine as the internal standard (Jacob *et al*. 1981). Mean (±SEM) plasma nicotine levels following the 5 and 10 µg/kg dose sessions were 2.3±0.1 and 3.4±0.2 ng/ml, respectively.

Procedures

All subjects provided informed consent after the nature and consequences of participation were explained. This research was approved by the Institutional Review Board of the University of Pittsburgh Medical Center. Subjects participated in four sessions, three to assess nicotine sensitivity to most responses and a fourth to assess nicotine reinforcement. Upon arrival to the lab for each session, subjects first provided expired-air carbon monoxide (CO) assessment (Vitalograph CO analyzer, Breathco, Inc., Lenexa KS), to verify absence of any recent smoking exposure (CO< 5 ppm), and breathalyzer assessment (Alco-Sensor III breathalyzer, Intoximeters Inc., St Louis MO) to verify no recent alcohol intake (BAL=0.00).

Nicotine Sensitivity Assessment—The first three sessions were virtually identical, differing only in the administered dose of nicotine spray (0, 5, 10 µg/kg). The order of doses across sessions was counter-balanced. Only one of the doses was presented on a given day, and the dose was administered three times per session, once every 30 mins. At the start of each session, subjects rested quietly, followed by a baseline assessment of mood, cardiovascular responses, sensory perception, and the performance measures, in that order. This sequence of measures was repeated for two more baseline trials, one every 30 mins, to habituate to testing. Saliva cortisol was obtained at the end of this baseline period. Then, this assessment sequence, along with the spray ratings (after mood items), was repeated another three times (3 dose trials), again once every 30 mins, with each assessment following nasal spray administration of the dose assigned for that session. A second saliva sample for cortisol analysis was obtained after the third and last spray dose trial.

Nicotine Reinforcement Assessment—At the start of the choice session, participants first engaged in two "sampling" trials. They self-administered the 0 µg/kg or 1.25 µg/kg/spray

bottles 8 times each (i.e. 0 and 10 μg/kg in all, respectively), waited 20 mins, then self-administered the other bottle 8 times, in blind fashion and counterbalanced order. In the subsequent four "choice" trials, one every 20 mins, participants were instructed to choose any combination of the two sprays, such that they self-administered a total of 8 sprays during each trial. The number of times nicotine was chosen (out of 32 total opportunities) was taken as the measure of reinforcement.

Genotyping

We performed the genotyping of the SNPs using the GoldenGate Assay on the Illumina platform (Illumina, San Diego, CA) at the Genomics Core Facility at USC/Norris Comprehensive Cancer Center (Director, David Van Den Berg). The assay utilizes a multiplexed oligonucleotide ligation assay (OLA) on genomic DNA and PCR amplification with universal primers. For additional details see http://www.illumina.com/products/prod_snp.ilmn. Data from the assay array is read by the BeadArray Reader (Illumina) and genotype calling is performed in BeadStudio software (Illumina, Inc.) using the genotyping module. The system includes automated protocols for the entire genotyping process utilizing robotics, barcoding, and extensive data and process tracking to ensure high call rates and accuracy. Additionally, genotyping quality control was further monitored by the inclusion of replicates and CEPH trios. Amplification by PCR was as described elsewhere (George *et al.,* 1993; Lesch *et al.,* 1996; Vandenbergh *et al*., 2002). Results were analyzed using GeneMarker® v1.5 (SoftGenetics) software.

The coding of genotypes for analysis, based on prior literature, was as follows: 1) DRD2 C957T, rs6277 (CC vs. CT vs. TT); 2) 5HTTLPR (or SLC6A4, presence or absence of the short allele); 3) DRD4 VNTR (presence or absence of the 7-repeat allele); 4) SLC6A3, rs27072 (presence or absence of the 9-repeat allele); 5) OPRM1 A118G, rs1799971 (presence or absence of the G allele); and 6) DRD2/ANKK1, rs1800497 (TT or CT vs. CC; note: T is the less common Taq1A allele).

Data Analysis

All analyses were conducted using SPSS 15.0. For most measures, response to each dose (0, 5, 10 μg/kg) was defined by change from pre-dose baseline to the post-dose mean of responses across the three dose administrations per session. Cortisol response was taken as the difference between the baseline sample and the single post-session sample. For spray ratings of reward and perception, and for startle and PPI, responses to each dose were examined directly, rather than as changes from baseline. Reinforcement was determined simply by the number of times nicotine spray (1.25μg/kg/spray) was chosen versus placebo spray on the separate session devoted to this measure.

We first determined any influence of the order of doses (6 orders) across sessions on responses, but found no such order effects, allowing us to collapse across order. Differences in responses to nicotine dose as a function of each gene were analyzed separately for each dependent measure using analysis of covariance (ANCOVA). Gene and sex were the between-subjects factors, and dose was the within-subjects factor. Only two effects were of interest: the interactions of gene x dose, which would suggest differential sensitivity across nicotine doses associated with genotype, and gene x dose x sex, indicating that the genetic association varies between men and women. The measure of sensory irritation from the spray was included as a covariate in all analyses. Because of the numerous mood and performance measures, analyses of effects on those responses were first conducted using multivariate ANCOVA (MANCOVA), to reduce the number of comparisons. Significant MANCOVA results were followed up with univariate ANCOVAs. For the reinforcement measure, there was no dose effect (i.e. subjects

chose either nicotine or placebo), and so ANCOVAs analyzed the influences of gene and gene x sex.

RESULTS

The distribution of genotypes for each gene, by sex, is presented in Table 1. Because no analyses were significant for the OPRM1 A118G SNP, this gene will not be discussed further.

Nicotine Reward and Perception

Nicotine reward and perception measures were associated with genetic variation, but only in men. The interaction of 5HTTLPR x dose x sex was significant for "liking", $F (2,190) = 3.20$, $p = .043$. As shown in Figure 1, compared to placebo, 5 μg/kg (but not 10 μg/kg) nicotine decreased liking more in men in the absence versus presence of the short allele. The nicotine perception measure of "feel the effects" was associated with the interactions of DRD2 C957T x dose, $F (4,184) = 2.49$, $p = .045$, DRD2 C957T x dose x sex, $F (4,184) = 2.55$, $p = .041$, and DRD4 x dose x sex, $F (2,190) = 4.26$, $p = .015$. As also shown in Figure 1, "feel the effects" was increased in men by 5 μg/kg nicotine more among those with the DRD2 C957T TT versus CT or CC genotype, and by 10 μg/kg nicotine more among those with the DRD2 C957T TT or CT versus CC genotype. Nicotine also increased "feel the effects" in men more in the presence versus absence of the DRD4 7 allele (Figure 1). Neither gene was associated with this response in women.

Mood

In the MANCOVAs of mood responses, DRD4 x dose, $F(24,360) = 1.63$, $p = .033$, and the interaction of DRD2 C957T x dose x sex, $F(48, 704) = 1.39$, $p = .045$, were significant. No other genetic associations with mood responses were apparent from the multivariate analyses. In follow-up univariate analyses, DRD4 x dose effects were significant for buzzed, positive affect, and vigor, $F(2,190)$'s= 6.15, $p = .003$, 4.44, $p = .013$, and 3.18, $p = .044$, respectively. As shown in Figure 2, nicotine increased buzzed and decreased positive affect and vigor more among those with the DRD4 7 allele versus those without the 7 allele. Regarding DRD2 C957T, the interaction effects of DRD2 x dose x sex were significant for fatigue and anger, $F (4,184)$'s = 2.56, p=.040, and 2.68, $p = .033$, respectively. In men, but not women, those with the TT allele showed decreased fatigue in response to 5 μg/kg nicotine and increased anger in response to both doses, compared to those with the CT or CC allele (Figure 2).

Physiological

No significant effects of gene x dose, or of gene x dose x sex, were seen for the cardiovascular responses to nicotine. An interaction of DRD4 x dose, $F(2,91) = 3.16$, p=.047, was seen for the change in saliva cortisol concentration. As shown in Figure 3, cortisol increased in response to 10 μg/kg in those with the DRD4 7 allele, compared to those without that allele.

Sensory processing

Startle response was associated with DRD4 and DRD2/ANKK1, while PPI was associated with DRD2 C957T, SLC6A3, and DRD2/ANKK1. Interactions of DRD4 x dose x sex were found for both startle measures: area under the curve (AUC), $F(2,152)= 3.51$, p=.032, and maximum peak (max), $F(2,152)= 5.32$, p=.006. Similarly, interactions of DRD2/ANKK1 x dose x sex were found for startle max, $F(2,140)=3.66$, p=.028. As shown in Figure 4, startle response of men to 5 μg/kg, but not 10 μg/kg (i.e. curvilinear), was greater in those with the presence of the DRD4 7 allele and in those with the DRD2/ANKK1 CC allele. (For DRD4, only max startle is shown in Figure 4, to save space. Results for AUC startle were virtually identical.)

For the PPI AUC measure, interactions of gene x dose were seen for DRD2 C957T, F(4, 146) = 2.56, p=.041, SLC6A3, F(2,152) = 4.32, p=.015, and DRD2/ANKK1, F(2,140)=4.55, p=.012. Similarly, for the PPI max measure, the gene x dose interaction was significant for SLC6A3, F(2,152) = 4.96, p=.008, and DRD2/ANKK1, F(2,140)=3.01, p=.052. PPI tended to worsen (i.e. reduced inhibition of startle) in those with the DRD2 C957T CT genotype (at 5 μg/kg only), with the absence of the SLC6A3 9 allele, and with the DRD2/ANKK1 CC genotype (at 10 μg/kg only), compared to the other genotypes (Figure 4). (For SLC6A3 and DRD2/ANKK1, only the AUC measure of PPI is shown in Figure 4, to save space. Results for max PPI were virtually identical.)

Performance tasks

MANCOVAs showed that only DRD4 x dose effects were significant for performance task responses, F(26,354) = 1.56, p=.043. In follow-up univariate analyses, DRD4 x dose effects were significant only for Sternberg task performance (correct rejections) under either the distraction, F (2,188) = 3.37, p=.037, or non-distraction (i.e. standard) conditions, F(2,188) = 3.01, p=.052, although the pattern of results differed depending on the distraction condition. As shown in Figure 5, compared to those with the DRD4 7 allele, absence of the 7 allele was associated with better performance in response to 5 μg/kg nicotine under the distraction condition, and with better performance in response to 10 μg/kg under the non-distraction condition.

Reinforcement

Nicotine choice was associated only with DRD4, F(1,95) = 5.83, p=.018. Those with an absence of the DRD4 7 allele chose nicotine more than those with presence of the 7 allele (13.4±1.3 vs 8.1±1.7 respectively). No gene or gene x sex effects were significant for the other genes examined. However, in an exploratory analysis, the interaction of 5HTTLPR x sex was marginally significant, F(1,95) = 3.02, p=.085, as men chose nicotine twice as often as did women among those homozygous for the long allele (15.9±2.9 vs 7.7±2.2), while there was no sex difference among those with the short allele (11.8±2.3 vs 11.6±1.6 for men and women, respectively).

DISCUSSION

To our knowledge, this study represents the first test of the association between genetic variation and initial sensitivity to acute nicotine responses in humans (i.e. in those essentially naïve to nicotine). Results should be considered very preliminary, given the relatively small sample and the number of genes and comparisons examined. However, our findings suggest that genetic variants purported to influence dopamine receptor function, particularly DRD4, were associated with initial sensitivity to mood and other effects of acute nicotine administration, and these effects often differed as a function of subject sex.

For DRD4, compared with those without the 7 allele, presence of the 7 allele was associated with increased sensitivity to mood (buzzed, decreased vigor and positive affect), nicotine perception ("feel effects"), and startle responses, but only in men. Regardless of sex, presence of the DRD4 7 allele was also associated with increased cortisol response and impaired rapid information processing performance. Together with the observed lower nicotine choice behavior, these findings suggest that presence of the DRD4 7 allele is associated with greater aversive responses to nicotine. Two prior studies in adult smokers have shown that the 7 allele is associated with increased risk of relapse in smoking cessation treatment (Shields *et al.,* 1998; David *et al.*, in press). Thus, our data suggest that greater aversive responses in the DRD4 7 group may reflect increased sensitivity to nicotine's effects, which may be protective with regard to smoking onset in teens. However, among those who become smokers, those carrying

the DRD4 7 repeat allele may become tolerant to these initially aversive responses, fostering greater subsequent risk of nicotine dependence. This notion is consistent with the "sensitivity" model (Pomerleau 1995) and with preclinical studies suggesting that rodent strains most sensitive to nicotine upon initial exposure tend to show the greatest and most rapid tolerance to such effects (Marks *et al.* 1991). Yet, far more research is needed before the "sensitivity" model, and DRD4 gene associations with sensitivity, can be adequately evaluated.

Associations of nicotine sensitivity with other genes believed related to dopamine function were less commonly found. In other analyses, we examined two putative functional SNPs related to the D2 dopamine receptor, DRD2 C957T and DRD2/ANKK1, and polymorphisms in the dopamine transporter, SLC6A3. Similar to DRD4, the DRD2 C957T variant was associated with anger and fatigue responses, as well as nicotine perception, across nicotine doses but only in men. The C957 allele has been associated with increased mRNA stability in vitro (Duan *et al.,* 2003), while the T957 allele has been associated with increased receptor binding potential in vivo (Hirvonen *et al.* 2004). Thus, our findings appear more consistent with prior in vivo data than prior in vitro data. The second SNP (DRD2/ANKK1), located about 10kb upstream of DRD2 in the kinase gene ANKK1, was related only to startle (in men) and PPI responses to nicotine. Similarly, the dopamine transporter SLC6A3 variant was related only to PPI response.

Very few significant associations with nicotine sensitivity were seen for the genes not believed to be linked to dopamine function. The serotonin transporter 5HTTLPR variant was associated with nicotine reward (liking) in men but not in women. Similarly, in an exploratory comparison, among those homozygous for the 5HTTLPR long allele, men tended to choose nicotine more than did women, while there was no sex difference among those with the short allele. Finally, none of the genetic factors was associated with cardiovascular responses to nicotine, and none of the phenotypes examined was associated with the OPRM1 A118G SNP. While the absence of a finding for OPRM1 A118G may appear inconsistent with prior associations with smoking behavior and cessation (Lerman *et al.*, 2003; 2004; Ray *et al.,* 2006), these previous studies focused on nicotine dependent adults while the present study focused on nicotine sensitivity in nicotine-naïve subjects. Thus, as with our findings regarding DRD4, discussed previously, the role of these genes may be dependent on the particular smoking phenotype examined, such as vulnerability to onset of dependence versus smoking persistence after the establishment of dependence.

Our results may have implications for understanding sex differences in the discriminative stimulus effects and perhaps other effects of nicotine, as some research with dependent smokers indicates such effects are less pronounced in women compared to men (Perkins 1999; in press). We found that DRD2 and DRD4 variants influenced nicotine perception ("feel effects"), as well as some mood ("anger", "fatigue") responses, but only in men and not women. These observations suggest that, rather than being due simply to sex per se, many differences in nicotine responses between men and women may be mediated, or at least moderated, by particular genes. Although other research suggests that sex differences in nicotine responses of smokers may be moderated by genes (Yudkin *et al.* 2004; Ray *et al.* 2006), our results go beyond those findings to indicate that genes may moderate sex differences in responses among nicotine naïve individuals, prior to the onset of dependence.

Aside from the novelty of our study focus, genetic associations with initial nicotine sensitivity in never-smokers, many of the methods of this study were novel and constitute key strengths. One strength was our conservative lifetime tobacco use cutoff for inclusion (<10 uses), as well as exclusion of anyone with any use in the prior 3 years, to minimize the possibility of chronic adaptation to nicotine effects (i.e. tolerance or sensitization; Perkins 2002), which would alter subjects' sensitivity across doses. Our criterion is more stringent than that used in most other

research, which typically defines "ever-smoker" as those with at least 100 lifetime uses (e.g. Giovino 2002), while those with less than 100 are non- or never-smokers. Another strength of the study was the prospective assessment of nicotine responses, which included standardized measures of responses to nicotine other than self-report, as opposed to the retrospective self-report of early responses to nicotine via smoking in past studies of initial sensitivity (e.g. Pomerleau *et al*. 1998). The broad array of responses was also a strength, allowing us to determine whether genetic associations may be specific to some but not other nicotine effects, although the large number of responses also raises concerns about the number of comparisons (see below). Other strengths of this study included the controlled dosing of nicotine and correction of doses for body weight, inclusion of placebo and more than one dose of nicotine, and use of nasal sensory irritation as a covariate to rule out differential irritation during testing as an explanation for differences in nicotine sensitivity.

On the other hand, this study had several clear limitations. One major limitation was the relatively small sample size of 101, which produced small genetic subgroups, although this sample is larger than past research assessing responses to nicotine in nonsmokers (see Perkins 2002). This problem reflects the practical conflict between studying a limited amount of data from a large sample versus extensive phenotyping of a smaller sample (e.g., prospectively assessing many different responses to multiple nicotine doses across sessions, as in this study). As such, we may have had inadequate power to detect other genetic influences on acute nicotine responses. Somewhat in contrast, another major limitation is that some of the findings could have occurred by chance, given that we examined 6 genes, multiplying the number of comparisons for each measure of interest. To reduce the likelihood of chance findings, we focused solely on just two effects for each gene, the interactions of gene x dose and gene x dose x sex, given our interest in determining the association of genes with response to nicotine dose and how sex may moderate that association. We also limited the number of comparisons for mood and performance measures by employing multivariate analyses, conducting analyses of individual mood and performance responses only when the overall multivariate results were significant for either of these interactions involving gene x dose. The fact that many of the significant interactions involved DRD4 suggests that results for that gene were not due to chance, although the few significant effects for the other genes could have been due to chance. Given the complete lack of prior research on genetic associations with prospectively assessed initial nicotine sensitivity, this study was not intended to be definitive but rather exploratory and heuristic in nature. This study should be replicated with larger samples to verify the findings, particularly those for DRD4.

In addition, despite its advantages, our nicotine administration procedure was also a limitation of the study. We chose this nicotine nasal spray procedure to provide a fairly rapid method of delivery of controlled nicotine doses. However, sensitivity to nicotine via nasal spray may not relate to sensitivity to nicotine via cigarette smoking, although one study with smokers suggested generally comparable acute mood responses to nicotine between these methods (Perkins *et al*. 1994b). Even more rapid uptake of nicotine by smoking, or stimuli accompanying smoking (e.g. taste, smell) but not nasal spray, may influence genetic associations with initial sensitivity to nicotine. Thus, although our method may have allowed for assessment of initial sensitivity to nicotine per se, sensitivity to nicotine via cigarette smoke inhalation may be affected differently by genetic factors. In addition, the doses used were fairly low, and greater influences of genetic factors on nicotine sensitivity may be more apparent in acute testing with larger doses of nicotine. Finally, we recruited young adults, rather than adolescents, due to ethical and practical concerns about giving nicotine to naïve adolescents. Adolescence is the age at which those who become smokers usually experience initial nicotine exposure, and genetic factors may have more influence on nicotine sensitivity at this age.

In summary, although these findings are preliminary and require replication, they suggest that initial sensitivity to some acute responses to nicotine is associated with specific genetic variants thought to be related to dopamine function, especially in men. In particular, these findings suggest that presence of the DRD4 7 allele is associated with increased sensitivity to nicotine's aversive effects. Gene variants thought to be linked to the function of the dopamine D2 receptor showed some association with other nicotine responses, but most of the genes examined here were not consistently associated with initial nicotine sensitivity. Also, these genetic associations may be specific to only this phenotype, initial sensitivity to nicotine, and may not be seen in other phenotypes related to tobacco exposure (e.g. persistence of smoking). Future research should examine whether these genetic factors influence initial sensitivity to nicotine via smoking, if possible to do ethically and practically in a naïve sample, and should explore whether these genes are related to risk of nicotine dependence in teens with any past exposure to tobacco. Such findings may demonstrate that variability in initial sensitivity to nicotine is a mechanism by which genetic factors promote vulnerability to onset of nicotine dependence.

Acknowledgments

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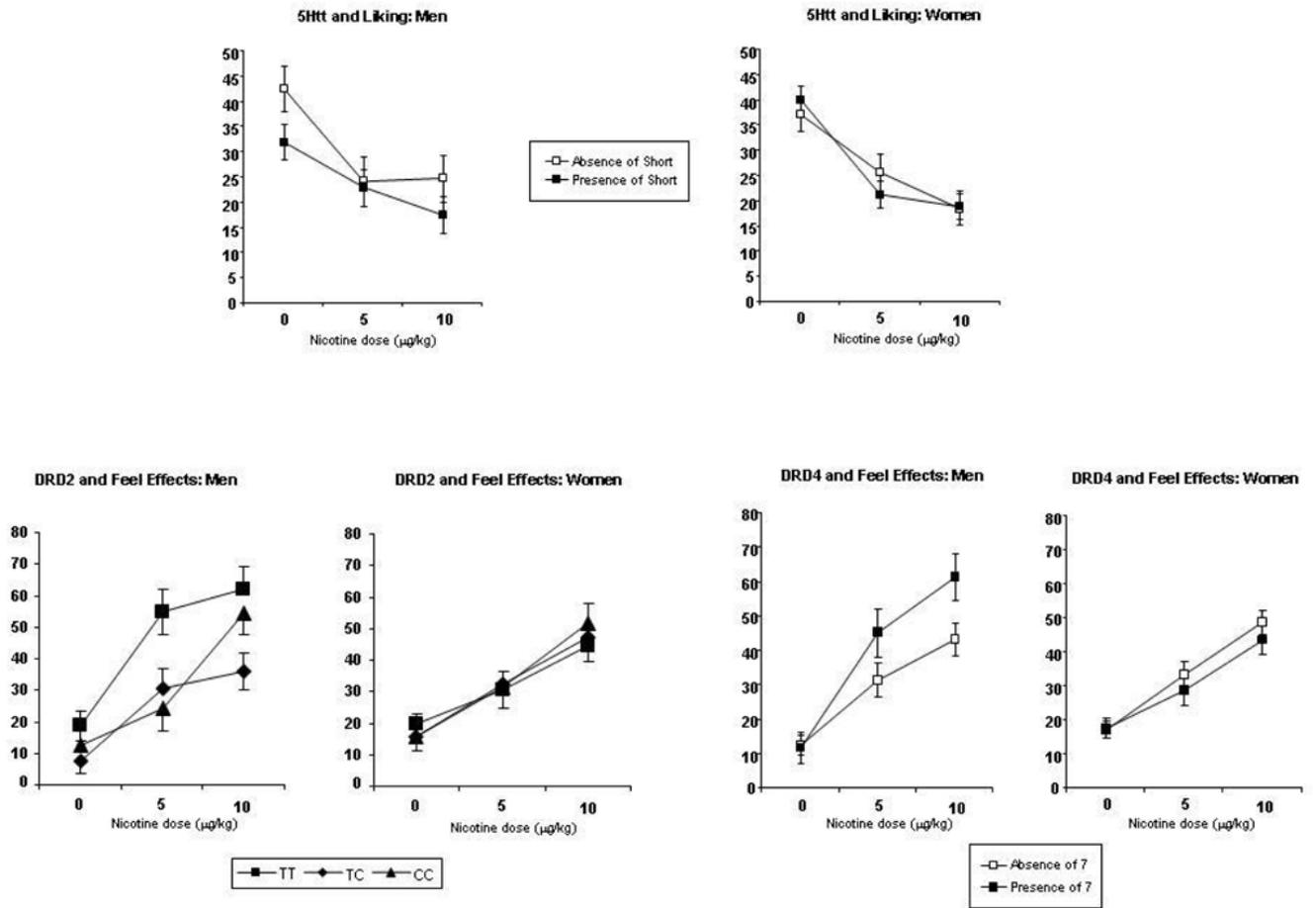
Figure 1.
Mean (SEM) dose-response effects of nicotine (0, 5, and 10 μg/kg) on nicotine spray reward ("liking") and perception ("feel the effects"), by sex and 5HTTLPR (SLC6A4), DRD2, and DRD4 genotypes.
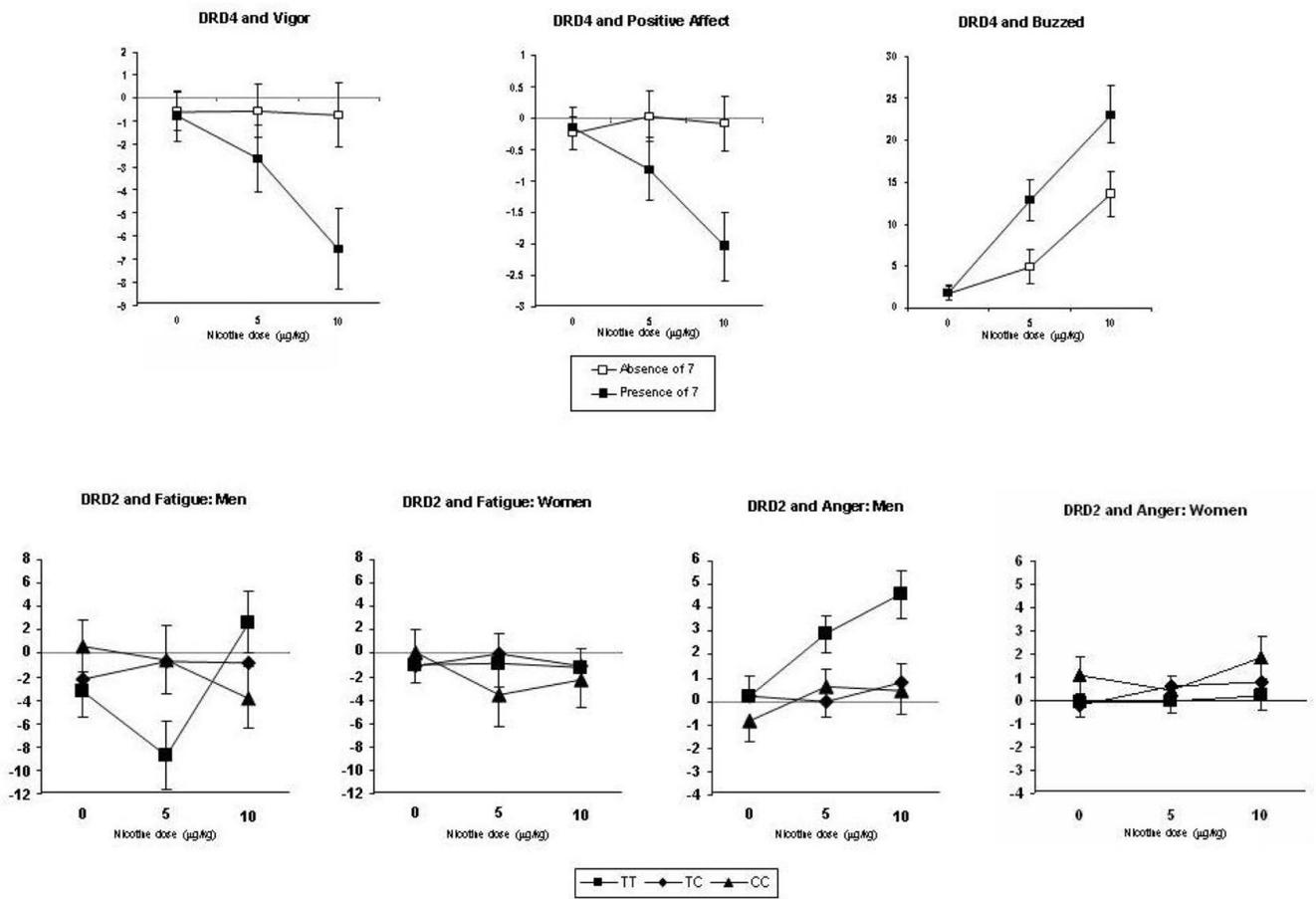
Figure 2.
Mean (SEM) dose-response effects of nicotine on mood responses, by DRD4 and DRD2 C957T
genotypes and, where relevant, by sex.

Figure 3.
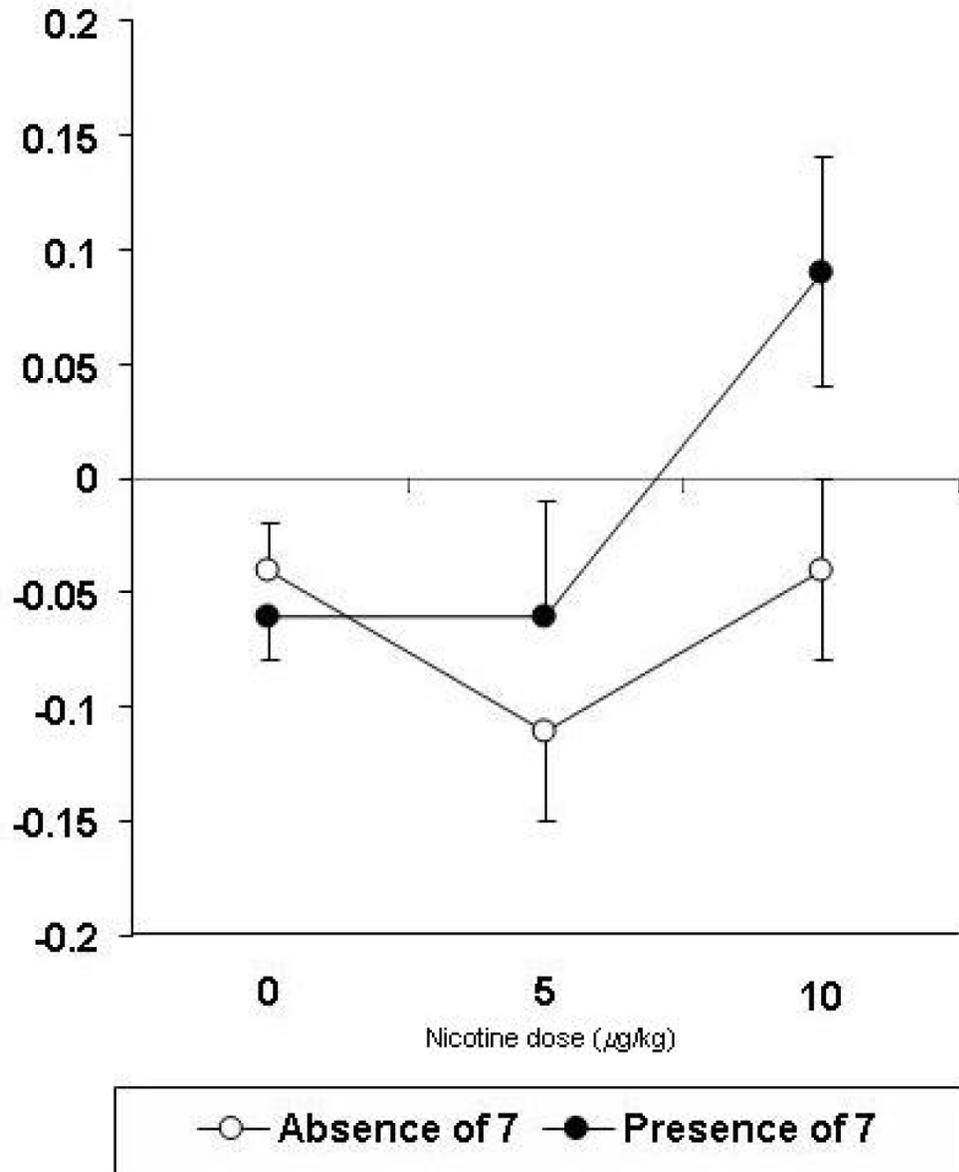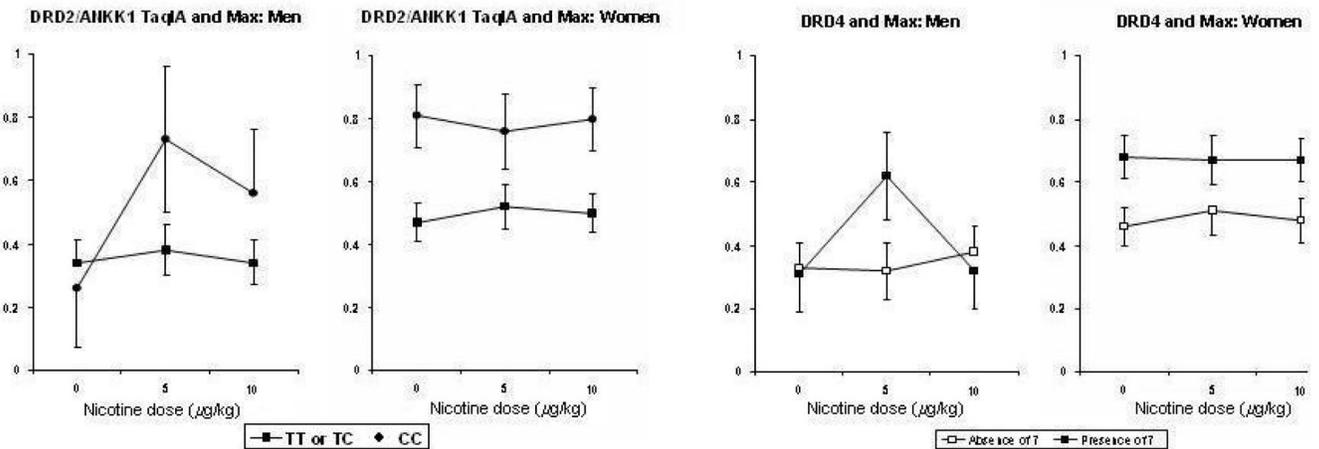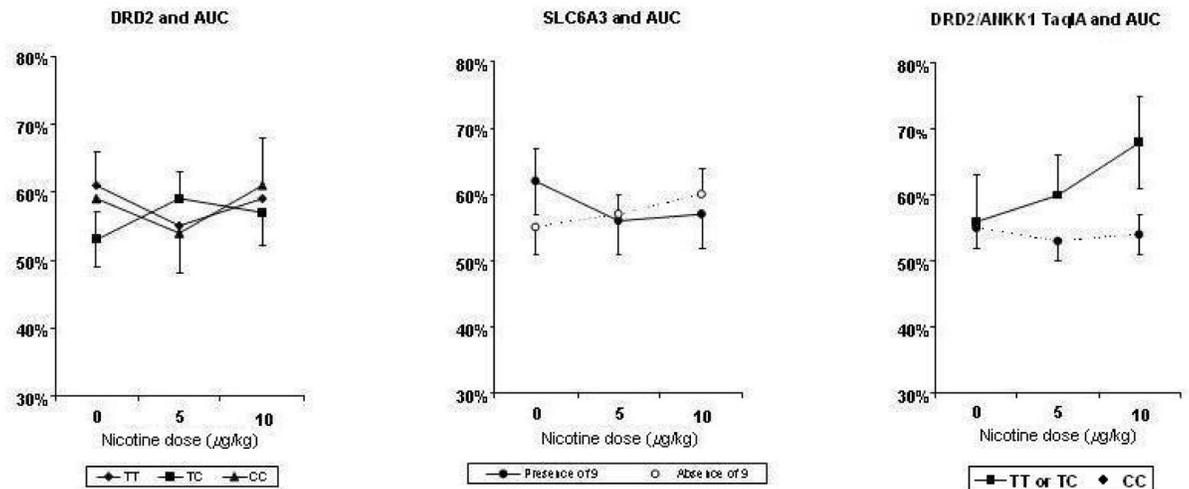Mean (SEM) dose-response effects of nicotine on cortisol responses (ng/ml) by DRD4 genotypes.

Figure 4.
Mean (SEM) dose-response effects of nicotine on startle response (top) and on pre-pulse inhibition (PPI) of startle (bottom), by DRD4, DRD2 C957T, SLC6A3, and DRD2/ANKK1 genotypes and, where relevant, by sex. Larger values indicate greater startle (top) and reduced inhibition of startle (bottom). DRD4 effects on the AUC measure of startle (not shown) were virtually identical to those shown here for max startle. Similarly, SLC6A3 and DRD2/ANKK1 effects on the max measure of PPI (not shown) were virtually identical to those shown here for the AUC measure of PPI.
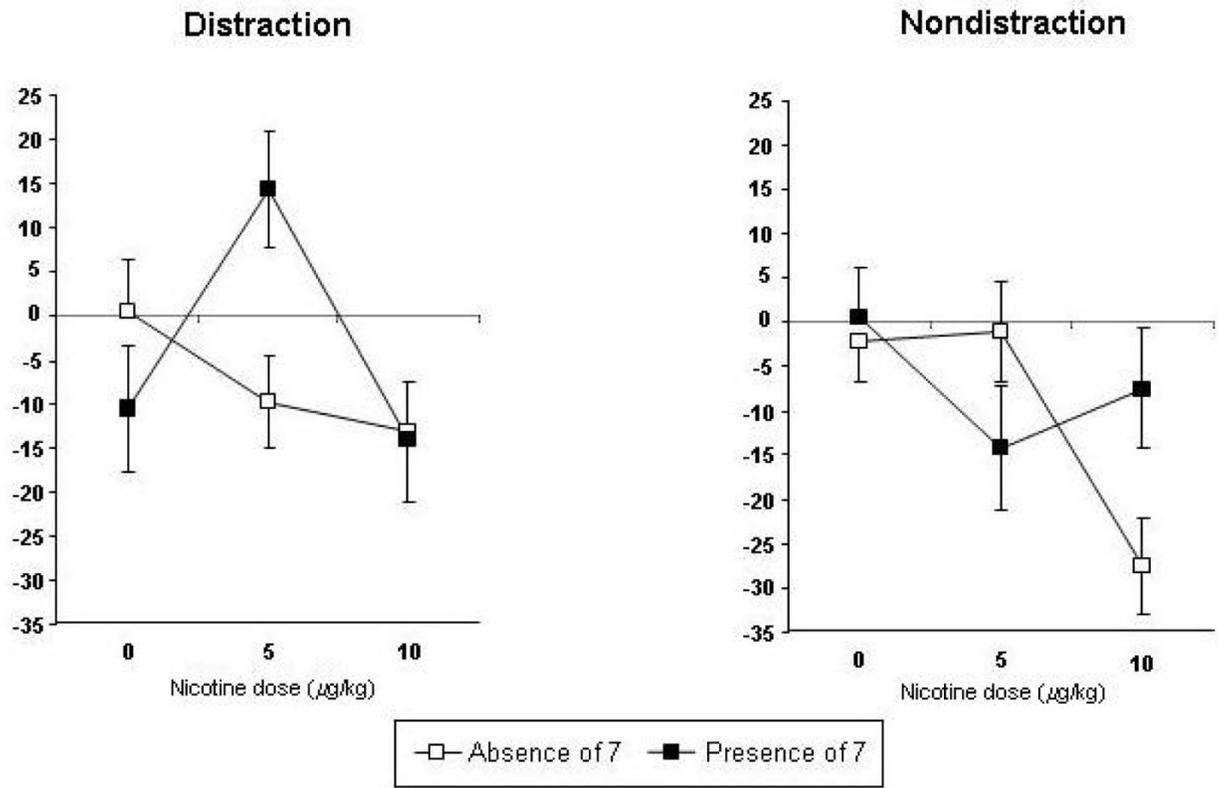
DRD4 and Sternberg information processing

Figure 5.
Mean (SEM) dose-response effects of nicotine on Sternberg rapid information processing performance (d-prime for "correct rejections" of non-targets), under non-distraction (standard) and distraction conditions, by DRD4 genotypes. Values are change from baseline in reaction time, in msec. Decreases indicate faster information processing.

Table 1

Frequencies of alleles for each gene.

		Males	Females	Total
DRD2 C957T	TT	10 (28.5%)	23 (36%)	33
	CT	15 (42%)	29 (45%)	44
	CC	10 (28.5%)	12 (19%)	22
5HTTLPR (SLC6A4)	Absence of Short	13 (37%)	23 (35%)	36
	Presence of Short	22 (63%)	42 (65%)	64
DRD4	Absence of 7	24 (69%)	37 (57%)	61
	Presence of 7	11 (31%)	28 (43%)	39
SLC6A3	Absence of 9	13 (37%)	24 (37%)	37
	Presence of 9	22 (63%)	41 (63%)	63
OPRM1	AA	30 (86%)	47 (73%)	77
	AG or GG	5 (14%)	17 (27%)	22
DRD2/ANKK1	TT or TC	10 (29%)	18 (30%)	28
	CC	24 (71%)	42 (70%)	66