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The Serotonin Transporter Gene and Startle Response During Nicotine Deprivation

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Abstract

Affective startle probe methodology was used to examine the effects of nicotine administration and deprivation on emotional processes among individuals carrying at least one s allele versus those with the l/l genotype of the 5-Hydroxytryptamine (Serotonin) Transporter Linked Polymorphic Region, *5-HTTLPR* in the promoter region of the serotonin transporter gene [Solute Ligand Carrier family 6 member A4 (*SLC6A4*) or SERT]. Smokers (n = 84) completed four laboratory sessions crossing deprivation (12-hour deprived vs. non-deprived) with nicotine spray (nicotine vs. placebo). Participants viewed affective pictures (positive, negative, neutral) while acoustic startle probes were administered. We found that smokers with the l/l genotype showed significantly greater suppression of the startle response when provided with nicotine vs. placebo than those with the s/s or s/l genotypes. The results suggest that l/l smokers, who may have higher levels of the serotonin transporter and more rapid synaptic serotonin clearance, experience substantial reduction in activation of the defensive system when exposed to nicotine.

Keywords

affect; *5-HTTLPR*; *SERT*; *SLC6A4*; smoking; startle probe

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The following IAPS slides were used: Positive, 4220, 4652, 4658, 4659, 4660, 4670, 5621, 5629, 8030, 8370, 8490, and 8500; Neutral, 7000, 7010, 7020, 7030, 7040, 7050, 7060, 7080, 7090, 7100, 7150, and 7170; Negative, 3010, 3060, 3100, 3120, 3130, 3150, 3170, 3500, 6230, 6350, 6560, and 9410. The subjects were asked to view each slide and then rate the slide on affective valence (positive, negative), arousal (low, high), and craving (low, high) by selecting a box on a Likert scale ranging from 1 to 9. Results are presented in Cinciripini et al. (2006).

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Introduction

Several neurotransmitters have been implicated in the effects of short-term nicotine administration and deprivation, including serotonin (5-HT; Benowitz, 2008). Serotonin is involved in the regulation of many brain functions, including sleep, cognition, sensory perception, motor activity, temperature regulation, mood, appetite, sexual behavior, and hormone secretion (Murphy et al., 2008). The diversity of the physiologic function of 5-HT is likely due to the fact that it orchestrates the activity and interaction of several other neurotransmission systems (Lesch & Mossner, 1998). Studies have shown that 5-HT function has broad developmental effects, promoting differentiation of serotonergic as well as glutaminergic neurons (Pezawas et al., 2005). Additionally, 5-HT levels are considered to be critical for the emotional development of the brain and can modify neural connections implicated in an increased risk for mood and anxiety-related disorders (Gross & Hen, 2004; Gaspar, Cases, & Maroteaux, 2003).

Within the central 5-HT system, the serotonin transporter (5-HTT) is thought to regulate the magnitude and duration of serotonergic signaling by governing the reuptake of serotonin from the synaptic junction. The transporter is encoded by a single gene, the solute ligand carrier family 6 (neurotransmitter transporter, serotonin) member A4 (*SLC6A4*), on chromosome 17q11.1-q12, the promoter region of which contains a 44-bp insertion/deletion polymorphism, also known as the *5-HTTLPR*. Research suggests that the long (l) allele of this polymorphic region is more than twice as active as the short (s) allele (Heils et al., 1996; Lesch et al., 1996), resulting in increased transcription of the serotonin transporter, and the potential for increased reuptake of serotonin from the synaptic cleft, and overall lower levels of serotonin in the synapse (Murphy et al., 2001), though not all studies have reported this functional relationship in humans (Shioe et al., 2003; Willeit et al., 2000). The *5-HTTLPR* has been linked to alcohol abuse, schizophrenia, depression, bipolar disorder, and smoking, though with inconsistent results (Serretti, Calati, Mandelli, & De, 2006). Based on the literature, it seems that individuals with extremely high or extremely low serotonin levels may be at risk for affective disorders, alcoholism, anxiety disorders, and personality characteristics such as neuroticism (Lesch & Mossner, 1998; Serretti et al., 2006; Gonda et al., 2009; Murphy et al., 2008).

A recent meta-analysis (Munafo, Clark, Johnstone, Murphy, & Walton, 2004) examining the roles of many candidate genes and smoking behaviors suggested that individuals with at least one copy of the s allele of the *5-HTTLPR* were less likely than those without to successfully quit smoking after behavioral treatment. Another study reported that individuals with at least one s allele and high levels of neuroticism were more nicotine dependent and more likely to report smoking to reduce negative affect than those with the l/l genotype and individuals with low levels of neuroticism (Hu et al., 2000). On the other hand, at least three studies have reported a positive association between the l allele of the *5-HTTLPR* and smoking (versus never smoking; Ishikawa et al., 1999; Kremer et al., 2005; Arinami et al., 1999), or no relationship at all (David, Munafo, Murphy, Walton, & Johnstone, 2007; Trummer et al., 2006; Gonda et al., 2009; Rasmussen, Bagger, Tanko, Christiansen, & Werge, 2009). Another study demonstrated that smokers exhibited higher 5-HT transporter availability in the brainstem than non-smokers (Staley et al., 2001), an area in which 5-HT transporter function may be altered in individuals with mood disorders (Willeit et al., 2000).

Given conflicting data associating the serotonin system with the regulation of emotion as well as the well-documented association between negative affect and smoking (Copeland, Kulesza, & Hecht, 2009; Leventhal, Waters, Kahler, Ray, & Sussman, 2009), it may be that the gene exerts an effect on smoking behavior in complex ways that are not completely understood but which at some level effect neurobiological pathways shared by both nicotine

and serotonin. Differences in behavioral phenotypes associated with smoking may be partly related to the effects of the gene on serotonin transport (and availability) which in turn, affect the activation/suppression of very fundamental neurobiological mechanisms related to processing of emotional information. These emotional processing systems and structures are likely to be very primitive in the sense that they are phylogenetically very old and are activated by motivationally relevant stimuli that predispose the organism toward appetitive (approach) or defensive (avoidance) behavior. A paradigm such as affective picture modulation using the acoustic startle response (ASR) may provide a sensitive measure of the functional impact the *5-HTTLPR* genotype on brain mechanisms associated with appetitive and defensive systems, particularly in response to events that have clear motivational relevance to the smoker: nicotine administration and deprivation.

The startle (eye blink) response is a defensive reflex, which occurs during the presentation of an unexpected auditory stimulus (probe). Its strength is measured by the electromyographic (EMG) changes in the orbicularis oculi region of the eye and is highly sensitive to ambient emotional cues. It is well established that the magnitude of the blink (EMG) response to the startling acoustic probe is enhanced when subjects view unpleasant or negative emotional pictures in comparison to pleasant and neutral pictures (e.g., Cook, Davis, Hawk, Spence, & Gautier, 1992; Vrana, Spence, & Lang, 1988), whereas the response is reduced during viewing of positive as opposed to negative or neutral emotional cues (Cuthbert, Bradley, & Lang, 1996). Startle potentiation by unpleasant stimuli is thought to reflect activation of the aversive/defensive motivational system, while startle inhibition by positive emotional cues may reflect activation of appetitive or approach motivational processes (Lang, Bradley, & Cuthbert, 1990; Lang, Bradley, & Cuthbert, 1992). The neural circuitry underlying the startle response may involve several structures also associated with nicotine administration and withdrawal, such as the prefrontal cortex, hippocampus, amygdala, and the nucleus accumbens (Swerdlow & Geyer, 1999; Hare, Tottenham, Davidson, Glover, & Casey, 2005; Phan et al., 2005; Stein et al., 1998).

A handful of studies have in fact investigated the cognitive-affective mechanisms associated with the *5-HTTLPR*. Beevers et al. (Beevers, Gibb, McGeary, & Miller, 2007) reported that depressed inpatients with at least one short allele exhibited more cognitive bias for anxiety-related words than those with two long alleles. Additionally, other studies have suggested that short allele carriers may more intensely process fear-related emotional stimuli (Bertolino et al., 2005; Surguladze et al., 2008; Osinsky et al., 2008), while Fox and colleagues (Fox, Ridgewell, & Ashwin, 2009) reported that individuals with the l/l genotype evidenced bias towards positive pictures and avoidance of threat related images. A study with adolescents revealed a linear relation between presumed serotonin availability (based on genotype) and the magnitude of attention towards angry and happy faces (Perez-Edgar et al., 2010). These studies were done with relatively small samples that are more typical of cognitive neuroscience studies rather than the traditionally larger genetic studies.

We are aware of only one study, not conducted in smokers, in which the effects of the *5-HTTLPR* was studied specifically in relation to the human startle response. That study showed that *5-HTTLPR* short allele carriers exhibited heightened startle responses compared to those with the l/l genotype (Brocke et al., 2006). However, the authors were unable to confirm their hypothesis that the s carriers would exhibit heightened fear-potentiated startle responses in comparison to those with the l/l genotype, as they found no interaction with picture content. Other studies have suggested that the manipulation of serotonin levels using pharmacological treatment may potentiate (Liechti, Geyer, Hell, & Vollenweider, 2001; Hayward, Goodwin, Cowen, & Harmer, 2005), attenuate (Murphy, Longhitano, Ayres, Cowen, & Harmer, 2006), or have no effect at all (Phillips, Langley, Bradshaw, & Szabadi, 2000; Quednow et al., 2004) on the startle response.

Hypothesis

In this study, acoustic startle probe methodology was used to examine the effects of nicotine administration and deprivation on emotional processes associated with drive and motivation (i.e., activation of the defensive system using the startle probe) with respect to genotype at the *5-HTTLPR* of the serotonin transporter gene, *SLC6A4*. The individuals in this study were a sub-sample of a larger study addressing the effects of deprivation and nicotine administration on the acoustic startle response more generally (Cinciripini et al., 2006). Specifically, our aim was to directly test the relationship between the three *5-HTTLPR* genotypes (i.e., s/s, s/l, and l/l), overnight nicotine deprivation, acute nicotine administration, and activation of the defensive system using the startle probe.

We hypothesized that the startle response in individuals with the *5-HTTLPR* l/l genotype would be notably suppressed in response to acute nicotine administration for overnight nicotine deprived smokers relative to non-deprived smokers. We further predicted that this suppression would be most pronounced when these individuals were exposed to negative emotional cues compared to positive or neutral cues. Method

Participants

Smokers were recruited from the Houston metropolitan area using newspaper advertisements and flyers. They were paid \$125 for attending 4 laboratory sessions and an additional five dollars each session for arriving promptly. Smokers who were between the ages of 18 and 59, fluent in English, smoked 10 or more cigarettes per day, had an expired carbon monoxide level greater than 8 ppm (or saliva cotinine >30 ng/ml), and reported no uncontrolled medical illness were included in the study. Individuals were excluded if they were taking psychotropic or narcotic medication, met criteria for a current psychiatric disorder, reported hearing loss, were using tobacco products other than cigarettes, or were involved in current smoking cessation activity. Smokers were not recruited based on their interest in quitting and no cessation treatment was provided. A total of 113 participants contributed to the main study (Cinciripini et al., 2006). A sub-sample of 84 individuals, restricted to African American and Caucasians smokers, provided genotypic data for the current analyses.

Design

This study used a within-subjects design. Smokers attended four 90-min laboratory sessions scheduled approximately three days apart. The four sessions provided a complete crossing of two pre-laboratory smoking deprivation conditions (12-hour deprived vs. non-deprived) with two conditions of acute nicotine spray administration (nicotine vs. placebo). During each session, participants viewed affective pictures (positive, negative, neutral) while startle probes were administered.

Two blocks of trials were presented in each session. The first was considered a habituation block used to acclimate the participant to the novel effects of the startle procedure, nasal spray administration, and general laboratory surroundings. All participants received placebo spray prior to this block. The second (test) block was similar to the habituation block in terms of slide content. Smokers received either nicotine or placebo nasal spray prior to the test block of trials. Self-report of nicotine withdrawal and nicotine craving was measured prior to each laboratory session. Before beginning the laboratory portion of the experiment, smokers were administered instruments assessing self-reported levels of nicotine use, acute stress, and affect. Subjects denied the use of nicotine products other than cigarettes.

Measures

We measured nicotine dependence using the Fagerström Test for Nicotine Dependence (FTND), a 6-item questionnaire that assesses various components of smoking behavior such as daily intake, difficulty in refraining from smoking, time to first cigarette, etc. (Fagerström, 1982; Heatherton, Kozlowski, Frecker, & Fagerström, 1991). We also assessed history of major depression using a brief interview consisting of items based on the DSM-IV (American Psychiatric Association, 1994) criteria for a history of major depressive disorder (MDD). This scale was developed and used in our previous studies. Finally, we assessed depressive affect at each visit using the Center for Epidemiologic Studies Depression Scale (CES-D), a 20-item self-report measure developed to assess depressive symptoms in community (non-clinical) populations (Ross and Mirowsky 1984).

Laboratory Sessions

DNA Sample Collection—In order to collect a buccal sample, volunteers were given a 1.5 fl. oz. bottle of Scope mouthwash (15 wt% of alcohol) and asked to swish the mouthwash vigorously throughout the mouth for one minute and then expel it into a provided plastic collection cup, where it was immediately frozen. DNA was extracted, purified by organic extraction and ethanol precipitation, and then dissolved to a final concentration of 10 ng/ul for amplification by Polymerase Chain Reaction (PCR) (Vandenbergh, Anthony & Whitfield, 2003). Amplification followed the published procedure of Serretti et al, 1999 (, 1999). Oligonucleotide primers flanking the 5-HTT-linked polymorphic region (5-HTTLPR) and corresponding to the nucleotide positions ranging from -1,416 to -1,397 (5'-GCGTTGCCGCTCTGAATTGC-3') and from -910 to -889 (5'-GAGGGACTGAGCTGGACAACCCAC-3') of the 5-HTT gene regulatory region were used to generate the long, 528-bp, fragment and the short, 484-bp, fragment. PCR amplification was carried out in a final volume of 30 µl consisting of 50 ng genomic DNA, 2.5 mM dNTPs, 0.1 µg of sense and antisense primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 5% dimethyl sulfoxide, and 1 U of Taq DNA polymerase. The reaction was then carried out with an initial denaturation for 2 min at 95°C, followed by 35 cycles of 95°C, 1 min/61°C, 1 min/72°C, and a final extension at 72° C for 7 minutes. Allele calls were verified by independent assessment from two investigators.

Smoking Deprivation—Prior to each deprivation session, participants were instructed to refrain from smoking for 12 hr. Compliance with nicotine deprivation instructions was assessed using expired CO (≤ 10 ppm) to confirm abstinence. Non-abstinent participants in the deprived condition were rescheduled. However, an exception was made where a CO above 10 ppm could be expected after a period of overnight deprivation. In this case, a value at or below 50% of their baseline CO was used as a cutoff point. The distribution of the CO values and our approach to the analyses involving expired CO and deprivation are discussed in the results section.

Physiological measurement—Measure of the startle eye-blink response was accomplished using EMG electrodes (Ag-AgCl) filled with saline gel, attached in the participant's right orbicularis oculi region (Fridlund & Cacioppo, 1986). This signal was acquired and amplified with BIOPAC Systems' (Goleta, CA) EMG100A module connected to BIOPAC Systems' MP100WSW bioamplifier with a time constant of 1000-ms. Data was recorded and displayed using BIOPAC Systems' AcqKnowledge III data acquisition software installed on a Pentium III computer. Following sensor attachment, participants rested quietly for 5 minutes to allow for initial adaptation to the hook-up procedure and assessment of signal quality. Startle response was scored offline manually in peak microvolts (µV) using the AcqKnowledge software.

Affective slide viewing—A total of 48 slides were presented. Thirty-six color slides, depicting a variety of events, were selected from the International Affective Picture System-IAPS (Center for the Study of Emotion and Attention (CSEA-NIMH), 1995). Twelve were selected from each of three valence categories of positive, neutral, and negative. Positive and negative slides were selected from the high arousal (interest) and neutral from the low arousal category. Our slides were selected based on their use in other studies demonstrating valence effects (Cuthbert et al., 1996; Greenwald, Bradley, Cuthbert, & Lang, 1998; Nitschke et al., 2002; Patrick, Berthot, & Moore, 1996; Bradley, Cuthbert, & Lang, 1996). An additional 12 slides related specifically to smoking cues were also presented and consisted of cues such as a burning cigarette in an ashtray, people smoking alone or in a social context, a pack of cigarettes, etc. (Carter et al., 2006).

Two separate blocks (habituation and test) of trials consisting of 24 slides each were presented using procedures similar to those used in previous studies (Cuthbert et al., 1996). Within each session, the blocks included 6 slides of each type (positive, negative, neutral, cigarette). Each slide was presented for 6 s followed by a randomly determined interslide interval that varied from 10 to 20 s. The order of slide presentation was counterbalanced, so that within a session, participants saw each picture equally often in the first through sixth positions within each block, and each block occurred first or second with equal frequency. Four separate slide orders, one for each session, were counterbalanced across subjects, though the slides were the same for each session. The procedure took approximately 45 min.

Startle probe presentation—The acoustic startle stimulus consisted of a 50-ms 100-dB presentation of white noise with instantaneous rise time. The stimuli were generated and amplified by Pentium III PC using Psychology Tools' (Pittsburgh, PA) Eprime software. The noise burst was presented binaurally using Sennheiser HD500 (Wedemark, Germany) headphones. A startle probe was presented during 16 of the 24 slides in each block, at a random time of 2.5-5 s after slide onset, such that a probe was presented during 4 of the 6 slides within each valence condition. Counterbalancing for slide order was arranged so that, across subjects, each slide would be associated with a startle probe equally often. In addition, to enhance unpredictability of the startle presentation, 8 startle probes were presented during interslide intervals in each block.

Acute nicotine (nasal spray) administration—As described above, each session consisted of a habituation and test block of trials. Participants received an administration of nasal spray prior to each block. Prior to the habituation block all participants received placebo spray. This provided an opportunity for adaptation to any aversive effects of the nasal spray (in addition to habituation to the probes). Prior to the test block, smokers received either nicotine (1 mg) or placebo (0mg) nasal spray counterbalanced within deprivation condition. In all cases the spray was administered following an initial 5-min rest period. Participants self-administered one spray (metered dose) per nostril (either 0 or 0.5 mg nicotine per spray). Participants were blind to the content of the spray. This was followed by a 5-minute absorption period, after which the trial block and physiological recording began. Peak arterial levels of nicotine of about 10 ng/ml have been reported to occur within five minutes of a single application of the nicotine nasal spray (0.5 mg/nostril) (Gourlay & Benowitz, 1997). The nicotine and placebo spray were provided by Pharmacia & Upjohn Inc, and the nicotine spray is marketed as a nicotine replacement product (Nicotrol NS).

Nicotine and cotinine assessment—Salivary samples were obtained on each session (both deprived and non-deprived), 5 minutes after spray administration prior to the habituation block (placebo only) and the test block (placebo/nicotine). The samples were

used to provide estimates of nicotine exposure between conditions and as a function of smoking deprivation. Assessments of cotinine in saliva samples were obtained on the first non-deprived session to provide an overall measure of tobacco exposure for characterizing the sample. The samples were analyzed for nicotine and cotinine using the High Performance Liquid Chromatography (HPLC) method of Hariharan & VanNoord (Hariharan & VanNoord, 1991). The assay provides sensitivity to <1ng/ml of nicotine and cotinine.

Results

Participant characteristics

Participant characteristics are summarized in Table 1. Four Asian participants were excluded from the secondary analysis, as the small number precluded the investigation of racial interactions with genotype. No significant differences (p 's>.05) between genotype were found for any of the baseline measures in Table 1, including race, gender, age, baseline CO, baseline cigarettes a day, FTND score, history of depression, or years as a smoker. Additionally, no significant differences were observed between African American and Caucasian participants for any of the baseline characteristics.

Allele frequency and genotype

Table 2 contains the allele frequencies for the *5-HTTLPR* polymorphisms. These frequencies are consistent with previous reports of smokers (Lerman et al., 2000). The frequency of the genotypes in the sample met criteria for Hardy-Weinberg equilibrium, ($\chi^2(df=1) = 0.31, p=ns$), as did the frequencies in Caucasians ($\chi^2(df=1) = 0.02, p=ns$) and African-Americans ($\chi^2(df=1) = 0.12, p=ns$), analyzed separately.

Population Stratification

Methods to detect population stratification evaluate data from a set of randomly selected loci (AIMS) not associated with the trait of interest for patterns of association indicating the likelihood of strata within a sample. We used the Structured Association (SA) method of Pritchard and colleagues (Pritchard, Stephens, & Donnelly, 2000) to adjust for stratification. This procedure uses a Bayesian clustering approach to identify populations within a sample on the basis of genotypic information at a set of markers in linkage equilibrium that are unlinked to the phenotype of interest. The procedure assigns individuals probabilistically to a population based on their genotypes. This assignment permits modeling of the candidate gene effect within populations without the need to assume that the effect is the same in all populations by testing for association conditional on the inferred ancestries of the individuals (Pritchard, Stephens, Rosenberg, & Donnelly, 2000). Our analysis revealed three distinct clusters within our population which we identified as African-American, Caucasian, and Other. Only three individuals were clustered in a group different than that of their self-reported race. Subsequent analyses revealed no significant population stratification within African American or Caucasian individuals on the *5-HTTLPR* gene (African Americans: $\chi^2(df=93) = 110.9, p=ns$; Caucasians: $\chi^2(df=83) = 102.7, p=ns$). There were too few individuals in the Other category to analyze in the current paper.

Smoking Deprivation, Expired CO, and Saliva Nicotine Levels

Smokers were instructed to abstain from smoking approximately 12 hours prior to a deprived session. Abstinence 12 hr was reported on 82.5% of the deprived sessions. In 17.5% of the deprived sessions, smokers reported being abstinent between 10 to 12 hr but were included in the analyses because they met the CO criteria. A 10-ppm CO cut-off value was used to verify self-reported abstinence. Average CO levels during deprivation sessions for this subset of the main sample were within this range ($M=9.3, SD=5.4$ versus $M=23.1$,

$SD=10.3$ for non-deprivation sessions). No significant differences in average CO levels were noted between genotypes. Because a significant percentage (approximately 32%) of the overall sample smoked over a pack a day, we allowed for the possibility that CO values above 10 ppm could occur even after 10 to 12 hr of deprivation. Thus, smokers exceeding the 10-ppm cut-off range were permitted to participate in the session if they reported at least 10 hr of deprivation and their CO value was less than 50% of their baseline CO (4.3 % of the main study sample). Analyses were performed in the main study that both included and excluded this subset of individuals, with no significant differences noted in the findings (Cinciripini et al., 2006). Consistent with our previous approach, this analysis included the entire sample of Caucasians and African Americans with genotyping information available for the SERT.

Saliva nicotine values were also available prior to nicotine spray administration. Saliva nicotine values have been reported to be 8.1 times higher than comparable assessments of nicotine in plasma (Rose, Levin, & Benowitz, 1993), but like plasma nicotine, saliva nicotine has been reliably related to withdrawal induced cigarette craving (Teneggi et al., 2002). Average saliva nicotine values were significantly lower between deprivation ($M=13.4$, $SD=16.7$) and non-deprivation ($M=489.4$, $SD=742.6$) conditions. There were no significant differences in average saliva nicotine values between genotypes.

Statistical Approach

The primary unit of analysis was the mean startle amplitude (peak), computed for each participant, within each session for each test block by spray by valence combination. Peak startle amplitude (orbicularis EMG) was integrated and rectified and defined as the largest peak from 20–120 ms following picture onset. A mixed models regression approach (Littell, Milliken, Stroup, Wolfinger, & Schabenberfer, 2006) was the primary analytic strategy for estimating the effects of *5-HTTLPR* genotype, nasal spray, smoking deprivation, and their interaction terms on the startle response in Caucasian and African Americans separately. Subject was included as a random effect. Covariates included deprivation status and slide valence. Where appropriate, significant interactions were further evaluated using least-square means and univariate t-tests for all relevant pair-wise comparisons. Likelihood ratio tests were conducted to evaluate model fit after the inclusion of additional variables and only models with significant increases were retained. An additional model then examined the variables of interest within the entire sample (African Americans and Caucasians) including race as a covariate. Subsequent iterations of the model involved including other potential variables of interest such as craving and negative affect, though none were significant and will not be reported here.

Genotype and Startle

The primary purpose of this secondary analysis was to evaluate the effects of *5-HTTLPR* genotype on startle responding. Accordingly, only main effects and interactions relating to *5-HTTLPR* genotype are reported here. Results from the entire sample with all variables of interest are presented in Cinciripini et al. (2006). Given the biracial distribution of our sample and possible concerns of population stratification in a mixed racial analysis, we analyzed our data in two ways. First, we conducted separate analyses on each of the racial groups. Second, we analyzed the combined sample, controlling for race and specifically testing for racial interactions. We felt that this second analysis provided more accurate estimates to describe the entire population of smokers.

A significant *5-HTTLPR* genotype by Spray interaction was observed for both Caucasian ($F(2,40) = 10.01$, $p = 0.003$) and African-American ($F(2,38) = 7.35$, $p = 0.002$) smokers. Subsequent pair-wise comparisons parsing the *5-HTTLPR* genotype X Spray interaction

indicated that smokers with the l/l genotype of the *5-HTTLPR* gene showed significantly greater startle suppression when provided with nicotine vs. placebo, compared with smokers carrying the s allele. There was no interaction with deprivation status or slide valence, though the main effect of valence was significant in African Americans ($F(3,38) = 6.95, p < 0.0008$) and Caucasians ($F(4,40) = 16.6, p < 0.0001$), confirming the expected ordering of the startle responses to each of the stimuli in both groups. Main effects due to deprivation and spray were not significant.

The results for the combined sample in the model including race, deprivation, valence, spray, the *5-HTTLPR* genotype, and their interactions showed a significant main effect of race ($F(1,80) = 5.49, p = 0.02$) indicating a higher overall startle for Caucasian vs. African American smokers. Additionally, the main effect of valence was significant ($F(2,80) = 10.01, p < 0.0001$) and revealed the expected ordering of the startle responses to each of the stimuli: negative ($M = 86.7 (6.6)$) > neutral ($M = 77.4 (6.2)$) > positive ($M = 71.5 (6.0)$). A significant *SLC6A4* genotype by Spray interaction was replicated in this analysis of the entire sample ($F(2,80) = 4.17, p = 0.02$; see Figure 1). No other significant interactions with race or *SLC6A4* genotype and other factors (e.g., valence, deprivation, sex) were detected. Both the separate race models and the combined racial analysis were repeated in an analysis that included startle responses that occurred during inter-slide intervals, as no significant interactions with valence were observed. The results of these analyses were identical to those reported above and will not be expanded upon here.

Finally, we ran identical analyses (both on each racial group separately and the combined sample) using the race variable generated from the SA method described earlier. Rather than using self-reported race to group individuals, we re-grouped individuals based on the randomly selected genetic loci into two racial categories using the Structured Association (SA) method (Pritchard et al., 2000). Because the results were identical to those reported here using self-reported race, we will not describe them further.

Discussion

Our results indicated that smokers with the l/l genotype of the *5-HTTLPR* gene, compared with smokers carrying the s allele, showed significantly greater acoustic startle response suppression when provided with nicotine vs. placebo nasal spray. This interaction did not differ by race, as it was found for both Caucasian and African-American smokers, and the effect was observed across all types of stimuli (i.e., there was no interaction with valence). In other words, l/l smokers experienced an overall decrease in activation of defensive pathways brought about by the startle probe, when given nicotine compared to smokers without the l allele, regardless of the type of foreground cue. These results suggest that nicotine may activate appetitive mechanisms that suppress the startle response to a greater degree among l/l smokers than smokers with the l/s or s/s alleles. However, this did not result in differential effects across valences; in other words, the effect seems to reduce startle responding irrespective of the valence of the foreground cue and was observed even startle responses in the absence of any emotional cue were included in the analysis.

If smokers carrying the l/l allele experience greater activation of appetitive (approach/reward) pathways during smoking than their s/s or s/l counterparts, this could indicate that nicotine plays a significant role in the activation of appetitive structures among l/l smokers. Research has found that the long (l) variant of the *5-HTTLPR* may be associated with increased transcription of the serotonin transporter, increased reuptake of serotonin from the synaptic cleft, and overall lower levels of serotonin in the synapse (Murphy et al., 2001), though not all research supports this (Javors et al., 2005; Johnson et al., 2008; Shioe et al., 2003; Willeit et al., 2000). One implication of these findings is that l/l smokers may use

nicotine to enhance serotonin levels compared to non-l allele smokers. Moreover, this may also suggest that l/l carriers are more likely to become dependent smokers, a contention which is supported by three studies (Ishikawa et al., 1999; Kremer et al., 2005). An additional implication is that l/l smokers might also be more likely to relapse after quitting smoking compared to non-l smokers.

The notion that l/l smokers use nicotine to compensate for serotonergic deficits is supported by the hypothesized interplay between nicotine and serotonin in the regulation of emotion. Nicotine may stimulate serotonin (5-HT) release at high doses (Ho & Tyndale, 2007; Mihaiescu, Palomero-Rivero, Meade-Huerta, Maza-Flores, & Drucker-Colin, 1998; Ribeiro, Bettiker, Bogdanov, & Wurtman, 1993) and its withdrawal may decrease 5-HT in limbic and forebrain structures, possibly by increasing the inhibitory influence of 5-HT_{1A} autoreceptors within the raphe nuclei (Kenny & Markou, 2001). In fact, drugs that increase serotonin by inhibiting the action of 5-HT_{1A} receptors block the increased startle response in animals undergoing nicotine withdrawal (Rasmussen, Kallman, & Helton, 1997). This loss of serotonergic stimulation could lead to an increase in emotional reactivity during nicotine withdrawal. Inhibiting the reuptake of serotonin also rapidly reverses the elevation in brain-stimulation reward thresholds observed in rats undergoing nicotine withdrawal (Harrison, Liem, & Markou, 2001). Balfour (Balfour & Ridley, 2000) has suggested, however, that nicotine may ultimately decrease 5-HT in the hippocampus (possibly by increasing 5-HT_{1A} receptors), an action that could be interpreted as depressogenic. Thus, it is possible that smokers or a subset of smokers with high *5-HTTLPR* expression (i.e., l/l genotype) and associated low serotonin levels may smoke to enhance serotonergic tone (Staley et al., 2001).

It should be noted that not all the evidence for relations between the s and l alleles of the *5-HTTLPR* and smoking are consistent. For example, a recent meta-analysis found that smokers carrying the s allele more likely to relapse than those homozygous for the l allele (Munafò et al., 2004). Differences between the samples may contribute to this discrepancy, (i.e., our population was not in treatment nor attempting to quit smoking). However, if indeed the s allele confers less 5-HTT and increased 5-HT, the biological plausibility of those carrying the s allele being more dependent seems less clear in light of the strong inverse association between depressive symptoms and quitting. One would expect those with higher levels of 5-HT to have reduced affective impairment during quitting and be more likely to succeed. In the absence of direct or indirect assessment of 5-HT, however, all these arguments must be considered speculative, albeit hypothesis generating.

Interestingly, while we found an interaction between the *5-HTTLPR* gene and nicotine nasal spray, we did not find an interaction between the *5-HTTLPR* gene, nasal spray, and deprivation as hypothesized. We also failed to find the hypothesized interaction with these terms and slide valence. However, when genotype is not accounted for, interactions between nicotine nasal spray and deprivation as well as deprivation and slide valence were observed in the analysis of the entire sample (Cinciripini et al., 2006; Robinson et al., 2003). Results of this analysis suggest that alterations in appetitive or defensive motivation by nicotine, given a particular genotype (i.e., l/l) are more global and not specific to the processing of certain cues, (i.e. negative stimuli) or observable only when smokers are deprived. Though this study found no differences in the relationship of the startle response to genotype between the two racial groups, it is possible that a larger sample size and more power might have revealed additional significant interactions that might have distinguished the two groups. For example, there appeared to be a similar trend among the s/s African American smokers that was similar to the l/l smokers, though this effect did not reach significance. It is unclear whether this trend has any theoretical significance and should be explored in future studies, though it will not be discussed further here.

Though there is little doubt that the serotonin system is a central player in the neurobiological response to nicotine, the precise role of the serotonin transporter or its associated alleles in smokers has yet to be elucidated. Researchers suggest that even pivotal regulatory proteins such as the serotonin transporter will only exhibit a moderate impact on complex behavioral phenotypes and may be obstructed by non-genetic influences, such as environmental factors (Lesch & Mossner, 1998). Indeed, some of the more robust relationships between the *5-HTTLPR* gene and depression have been reported only in the presence of high levels of environmental stress (Caspi et al., 2003; Kendler, Kuhn, Vittum, Prescott, & Riley, 2005), though a recently published meta-analysis was unable to validate this relationship when combining the results of 14 studies comprised of over 14,000 participants (Risch et al., 2009). The authors reported that the relationship between stressful life events and depression was highly robust, but was not moderated by *5-HTTLPR* genotype. However, the role of nicotine in these studies is unknown. The effect of genotype on smoking behavior and/or withdrawal is likely more complex and may require more in-depth investigation than basic phenotypes typically allow (i.e., smoker versus non-smoker or quitter versus non-quitter). There may be more subtle neurobiological pathways that are influenced by genotype which in turn are related to withdrawal symptoms or inability to quit smoking. It is also possible that l/l smokers are vulnerable for different reasons than s-carrier smokers, as there are many variables associated with smoking and withdrawal behavior (affect, cognitive functioning, etc.).

In conclusion, our study is novel in that it is the first to demonstrate a relationship between a genotypic marker of 5-HT reuptake efficiency, and neural mechanisms associated with appetitive and defensive activation. Smokers with the l/l genotype experienced a decrease in defensive responding (as measured by the startle response) when given nicotine compared to smokers without the l allele, suggesting that l/l allele smokers experienced enhanced activation of appetitive mechanisms, which may be related to nicotine's reinforcing effects and mood modulating properties. These results suggest that l/l smokers could be more likely to smoke to offset a potential deficit in serotonin by activating appetitive structures with the consumption of nicotine. The small sample size in the current study represents a major limitation. It is possible that a larger sample would have revealed additional interactions between the variables of interest that might further elucidate the interplay between genotype and neural processes in smokers. However, given that large genetic studies often employ crude phenotypes such as smoker versus non-smoker that are so distal from specific neural processes in smokers, we believe that the current study offers a preliminary but compelling addition to the smoking literature. Future work would benefit by more precisely identifying the relationship between nicotine and 5-HT in the brain, possibly by using imaging technology to examine 5-HT activity in the mesolimbic system during affect relevant manipulations.

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Figure 1.
Nasal spray reduces startle amplitude in l/l smokers in African American and Caucasian participants

Figure 2.
Nasal spray reduces startle amplitude in l/l smokers

Table 1Demographics and baseline characteristics, by genotype; $N = 84$.

Characteristic	Genotype		
	s/s	s/l	l/l
	<u>N (%)</u>	<u>N (%)</u>	<u>N (%)</u>
Female	6 (33.3)	21 (53.9)	9 (33.3)
African American	6 (33.3)	18 (46.1)	17 (63.0)
Positive Depression History	4 (22.2)	7 (18.0)	5 (18.5)
	<u>Mean (SD)</u>	<u>Mean (SD)</u>	<u>Mean (SD)</u>
Age	35.9 (12.6)	37.7 (10.0)	38.5 (10.8)
Baseline CO	22.4 (11.1)	21.1 (10.3)	19.4 (7.7)
CESD D Score ^a	2.0 (2.2)	2.3 (3.2)	3.6 (3.9)
Cigarettes/day	19.0 (9.8)	21.8 (9.5)	22.7 (11.0)
FTND Score	4.3 (2.1)	5.3 (2.2)	4.9 (2.2)
Years of Smoking	17.6 (11.7)	20.4 (10.2)	20.0 (10.5)

^a CESD Depression Scale; consists of 7 items

Table 2

Allele frequency and genotype.

Allele	N	%
<i>SLC6A4</i> short (s)	75	44.6
<i>SLC6A4</i> long (l)	93	55.4
Total alleles	168	
Genotype		
s/s	18	21.4
s/l	39	46.4
l/l	27	32.1
Total N	84	