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DAT Genotype Modulates Brain and Behavioral Responses Elicited by Cigarette Cues

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We previously demonstrated differential activation of the mesocorticolimbic reward circuitry in response to cigarette cues independent of withdrawal. Despite robust effects, we noted considerable individual variability in brain and subjective responses. As dopamine (DA) is critical for reward and its predictive signals, genetically driven variation in DA transmission may account for the observed differences. Evidence suggests that a variable number of tandem repeats (VNTRs) polymorphism in the DA transporter (DAT) *SLC6A3* gene may influence DA transport. Brain and behavioral responses may be enhanced in probands carrying the 9-repeat allele. To test this hypothesis, perfusion fMR images were acquired during cue exposure in 19 smokers genotyped for the 40 bp VNTR polymorphism in the *SLC6A3* gene. Contrasts between groups revealed that 9-repeat (9-repeats) had a greater response to smoking (*vs* nonsmoking) cues than smokers homozygous for the 10-repeat allele (10/10-repeats) bilaterally in the interconnected ventral striatal/pallidal/orbitofrontal cortex regions (VS/VP/OFC). Activity was increased in 9-repeats and decreased in 10/10-repeats in the VS/VP/OFC ($p<0.001$ for all analyses). Brain activity and craving was strongly correlated in 10/10-repeats in these regions and others (anterior cingulate, parahippocampal gyrus, and insula; $r^2=0.79$–0.86, $p<0.001$ in all regions). Alternatively, there were no significant correlations between brain and behavior in 9-repeats. There were no differences in cigarette dependence, demographics, or resting baseline neural activity between groups. These results provide evidence that genetic variation in the DAT gene contributes to the neural and behavioral responses elicited by smoking cues.
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INTRODUCTION

Cigarette smoking persists as the premier cause of preventable death in the United States and is the direct cause of most lung cancer, chronic obstructive pulmonary disease, emphysema, and numerous other devastating and fatal diseases (Centers for Disease Control and Prevention, 1993; Cancer Facts and Figures, 2007). In total, 40% of the approximately 36 million smokers in the United States attempt to quit each year however less than 7% succeed, even when assisted with nicotine replacement (Baillie *et al*, 1995; Hughes *et al*, 2003). During abstinence, smokers are often obsessively fixated on smoking to alleviate intolerable cravings, induced by nicotine withdrawal and/or reminders to smoke, both of which contribute to relapse (Baker *et al*, 1986; Shiffman *et al*, 1996). Nicotine withdrawal symptoms abate within 1 month of quitting (Hughes, 2007). However, smokers report that reminders to smoke such as the smell of a burning match, another person smoking, and even internal mood states repeatedly associated with smoking can trigger relapse months or even years after quitting.

A wealth of preclinical literature examining the neurobiology underlying drug dependence and the cues that predict their availability point to a final common brain substrate, the mesolimbic dopaminergic reward system (Di Chiara and Imperato, 1988; Di Ciano *et al*, 1998; Duvauchelle *et al*, 2000; Gratton and Wise, 1994). Current neuroimaging techniques provide the opportunity to examine dopaminergic systems in humans. Volkow *et al* (2006), followed by Wong *et al* (2006) were the first to provide direct evidence that cocaine cues on their own elicit craving and increased dopamine (DA) release in the striatum of cocaine-addicted individuals. This work was followed by the study of Boileau *et al* (2007) who showed

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that only three pairings with amphetamine produced conditioned DA release in the ventral striatum (VS) of normal volunteers accompanied by amphetamine-like behavioral responses.

We showed robust and consistent brain perfusion, independent of withdrawal, to smoking cues (SCs) in reward-related mesocorticolimbic circuitry (amygdala, VS, orbitofrontal cortex, parahippocampus, medial thalamus, and insula) that correlated with craving in posterior cingulate and dorsolateral prefrontal cortex (Franklin *et al*, 2007) supporting hypotheses implicating this circuitry in stimulus-evoked craving and relapse (Corrigall *et al*, 1992; Di Chiara, 2000; Franklin and Druhan, 2000a, b). Despite a robust overall cue effect, there was considerable interindividual variability in the brain response. As heritability estimates for various smoking behaviors range from 46 to 84% (Batra *et al*, 2003; Heath and Madden, 1995), and as DA is a critical neurotransmitter for drug reward and is released by its signals, we hypothesized that genetically driven variation in DA transmission contributes to differences in the strength of the attribution of incentive salience to cues associated with smoking cigarettes and may be reflected in different neural activation patterns and/or intensity of activations.

In the present studies, we linked functional neuroimaging and behavioral measurements of subjective craving with a candidate gene approach to evaluate the impact of genetic variation in the dopamine transporter (DAT) gene (*SLC6A3)* on brain perfusion to SCs. The DAT is abundantly present in the VS and rapidly clears DA from the synapse after phasic release that occurs in response to primary rewards and predictive stimuli (Jaber *et al*, 1997; Mash *et al*, 2002; Zahniser and Sorkin, 2004). Evidence suggests that a 40 bp variable number of tandem repeat (VNTR) polymorphism in the 3′-untranslated region of the *SLC6A3* effects DAT expression (Vandenbergh *et al*, 1992). The 9-repeat allele (9-repeats) is associated with lower DAT expression (Fuke *et al*, 2001; Mill *et al*, 2002) (although see van Dyck *et al*, 2005), which may result in slow DA clearance potentially reflecting prolongation of the DA-related 'reward message'. In support of this view, Erblich *et al* (2005) found that smokers carrying a 9-repeat reported stronger SC-induced cravings. Further support is provided by a study in which ^{11}C raclopride competition in striatal regions was greater in 9-repeats immediately after smoking (Brody *et al*, 2006), which might indicate either compromised DAT function or reduced availability in the 9-repeat probands.

These and other studies provide the foundation for our hypothesis that synaptic DA levels are sustained in smokers with the less 'active' or less available form of the DAT and may be reflected in a heightened neural response to SCs. Particularly, as in our previous study, we expected carriers of a 9-repeat to exhibit increased activity in the VS, orbitofrontal cortex (OFC), amygdala, parahippocampal gyrus, thalamus and insula, and would report greater craving to the SCs compared to probands homozygous for the 10-repeat allele (10/10-repeats). To test this hypothesis, we utilized the technique of continuous arterial spin-labeled (CASL) perfusion fMRI, which is quantitative and stable over time and thus well suited for the study of low frequency (sustained) brain changes (Detre *et al*, 1992), such as drug craving, which once triggered, can persist for several minutes (Childress *et al*, 1999; Franklin *et al*, 2007; Volkow *et al*, 2006).

Subjects

The study, approved by the University of Pennsylvania Institutional Review Board, adhered to the Declaration of Helsinki. Smokers were compensated $100 for completion of both MRI scans. Subjects ($\sim$75%) were recruited from those presenting for treatment for nicotine dependence at the University of Pennsylvania Treatment Research Center and through word of mouth ($\sim$25%). Subjects were screened, tested on study knowledge, and consented prior to psychological and physical evaluations. The Minnesota International Neuropsychiatric Interview (Sheehan *et al*, 1998) is a short accurate structured diagnostic psychiatric interview, which was used to determine current DSM-IV diagnosis of psychoactive substance dependence other than nicotine and to diagnose current severe psychiatric symptoms. Individuals with other current psychoactive substance dependence or current DSM-IV psychiatric diagnoses were excluded. Severity of nicotine dependence was determined from a laboratory-developed Smoking History Questionnaire that included a modified Fagerstrom Test for Nicotine Dependence (FTND; Fagerstrom and Schneider, 1989).

Individuals with an abnormal structural MRI, a history of head trauma or injury causing loss of consciousness lasting greater than 3 min or associated with skull fracture or intercranial bleeding, or had magnetically active objects on or within their body were excluded.

DNA samples were acquired and functional scanning was performed on 22 subjects, 3 of whom were excluded due to incomplete image acquisition. A subgroup of these subject's imaging data has been published previously (Franklin *et al*, 2007). Thus, the final sample consisted of 19 ($N = 10$ men, 32% African American, 43% European American, 21% multiple ethnicity) physically healthy and mentally stable smokers between the ages of 18 and 60 (35.8 ± 2.38) who met DSM-IV criteria for nicotine dependence (FTND: 4.77 (± 0.52); indicating moderate dependence). Subjects smoked from 12 to 35 cigarettes per day (21.4 ± 1.61) and averaged 13.3 (± 0.47) years of education.

METHODS

Imaging Approach

Continuous arterial spin-labeled perfusion fMRI, an indirect measurement of neural activity (Floyd *et al*, 2003), was used to test whether brain and behavioral responses to SCs would be heightened in probands carrying an *SLC6A3* 9-repeat (9-repeats) *vs* probands homozygous for the 10/10-repeat (10-10 repeats). This technique has been previously described in detail (Detre *et al*, 1992; Detre and Wang, 2002). Perfusion fMRI provides a quantitative measurement of cerebral blood flow (CBF; ml of blood per 100 g of tissue per minute) permitting comparisons of changes in regional activity across multiple scanning sessions. This feature is beneficial for the study of longitudinal or very low-frequency brain changes, such as the drug craving state.

Thus, it is particularly useful in our SC paradigm wherein smokers enter and exit the scanner between cue sets to smoke a cigarette.

Scanning Procedures and Stimulus Description

The cue-reactivity procedure exposes subjects to a variety of smoking-related stimuli whereas subjective reports of craving and functional MRI data are acquired.

Two separate scanning sessions (counterbalanced with respect to SC and non-SCs) were administered, 1 h apart and preceded by smoking to minimize 'carry over' craving and pharmacologic withdrawal that can accrue throughout scanning. For each session, a 10 min CASL scan was acquired during cue exposure approximately 20–25 min after smoking to ensure dissipation of the acute cardiovascular effects of smoking. Subjects were informed of the opportunity to smoke after each scan as expectancy enhances subjective craving and physiological responses (Droungas *et al*, 1995; McBride *et al*, 2006). Craving and withdrawal reports were obtained before and after each scan using the Craving and Withdrawal Questionnaire (Table 1).

Cue exposure consisted of two 10-min stimulus sets: a non-SC audiovisual clip comprised of several individuals (differing in race, age, and sex) relating interesting short stories or anecdotes, or a SC video containing similar footage but featuring smoking, cigarette cues, and explicit language designed to induce appetitive desire for a cigarette. Smokers held one of their own cigarettes during the SC scan and a pencil during the non-SC scan. Cue sets have been previously validated in Franklin *et al* (2007).

Imaging Parameters and Data Processing

MR scanning parameters were previously described in detail in Franklin *et al* (2007). Briefly, data was acquired on a 3.0 T Trio whole-body scanner (Siemens AG, Erlangen, Germany), using a standard volume coil. A T1-weighted three-dimensional HR MPRAGE scan was acquired (FOV = 250 mm, TR/TE = 1620/3 ms, 192 × 256 matrix, slice thickness 1 mm) for anatomical coregistration and spatial normalization. CASL perfusion fMRI was used to acquire the resting baseline (5 min) and SC and non-SC (10 min) scans. Interleaved images with and without labeling were obtained using a gradient echo and echo-planar-imaging sequence with a delay of 700 ms inserted between the end of the labeling pulse and image acquisition (FOV = 220 mm, matrix = 64 × 64 × 14, TR/TE = 3000/17 ms, flip angle = 90°, slice thickness = 8 mm with a 2 mm interslice gap.

Table 1 Table of Demographics (Means ± SEMs)

Demographic	9-repeats	10/10-repeats
Age	37.9 (±4.1)	33.8 (±2.8)
Education	13.89 (±0.68)	12.69 (±0.64)
Sex	Male (6), Female (4)	Male (4), Female (5)
Pack years	22.88 (±5.51)	20.92 (±6.04)
Cigarettes per day	19.89 (±2.64)	23.13 (±1.42)
FTND scores	4.29 (±0.77)	5.19 (±0.89)
Race	Black (4), White (4) Multiple (2)	Black (3), White (4) Multiple (2)

There were no significant differences in any of the above characteristics between groups.

Imaging Data Processing and Statistical Analyses

An SPM2-based (Wellcome Department of Cognitive Neurology, London, UK) ASL data processing toolbox (Wang *et al*, 2008) was used for data analyses as described previously (Franklin *et al*, 2007). Briefly, ASL image pairs were realigned to the mean of all control images and spatially smoothed with a three-dimensional isotropic Gaussian kernel (FWHM, 10 mm). CBF image series were generated using a simplified two-compartment model with the simple-subtraction method for CBF calculations (Aguirre *et al*, 2005). The mean control image of each subject's data was coregistered to the structural image using the mutual information based coregistration algorithm provided by SPM2. The same coregistration parameters were also used to coregister the CBF maps to the structural image. The structural image was then spatially normalized to the Montreal Neurological Institute (MNI) standard brain. The same parameters were used to normalize the CBF images to the MNI standard space. Each subject's normalized mean control images were segmented using SPM2. The segmented gray matter masks were averaged and the overlap of subject's gray matter was extracted. This final mask was used for calculating global CBF for each session.

Statistical Analyses

Voxel-wise analyses of the CBF data were conducted on each subject, using a general linear model (GLM) with global CBF time course and age as nuisance covariates added into the model. There was no temporal filtering or smoothing. All preprocessing was done blinded and prior to subsequent group assignment. Contrasts between cue sets were defined in the GLM model to assess the voxel-by-voxel CBF difference. Using the corresponding parametric maps of this contrast (the β maps), random effects analysis was employed to test for a significant main effect of condition (SC *vs* non-SC) in each genotype with a statistical parametric map of the *T*-statistic at each voxel for population inference (second-level analysis). This two stage analysis is theoretically equivalent to a two way ANOVA (Penny *et al*, 2003).

Simple regression analyses were conducted on CBF with the change in craving scores as the covariate of interest (post- (−) pre-SC craving scores)) to test for brain/behavioral correlations.

Genotyping

Genomic DNA was extracted from anti-coagulated venous blood samples using a standard salting out method (Lahiri and Nurnberger, 1991). Genotyping of the *SLC6A3* 40 bp repeat polymorphism (rs28363170) was performed as previously described (Vandenbergh *et al*, 1992). Briefly, PCR was performed using a mix of 100 ng genomic DNA, 1 × AmpliTaq buffer containing $MgCl_2$, 200 nM dNTP mix, 150 nmol for each primer (forward: 5′-TGTGGTGTAGG

GAACGGCCTGAG-3′ and reverse: 5′-CTTCCTGGAGGTC ACGGCTCAAGG-3′) and 2.5 U AmpliTaq per reaction. The PCR conditions included an initial melting step (94°C; 4 min) followed by 40 cycles of melting (94°C; 1 min), annealing (65°C; 1 min), and extending (72°C; 1 min). A final extension step was used (72°C; 5 min). Reaction products were separated by agarose gel electrophoresis, and product sizes were determined by comparison to molecular weight standards and known sequenced samples. In addition, four samples were confirmed by sequencing. All samples were run in duplicate with 100% concordance rate and read independently by two blinded investigators.

RESULTS

Group Assignment

Nineteen smokers were genotyped for variance in the *SLC6A3*. Of which, 9 subjects were homozygous for the 10/10-repeats whereas 8 were heterozygous and 2 homozygous for the 9-repeat. Genotype frequencies did not deviate from that expected under Hardy–Weinberg equilibrium ($\chi^2 = 0.1067$). As noted previously, smokers who carried at least one 9-repeat were classified and grouped together for analyses as 9-repeats and 10/10-repeat probands were classified as 10/10-repeats resulting in allele frequencies of 0.32 for 9-repeats and 0.68 for 10/10-repeats, which is similar to that found in other studies (Vandenbergh *et al*, 2002). Both groups contained similar numbers of treatment seekers *vs* nontreatment seekers.

Demographics

As shown in Table 1, there were no significant differences between genotypes in ethnicity, sex, cigarettes smoked per day, pack years (a measure to quantify intensity of chronic cigarette exposure since smoking initiation) or other general demographic items.

Subjective Measures

Subjects rated their craving, withdrawal, mood state, and interest in the video before and after stimulus presentations using a 9-item Craving and Withdrawal Questionnaire. For each item, reported scores are the post- (−) pre-stimulus scores. All items were analyzed using paired *t*-tests except for item 3, intended to assess craving, which we predicted would be greater following SC exposure. There were no significant differences in responses to any of the items for either the SCs (Table 2) or the non-SCs (not shown). Of note, overall craving reports to SCs approached significance and were positive in 9-repeats and negative in 10/10-repeats (Table 2, item 3, $p < 0.11$).

Imaging Results

For comparisons between conditions and/or groups and regression analyses only clusters with voxels having a height threshold exceeding $p < 0.001$ (uncorrected), an extent threshold of 20 contiguous voxels, and significant at the cluster level are reported. Coordinates are in MNI as provided by SPM, however, can be converted to Tailarach coordinates (http://www.mrc-cbu.cam.ac.uk/Imaging/mnispace.html). Coordinates listed are those chosen from the suprathreshold voxel of each ROI using the Duvernoy Brain Atlas as a reference (Duvernoy, 1999).

Smoking cue reactivity in 9-repeats vs *10/10-repeats*. In comparison to 10/10-repeats, 9-repeats exhibited significantly greater activity in response to SCs *vs* non-SCs bilaterally in the OFC and parahippocampus; in the left VS/ventral pallidal (VP) region, and dorsolateral PFC; and in the right ventral medial PFC, fusiform gyrus, and superior temporal and frontal gyri. Activity was reduced in the right superior frontal gyrus. Representative axial and coronal sections coregistered to the MNI brain are shown in Figure 1. See Table 3 for *T*-values and MNI coordinates, as provided by SPM.

Smoking cue reactivity in separate allelic groups. The brain's response to SCs was also examined separately in 9-repeats and 10/10-repeats. 9-repeats exhibited enhanced activity bilaterally in OFC, VS/VP, and parahippocampal gyrus; and in left amygdala, anterior cingulate, and superior frontal gyrus. Notably, activity was decreased in 10/10-repeats bilaterally in the OFC and in the left VS/VP, ventral

Table 2 Table of Craving and Withdrawal Questionnaire Scores Generated from the Changes Elicited by the Smoking Stimuli (Post-Scores−Pre-Scores) in 9-Repeats ($N = 10$) *vs* 10/10-Repeats ($N = 9$)

Craving and withdrawal questionnaire	9-repeats	10/10-repeats	*P*-value
(1) How calm are you right now?	0.89 ± 0.73	−0.02 ± 0.36	0.19
(2) How content do you feel right now?	0.56 ± 0.47	−0.22 ± 0.40	0.23
(3) How much do you desire a cigarette right now?	1.20 ± 0.64	−0.11 ± 0.80	0.11
(4) Right now, if you could, would you smoke a cigarette?	1.11 ± 0.54	1.22 ± 0.21	0.21
(5) Right now, do you feel as though you have had a cigarette?	0.30 ± 0.76	−0.33 ± 0.65	0.91
(6) Right now, do you feel edgy, as if you have not had a cigarette in a while?	0.33 ± 0.76	0.89 ± 0.89	0.64
(7) How much of an urge or desire do you have to smoke right now for pleasure?	0.89 ± 0.61	0.78 ± −0.22	0.45
(8) How much do you need to smoke right now for relief?	0.22 ± 0.49	−0.22 ± 1.02	0.70
(9) How interested were you in the video you just watched?	4.11 ± 0.82	5.33 ± 0.50	0.22

Each question was preceded by the phrase 'On a scale from 1 to 7…'. Data were analyzed using two-tailed *t*-tests for all items except item 3. There were no significant differences across groups in any of the nine items.

Table 3 Brain Activity in 9-Repeat *vs* 10/10-Repeats, 9-Repeats, and 10/10-Repeats

	9- *vs* 10/10-repeats				9-repeats				10/10-repeats			
Region	***x***	**y**	**z**	***T* value**	***x***	**y**	**z**	***T* value**	***x***	**y**	**z**	***T* value**
Amygdala												
L	NS	NS	NS	NS	−18	−4	−18	+4.59	NS	NS	NS	NS
VS/VP												
R	NS	NS	NS	NS	4	6	−6	+3.07	NS	NS	NS	NS
L	−16	2	−16	+4.88	−18	11	−14	+3.72	−14	12	−19	−4.84
OFC												
R	16	22	−20	+4.11	12	40	−18	+3.53	14	16	−24	−4.51
L	−14	16	−18	+9.11	NS	NS	NS	NS	−14	16	−18	−10.31
L	−23	27	−24	+3.84	−18	18	−15	+5.35	NS	NS	NS	NS
Parahippo G												
R	41	−41	−24	+3.41	24	−10	−36	+4.84	38	−24	−18	+5.69
L	−24	4	−32	+3.27	−22	−10	−30	+7.06	−32	−22	−18	+3.68
A Cing												
L	NS	NS	NS	NS	−6	30	24	+4.13	00	14	24	+4.38
DLPFC												
L	−24	60	3	+6.22	NS	NS	NS	NS	NS	NS	NS	NS
Fusiform G												
R	43	−34	−24	+4.37	NS	NS	NS	NS	NS	NS	NS	NS
VMPFC												
R	6	42	−21	+3.76	NS	NS	NS	NS	NS	NS	NS	NS
VLPFC												
L	NS	NS	NS	NS	NS	NS	NS	NS	−26	58	−3	−5.88
Sup Temp G												
R	48	24	−25	+3.76	NS	NS	NS	NS	NS	NS	NS	NS
Sup frontal												
R	14	56	18	−3.57	NS	NS	NS	NS	NS	NS	NS	NS
L	NS	NS	NS	NS	−6	68	6	4.50	NS	NS	NS	NS
Med Thal												
L	NS	NS	NS	NS	NS	NS	NS	NS	−14	−10	0	−5.20
Insula												
V. Ant R	NS	NS	NS	NS	NS	NS	NS	NS	40	18	−5	+3.27
D. Ant L	NS	NS	NS	NS	NS	NS	NS	NS	−48	6	6	+7.76
Inf Temp G												
R	NS	NS	NS	NS	NS	NS	NS	NS	42	−28	−12	+3.65
Inf frontal G												
R	NS	NS	NS	NS	NS	NS	NS	NS	54	14	18	+5.87
Cb	NS	NS	NS	NS	NS	NS	NS	NS	−4	−64	0	−4.16

Abbreviations: A Cing, anterior cingulate; Ant, anterior; Cb, cerebellum; D, dorsal; DLPFC, dorsolateral prefrontal cortex; G, gyrus; Inf, inferior; L, left; Med Thal, medial thalamus; Parahippo, parahippocampal; N, nonsignificant; R, right; Sup, superior; Temp, temporal; V, ventral; VLPFC, ventral lateral prefrontal cortex; VMPFC, ventral medial prefrontal cortex; VP, ventral pallidal; VS, ventral striatum.
Coordinates are in MNI.

lateral prefrontal cortex, medial thalamus and cerebellum; and was greater bilaterally in the insula and parahippocampal gyrus and in the right inferior temporal and left inferior frontal gyri and anterior cingulate (Table 3; Figure 1). There were no differences between groups in resting baseline blood flow at this threshold indicating that the differences were unrelated to variation in neurophysiology present before cue exposure. There were no differences between groups in brain responses to non-SCs.

Brain and behavioral correlates by genotype. Linear regression analyses were conducted separately for each genotype on the change in craving scores to SCs and brain activity at each voxel (Figure 2). Although brain activity and craving was reduced following SC exposure in 10/10-repeats, strong correlations between subjective craving responses and brain activity were observed in the left OFC, $r^2 = 0.86$, $T = 5.92$; left VS, $r^2 = 0.85$, $T = 3.81$; right VS, $T = 3.72$, $r^2 = 0.86$; anterior cingulate (rostral supragenual region), $r^2 = 0.89$, $T = 4.72$; left parahippocampal gyrus, $r^2 = 0.85$, $T = 4.46$; left insula, $r^2 = 0.85$, $T = 5.15$; and right insula $r^2 = 0.85$, $T = 3.40$ (anterior ventral). In contrast, there were no significant correlations between brain responses and craving in 9-repeats; however, a trend was observed in VS ($r^2 = 0.29$). This effect was statistically significant between groups at each region (T-values ranged from 4.42 to 5.69 at $p < 0.01$, 20 contiguous voxels). Notably, the brain responses in individual 9-repeat subjects were inconsistent and quite variable across regions. For example, the smoker with a change in craving score of '2' and who had the greatest brain activity compared to other smokers in the VS is not the same individual whose score was also '2' and had the greatest perfusion in the OFC. Also, one subject had extremely high activity in the OFC. The lack of a relationship between craving and brain activity was still present using Spearman's ρ correlation analysis and when that subject was excluded from the analysis. Brain/behavioral correlations were not observed in any other regions in either group.

DISCUSSION

Summary of Results

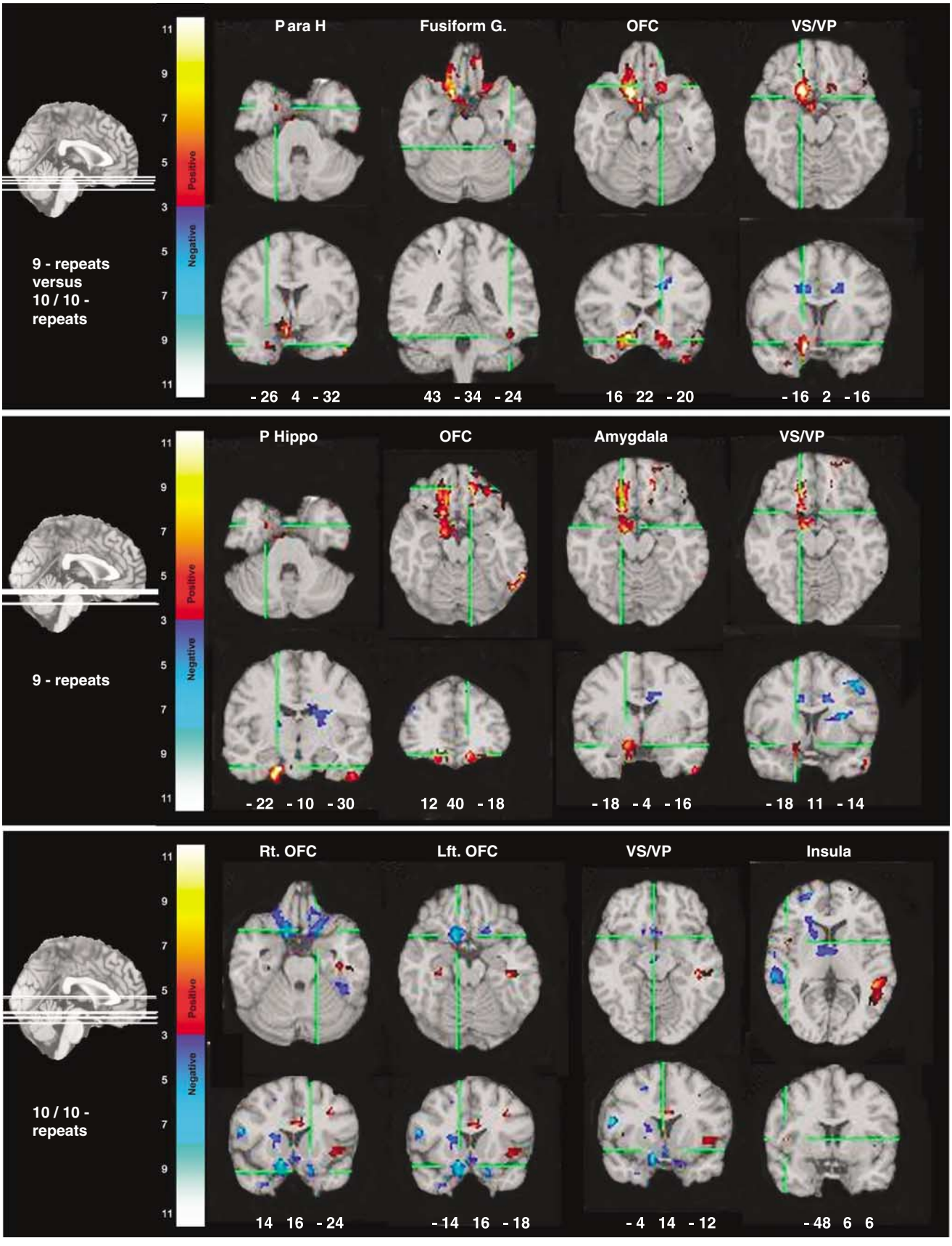
Here we report that genetic variation in dopaminergic transmission contributes to the brain and behavioral responses elicited by SCs. We observed increased brain activity during exposure to SC *vs* non-SCs in smokers carrying at least one 9-repeat-VNTR allele of the DAT *SCL6A3* gene (9-repeats) in comparison to 10-repeat-VNTR homozygotes (10/10-repeats) in the interconnected OFC/VS/VP. Subsequent analyses after grouping smokers by genotype produced quite different patterns of activity. Brain activity was heightened bilaterally in the OFC/VS/VP in 9-repeats and reduced in the same regions in 10/10-repeats. Further, no significant correlations were found between brain activity and craving in 9-repeats, whereas neural activity strongly correlated with craving in several *a priori* regions in 10/10-repeats: OFC, VS, anterior cingulate, parahippocampal gyrus, and insula.

As expected, 9-repeats exhibited increased neural activity to SCs in reward-related circuitry (amygdala, VS/VP, parahippocampal gyrus, OFC, and anterior cingulate) compared to 10/10-repeats. These results are analogous to our earlier findings characterizing the neural correlates of withdrawal-free SC reactivity (Franklin *et al*, 2007). To delineate further the influence of DAT genotype on cue reactivity, separate analyses were conducted on 9-repeats and 10/10-repeats. We expected SCs to elicit increased activity in reward-related circuitry in both groups, albeit greater in 9-repeats. Contrary to expectation, we observed dramatically different brain activation patterns in the separate groups. 9-repeats exhibited increases in activity in amygdala, OFC/VS/VP, parahippocampal gyrus, and anterior cingulate whereas 10/10-repeats revealed equivalent but opposite responses in VS/VP/OFC and increased activity in parahippocampus, anterior cingulate, and insula.

Possible Role for the DAT in Cue Reactivity

The repetitive intermittent surges in VS DA stimulated by nicotine and other addictive drugs strengthen stimulus–drug associations and the pathways that mediate them (Balfour *et al*, 2000; Di Chiara *et al*, 1999; Pontieri *et al*, 1998). Thus, discrete stimuli or contexts predictive of drug availability acquire powerful incentive properties and motivate drug-seeking behavior (Parkinson *et al*, 2002; Stuber *et al*, 2005). The phasically released DA in response to rewards and their predictors is rapidly removed by the DAT. The 9-repeat-VNTR allele has been associated with lower DAT expression in the VS in molecular studies (Fuke *et al*, 2001; Mill *et al*, 2002; VanNess *et al*, 2005); however, *in vivo* studies are less consistent. Studies quantifying DAT protein availability report that 9-repeats exhibit higher striatal availability in healthy subjects (van Dyck *et al*, 2005); lower availability in alcoholics and controls, irrespective of alcohol dependence (Heinz *et al*, 2000); and no change in DAT density in schizophrenics and controls, also irrespective of schizophrenia (Martinez *et al*, 2001). In our view, DAT availability does not imply DAT function and we suggest the increased perfusion in 9-repeats during SC exposure in the VS and related circuitry is indirect evidence of reduced DAT activity and support our working hypothesis that extending the time between DA release and DA reuptake increases the incentive salience of smoking stimuli

Figure 1 Representative fMR axial and coronal brain slices analyzed in SPM2 and displayed in MRIcro (two-tailed) overlaid on the Montreal Neurological Institute (MNI) brain showing brain activity to the smoking cues greater than to the nonsmoking cues in 9-repeats *vs* 10/10-repeats, 9-repeats, and 10/10-repeats. *T*-values range from negative (−) 10.31 to positive (+) 9.11. Crosshairs are centered on the suprathreshold voxel of representative significant clusters. Note that not all regions showing significance are centered under crosshairs. See Table 3 for a complete list of coordinates and *T*-values of regions showing significant activations in response to smoking cues. Images are displayed neurologically (left is left).


P ara H
Fusiform G.
OFC
VS/VP
9 - repeats versus 10 / 10 - repeats
Positive
Negative
11 9 7 5 3 5 7 9 11
- 26 4 - 32
43 - 34 - 24
16 22 - 20
- 16 2 - 16
P Hippo
OFC
Amygdala
VS/VP
9 - repeats
- 22 - 10 - 30
12 40 - 18
- 18 - 4 - 16
- 18 11 - 14
Rt. OFC
Lft. OFC
VS/VP
Insula
10 / 10 - repeats
14 16 - 24
- 14 16 - 18
- 4 14 - 12
- 48 6 6

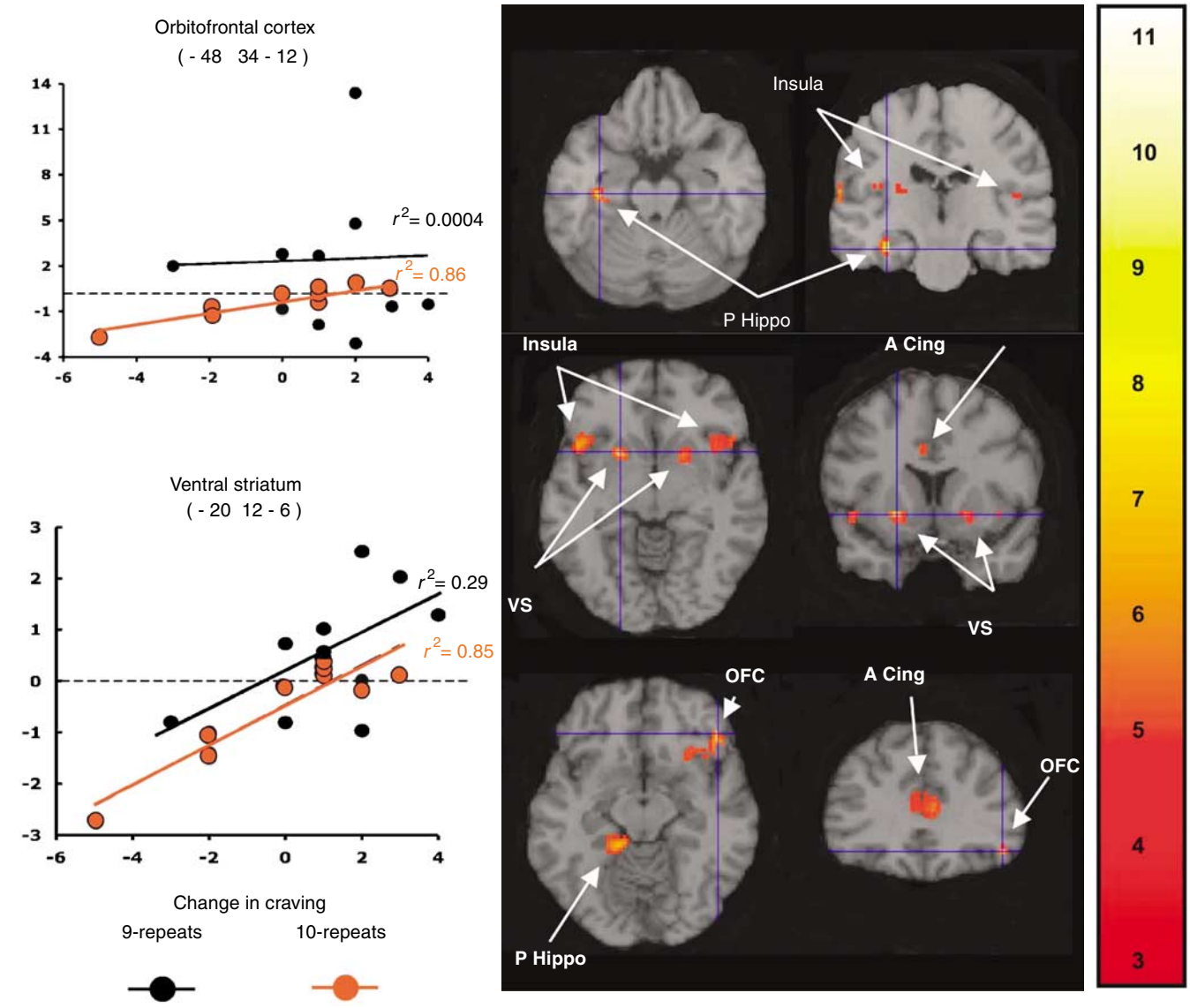


Figure 2 Graphs of individual perfusion fMRI responses (β-coefficients) as a function of the change in craving elicited by the smoking cues in the orbitofrontal cortex (OFC) and ventral striatum (VS). Strong correlations in 10/10-repeats contrast with weak or insignificant correlations in 9-repeats. Imaging data were analyzed in SPM2 and is displayed in MRIcro overlaid on the Montreal Neurological Institute (MNI) brain. As shown in representative brain slices, correlations with craving in 10/10-repeats were also observed in anterior cingulate, $r^2 = 0.89$; right ventral striatum, $r^2 = 0.85$; left parahippocampal gyrus, $r^2 = 0.85$; and bilaterally in the insula, $r^2 = 0.81$ on right and 0.85 on left. Coordinates are in MNI. Note that the abscissas are adjusted in relation to the intensity of perfusion in each region. Slices are displayed neurologically in (left is left). *T*-values are shown in the graph (right) and ranged from 4.42 to 5.69 at $p < 0.01$, 20 contiguous voxels.

and may increase the probability of relapse associated with cue exposure.

Interpretation of Results

Differences between groups in underlying motivational processes, possibly genetically mediated, may partially explain the differences in brain activation. As the two primary motivators to smoke are pharmacological withdrawal from nicotine and exposure to smoking-related stimuli, and as smokers were satiated prior to cue sessions, we speculate that the pharmacological and physiological state produced by recent smoking eliminated the power of the cues to elicit a brain or behavioral response in 10/10-repeats, whose smoking behavior may be motivated to a larger extent by the absence of nicotine in the brain. As the OFC is involved in assigning the relative motivational significance to rewards and their signals (Salzman *et al*, 2007), a reduction in activity in that region may signal reduced cue value in the sated condition. Alternatively, activity in the OFC and related regions subserving motivational responses to emotional stimuli may be sustained even in the sated state in 9-repeats with a less active or less available form of the DAT.

The above interpretation may explain some of the variance but does not address the finding of a decreased response in VS/VP/OFC in 10/10-repeats. It is unlikely that they were uninterested or able to ignore the SCs as the

parahippocampal region, important in episodic memory (conscious recall of autobiographical events), was strongly activated in both groups, and subjective responses assessing cue interest did not differ between groups.

Were SCs aversive to 10/10-repeats in the satiated condition? It has been shown that even chocolate, as palatable as it may be to professed chocolate lovers, becomes increasingly unpleasant as more of it is consumed, which correlates dose dependently with changes in the location and direction of neural activity (Small *et al*, 2001). Further, 10/10-repeats exhibited strong bilateral activation in the insula, not observed in 9-repeats. This is consistent with a wealth of literature wherein insula activation is associated with increased feelings of disgust to aversive stimuli (Schafer *et al*, 2005; Schienle *et al*, 2002; Stark *et al*, 2007; Wright *et al*, 2004). This interpretation is supported by recent work in our laboratory with cocaine-addicted patients wherein we demonstrated a positive correlation between brain activation bilaterally in the insula to 33 ms unseen aversive targets and negative affective bias to visible versions of the same stimuli in an off-magnet task (Childress *et al*, 2008). It is also supported by the finding here of an overall decrease in craving elicited by SCs only in 10/10-repeats. There is also an extensive literature wherein heightened insula activity is implicated in mediating responses to appetitive stimuli, including our previous study examining responses to smoking stimuli explicitly designed to be highly appetitive to smokers (Bjork *et al*, 2004; Franklin *et al*, 2007; Hinton *et al*, 2004; Wang *et al*, 2004). As the insula is a large and heterogeneous structure and is implicated in mediating both aversive and appetitive responses, its continued study focusing on discrete regional activity is warranted before a clear interpretation can be made. The addition of an affective bias task will test the above hypothesis in future work.

Although no significant correlations with craving and brain response was observed in 9-repeats, 10/10-repeats exhibited robust correlations between brain and behavior in several reward-related regions. We tentatively suggest that processing in the frontostriatal regulatory network is disturbed in 9-repeats such that they are less able to correctly label their craving experience. The inability to label craving may confer enhanced vulnerability to the effects of cues and therefore increased probability of relapse in their presence. In other words not being able to identify feelings correctly may make it harder to control behavior. Although not tested here and purely speculative at this juncture, the inability to describe or identify the craving state may reflect an underlying and genetically mediated personality trait that generalizes to other emotions such that 9-repeats are exhibiting an 'alexithymic' response to cues. Alexythymia is a personality trait characterized by deficits in cognitive processing of emotions and is highly prevalent in various psychological disorders (Alvarez and Shipko, 1991; Shipko *et al*, 1983) including drug addiction (Taylor *et al*, 1990). Alexithymic individuals are deficient in understanding and describing emotions and are at high risk for poor treatment response using conventional methods (Haviland *et al*, 2000). In support of this theory, the neural responses in 9-repeats concur with those of a PET study comparing alexithymic and normal responses to emotional stimuli. As in 9-repeats, alexithymics had less activation in superior frontal gyrus and anterior cingulate and greater activation in ventral medial prefrontal gyrus (Karlsson *et al*, 2008). However, the literature examining brain correlates of alexithymia is sparse and somewhat inconsistent, with the exception of a general consensus on the lack of activation in the anterior and superior frontal gyri in alexithymic individuals (Berthoz *et al*, 2002; Huber *et al*, 2002; Kano *et al*, 2003).

An alternative explanation for the lack of association between brain activity and craving in 9-repeats is that the cues had induced a maximal response in brain blood flow to reward-related regions. This interpretation is unlikely as examination of the pattern of activation in individual 9-repeats is quite variable across subjects and across regions. This interpretation is further undermined as pre-cue craving scores were similar in both groups. Nevertheless, this possibility cannot be ruled out.

Notably, overall craving difference scores to the SCs approached significance and were positive in 9-repeats and negative in 10/10-repeats. These same sets of cues elicited craving in a previous study, demonstrating that the SCs do elicit a robust subjective craving response in sated smokers (Franklin *et al*, 2007). Although the brain's response to emotionally laden processes is not under subjective control, neurophysiological measures are presumed to be more direct and more sensitive than subjective measures of the same processes, requiring much smaller sample sizes to demonstrate effects (Hariri *et al*, 2006). Our previous study had twice the number of subjects, which appears to be necessary to achieve significant overall craving difference scores in sated smokers. There were also no differences in other behavioral measures such as cigarette dependence as measured by the FTND or number of cigarettes smoked per day. Again, this may be a function of small sample size. Alternatively, genotype may not predict the degree of dependence, cigarettes smoked per day or even smoking cessation outcome. It may be related to differential underlying motivations to continued smoking and relapse as discussed above, and if true, could aid in our efforts to reduce relapse rates by using genotype to predict medication response. For example, if 9-repeats' smoking behavior is affected more by SCs and less by WD symptoms, nicotine replacement therapy may not be a viable treatment option.

Study Limitations

These results should be interpreted with caution given the modest sample size. In addition, the use of probands of various ethnic backgrounds might lead to issues of population stratification as global population studies have demonstrated varying frequencies of the two alleles (Gelernter *et al*, 1998; Palmatier *et al*, 1999). Future studies will have to be carefully matched for ethnic background and might have to include genomic controls to address issues of population stratification.

Further investigation into the genetics underlying DA signaling in reactivity to SCs is warranted since it is likely that additional polymorphisms contribute to different responses. Indeed, a DA D4 receptor subtype polymorphism has been implicated in influencing responses to SCs (Hutchison *et al*, 2002; McClernon *et al*, 2007). The DA

receptor subtype 2 (DRD2) is also a likely candidate as genetic load has been associated with an increased risk of substance abuse and other addictive behaviors, including smoking (Batra *et al*, 2003; Comings *et al*, 1996; Noble *et al*, 1994), however see (Bierut *et al*, 2000; Singleton *et al*, 1998). In addition, genetic variance in the DRD2 Taql RFLP and the DAT *SLC6A3* had an additive effect on subjective craving scores generated by SC exposure (Erblich *et al*, 2005). Interactions between dopaminergic polymorphisms may also be important. For instance, individual differences in VS sensitivity to reward were partly explained by an epistatic gene–gene interaction between the *SLC6A3* and the catechol-*o*-methyltransferase (*COMT*) genes (Yacubian *et al*, 2007). Because of these and other studies we will broaden our examination to determine whether SC reactivity is modulated by genetic variance in the DRD2 and DRD4 receptor subtypes and in COMT.

Conclusions

Undeniably, dependence on cigarettes is the number one cause of premature death and exacts a massive economic burden on families and society as a whole. Though relapse to smoking and other psychoactive drugs is multi-determined, a commonly cited state that precedes relapse is cue-induced craving. It is imperative that we continue to strive to understand the brain mechanisms underlying craving and its link to relapse. These results support our hypothesis that the brain's response to SCs is partially genetically mediated through variance originating in dopaminergic components.

They point to an underlying preexisting mechanism in the etiology of addiction processes by providing evidence that the neural and behavioral responses to smoking-related stimuli are mediated by at least one dopaminergic gene variant. These results are a significant contribution to the field of cigarette and other addictions, as they may prove useful in identifying endophenotypes, which may have immediate implications for clinical outcome and for medications development. Further, they indicate that the combined use of neuroimaging and genomics offers the potential to develop predictive markers of relapse vulnerability and identify novel targets for individualized treatment.

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DISCLOSURE/CONFLICT OF INTEREST

Dr JA Detre has received royalties for the commercial licensure of ASL perfusion fMRI. None of the other authors have reported any potential conflicts of interest.

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