

Original investigation

# Evidence of Altered Brain Responses to Nicotine in an Animal Model of Attention Deficit/Hyperactivity Disorder

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## Abstract

**Introduction:** Individuals with attention deficit/hyperactivity disorder (ADHD) are susceptible to earlier and more severe nicotine addiction. To shed light on the relationship between nicotine and ADHD, we examined nicotine's effects on functional brain networks in an animal model of ADHD.

**Methods:** Awake magnetic resonance imaging was used to compare functional connectivity in adolescent (post-natal day  $44 \pm 2$ ) males of the spontaneously hypertensive rat (SHR) strain and two control strains, Wistar-Kyoto and Sprague-Dawley ( $n = 16$  each). We analyzed functional connectivity immediately before and after nicotine exposure (0.4 mg/kg base) in naïve animals, using a region-of-interest approach focussing on 16 regions previously implicated in reward and addiction.

**Results:** Relative to the control groups, the SHR strain demonstrated increased functional connectivity between the ventral tegmental area (VTA) and retrosplenial cortex in response to nicotine, suggesting an aberrant response to nicotine. In contrast, increased VTA-substantia nigra connectivity in response to a saline injection in the SHR was absent following a nicotine injection, suggesting that nicotine normalized function in this circuit.

**Conclusions:** In the SHR, nicotine triggered an atypical response in one VTA circuit while normalizing activity in another. The VTA has been widely implicated in drug reward. Our data suggest that increased susceptibility to nicotine addiction in individuals with ADHD may involve altered responses to nicotine involving VTA circuits.

**Implications:** Nicotine addiction is more common among individuals with ADHD. We found that two circuits involving the VTA responded differently to nicotine in animals that model ADHD in comparison to two control strains. In one circuit, nicotine normalized activity that was abnormal in the ADHD animals, while in the other circuit nicotine caused an atypical brain response in the ADHD animals. The VTA has been implicated in drug reward. Our results would be consistent with an interpretation that nicotine may normalize abnormal brain activity in ADHD, and that nicotine may be more rewarding for individuals with ADHD.

## Introduction

Clinical studies indicate that the earliest symptoms of nicotine addiction can appear after the first cigarette.<sup>1</sup> This has focused attention on the potential effects of a single dose of nicotine. In terms of biochemical effects, a single dose of nicotine increases glutamate transmission in various brain regions both *in vivo*<sup>2,3</sup> and *in vitro*.<sup>4-6</sup> In terms of behavioral effects, locomotor sensitization may be apparent with the second dose, suggesting that neurologic alterations associated with sensitization can begin with the first dose.<sup>7,8</sup>

The first doses of nicotine do not initiate addiction in all tobacco users; in fact, there is significant individual variability in susceptibility to the development of nicotine addiction which is attributed in part to genetic influences.<sup>9</sup> At particular, risk of addiction are youths with attention deficit hyperactivity disorder (ADHD). Adolescents diagnosed with ADHD begin smoking at a younger age and are more than eight times more likely to develop nicotine addiction than are healthy controls.<sup>10</sup> There is interest in identifying the characteristics of the brains of individuals with ADHD that might facilitate nicotine addiction. Aside from addiction issues, it has been hypothesized that nicotine might help to normalize brain function in youths with ADHD.<sup>11</sup> Therefore, we were interested in determining whether the ADHD brain reacts to nicotine in a way that suggests that brain function is normalized or that suggests that nicotine may be more rewarding.

In order to advance our understanding of ADHD, a variety of animal models have been developed. The most extensively studied model is the adolescent spontaneously hypertensive rat (SHR). It is an inbred strain originating from outbred Wistar rats selected for high blood pressure.<sup>12</sup> It develops hypertension beyond puberty, prior to which it has been used and validated as a model of ADHD.<sup>12,13</sup> The SHR line shows dopaminergic alterations,<sup>14,15</sup> and cholinergic alterations<sup>16-18</sup> which are of interest given associations between prenatal maternal smoking and ADHD and cholinergic dysregulation.<sup>19,20</sup>

Although the role of nicotinic receptor up-regulation in addiction is not fully elucidated, nicotine induces receptor up-regulation in rats and humans,<sup>21</sup> but not in the SHR.<sup>22</sup> The SHR also shows dampened nicotine-induced norepinephrine release.<sup>23</sup> The SHR also differs from other strains in relation to resting state functional connectivity (rsFC).<sup>24,25</sup> rsFC analysis identifies correlations in activity in different parts of the brain by detecting temporal correlations of low-frequency spontaneous fluctuations of the blood oxygenation level-dependent signal.<sup>26-28</sup> We have found marked functional connectivity differences in mesocorticolimbic reward circuits in the SHR.<sup>25</sup> To investigate whether ADHD involves altered responses to nicotine, we used rsFC to study brain responses to the initial exposure to nicotine in the SHR and two control strains, the Wistar-Kyoto parent strain and the outbred Sprague-Dawley.

## Methods

### Animals

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Massachusetts Medical School and carried out in accordance with the guidelines published by the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. In total, 48 adolescent male rats (postnatal day 25–28) were obtained from Harlan Laboratories, Inc., which included 16 Sprague-Dawley (SD), 16 SHR (SHR/NHsd) and 16 Wistar-Kyoto (WKY/NHsd), each strain later randomly separated into two equal drug groups. The SHR/NHsd line was selected for (1)

its repeatedly demonstrated open field locomotor hyperactivity<sup>29-32</sup> and atypical attention patterns<sup>29,33-35</sup>; (2) its genetic polymorphism in neuronal nicotinic receptor  $\alpha 3$ ,<sup>36</sup> a gene that has been associated with nicotine use in subjects with ADHD,<sup>37</sup> and (3) our data showing rsFC alterations in mesocorticolimbic reward circuits.<sup>25</sup> Animals were housed in pairs and the environment was maintained at 22–24°C with a reversed 12 hour light–12 hour dark schedule (lights on at 9:00 PM and off at 09:00 AM). Food and water were provided *ad libitum*.

### Drugs

Nicotine hydrogen tartrate (Sigma) was dissolved in saline, pH adjusted to  $7.2 \pm 0.2$ , and delivered subcutaneously at a dose of 0.4 mg nicotine base/kg in a volume of 1 mL/kg. This commonly used dosage reflects human cotinine plasma levels after smoking<sup>38</sup> and produces locomotor sensitization. Being a moderate dose, it allows the potential observation of both amplified and dampened strain effects.<sup>39</sup>

### Acclimation for Imaging

Magnetic resonance imaging was conducted with awake animals. In spite of general network similarities between anesthetized and awake imaging,<sup>40</sup> there are qualitatively different functional connectivity patterns<sup>41,42</sup> and substantial dampening in the regional recruitment by nicotinic agonists.<sup>43</sup> We employed our validated procedure for the acclimation of rats to restraint and noise.<sup>44</sup> Briefly, rats were anesthetized with isoflurane and EMLA cream (lidocaine 2.5% and prilocaine 2.5%, Hi-Tech Pharmacal Co., Inc.) was applied topically to minimize pain from mechanical restraint. The animals were then secured in Plexiglas stereotaxic head holders using plastic ear bars. Animals were then placed in a black opaque tube “mock scanner” and exposed to recorded scanner noises. Animals were acclimated for 8 days, one session per day. The time of acclimation increased from 15 minutes on the first day to 90 minutes on days 6, 7, and 8, at increments of 15 minutes per day.

### Animal Preparation for Imaging

The animals were briefly anesthetized using isoflurane. The head was fitted into a head restrainer with a built-in coil, with the incisors secured over a bite bar. The nose was secured with a nose clamp and ear bars were positioned inside the head-holder with adjustable screws fitted into lateral sleeves. The body of the animal was then placed into a body restrainer, previously demonstrated to successfully minimize head movement.<sup>44</sup> Isoflurane was removed after this setup and the restraining system was positioned in the magnet. Imaging sessions started following signal optimization approximately 15 minutes after animals were placed in the magnet.

### Magnetic Resonance Imaging

Magnetic resonance imaging experiments were performed on a 4.7 T/40 cm horizontal magnet equipped with a Biospec Bruker console (inner diameter 12 cm). A surface coil (internal diameter 2.3 cm) was used for brain imaging.

For each rat, anatomical images were obtained using rapid acquisition relaxation-enhanced (RARE) sequence with the following parameters: relaxation time (TR) = 3000 ms, RARE factor = 8, echo time (TE) = 12 ms, resolution matrix =  $256 \times 256$ , field of view (FOV) = 32 mm  $\times$  32 mm, slice number = 18, and slice thickness = 1 mm.

Two functional scans were subsequently performed using echoplanar imaging (EPI) sequence with the following parameters: TR = 1 s, TE = 30 ms, flip angle = 60°, resolution matrix = 64 × 64, FOV = 32 mm × 32 mm, and slice thickness = 1 mm. The first EPI (EPI1) was 20 minutes (1200 repetitions) and the second EPI scan (EPI2) was 30 minutes (1800 repetitions). EPI1 was acquired without drug or saline administration. EPI2 had a 1-minute baseline period, followed by a subcutaneous injection over 3 seconds of saline or nicotine and followed by 29 minutes of continuous data acquisition. EPI1 and the final 20 minutes of EPI2 from all animals were used for the rsFC analyses.

### Preprocessing of Imaging Data

The imaging data were preprocessed using Medical Image Visualization and Analysis (MIVA, <http://ccni.wpi.edu/>), MATLAB (The Mathworks Inc., Natick, MA), and SPM8 (Wellcome Department of Cognitive Neurology, London, UK). Each 20-minute resting-state session was then divided into six short runs (200 repetitions per run) for functional connectivity analysis. All EPI sessions were first aligned and coregistered to a fully segmented rat brain atlas in MIVA.<sup>28</sup> After registration, all sessions went through the following pre-processing steps: motion correction, spatial smoothing (full width-half maximum = 1 mm), and 0.002–0.1 Hz band-pass filtering. Eight runs with excessive motion (>0.2 mm in each direction) were discarded (two runs each from two animals and one run from four animals). The time course for each individual voxel was further corrected for head movement by regression on the six motion parameters (translations and rotations) estimated in the procedure of motion correction. Furthermore, white matter and ventricle signals were estimated by averaging the time courses of all voxels inside the white matter and ventricles and then regressed out from time courses for rsFC analysis.

### rsFC analysis

rsFC was evaluated using a region-of-interest (ROI)-based correlational analysis on a voxel-by-voxel basis.<sup>28</sup> Sixteen ROIs were chosen representing reward, emotion, and executive function areas: the prelimbic area (PL), infralimbic area (ILA), anterior cingulate cortex (ACC), nucleus accumbens (NAcc), ventral tegmental area (VTA), insula (INS), amygdala (AMG), retrosplenial cortex (RSP), hippocampus (HP), caudoputamen (CPu), substantia nigra (SNr), visual cortex (VIS), substantia innominata/ventral pallidum (SI), medial habenula (MH), and the interpeduncular nucleus (IPN). For each animal, regionally averaged time courses from all voxels inside the ROI regions were used as time courses to correlate. Pearson cross-correlation coefficients (CCs) between each two ROI time courses were calculated. CCs were transformed using Fisher's Z transformation. Z- scores from each of the six short runs were averaged and then transformed back to CCs to produce representative rsFC of the animal.

### Group Analysis

For each of the two EPI scans, the strength of rsFC for each ROI couple was compared using mixed-design analyses of variance (ANOVA) with the three strains (SD, SHR, or WKY) and two drug groups (saline or nicotine) as between-subject factors, as well as the imaging time points (pre- and post-drug administration) as a within-subject factor. To identify unique responses to nicotine in the SHR strain, the impact of this substance had to differ between the SHR and the

other two strains. Specifically, any significant three-way interactions, adjusted for multiple testing according to false discovery rate procedure (FDR,  $p < .05$ , according to the number of regional couplings tests)<sup>45</sup> were followed up with simple effects analyses adopting the least significant difference adjustment for multiple comparisons.

### Results

The ANOVAs identified 32 circuits in which rsFC alterations were identified in relation to either differences between strains, differences between saline and nicotine, and/or differences before and after drug administration (Table 1). However, our a priori criterion for results of interest according to a significant three-way interaction was met in only two circuits: the RSP-VTA ( $F = 3.7, p = .034$ ) and VTA-SNr ( $F = 3.4, p = .045$ ; FDR-adjusted to 0.047). As shown in Figure 1, RSP-VTA functional coupling was significantly greater after nicotine administration in the SHR, with the SHR showing increased rsFC in this circuit while the opposite effect was seen in the two control strains ( $F_{(2,41)} = 5.0, p = .012$ ). For VTA-SNr coupling, the SHR showed increased rsFC relative to both control strains in response to a saline injection ( $p < .05$ ) but no strain differences were seen after a nicotine injection (Figure 2). A small difference between the SHR and one of the control strains, WKY, in the before-saline scan was not observed in the equivalent before-nicotine scan, yet overall strain ranking was maintained across these conditions and no significant within-strain differences between “before” conditions of each drug were observed.

### Discussion

We were interested in determining whether the brain responds to nicotine differently in an animal model of ADHD. As ROIs, we chose brain regions associated with reward, emotion, and executive function.<sup>46</sup> Two circuits involving the VTA demonstrated altered rsFC in response to the first dose of nicotine in the SHR as compared to both control strains. Nicotine acts on dopaminergic neurons in the VTA, stimulating the release of dopamine in the NAcc which is hypothesized to contribute to incentive salience.<sup>47</sup> In the current study, significant differences in rsFC were identified in 32 circuits (Table 1). Alterations in rsFC in response to nicotine were observed in a number of circuits previously associated with nicotine effects or conditioned responses,<sup>18,46,48–52</sup> including VTA-MH, SN-MH, INS-SN, RSC-HP, and RSC-ACC (Table 1, drug main effects). Selective strain differences in the impact of nicotine (pre- vs. post-administration) included an increase in rsFC in the RSP-VTA circuit in the ADHD model, the SHR, while having the opposite effect in the control strains. It is tempting to speculate that this enhancement of VTA functional connectivity may underlie enhanced rewarding properties of nicotine,<sup>53,54</sup> or even the dampened aversive properties,<sup>55</sup> even though we did not observe rsFC changes in the VTA-NAcc circuit (Table 1).

This absence of altered VTA-NAcc connectivity is notable, even though rewarding properties of nicotine in the VTA have also been associated with engagement of other brain regions.<sup>56</sup> Although we observed changes in rsFC in response to nicotine in several circuits, there might be limitations in the sensitivity of rsFC, as examined using a seed-based approach, in relation to NAcc subregional<sup>57</sup> or even cell population heterogeneity of response to nicotine.<sup>56</sup> Another consideration is that we examined only one dose, 0.4 mg/kg nicotine base. This is the most commonly used dose of nicotine

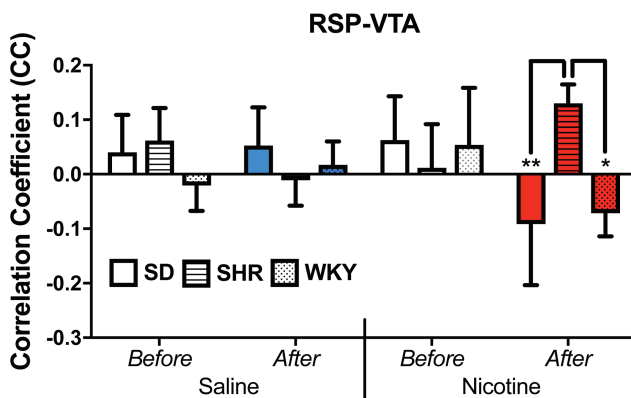
**Table 1.** Main Effects, Two-way, and Three-way Interactions for Each Regional Coupling

Regional coupling		Strain	Time	Drug	Strain × drug	Strain × time	Drug × time	Strain×drug × time
VTA-SN	F	/	/	/	/	/	4.082	3.356
	p	/	/	/	/	/	.050	.045
VTA-IPN	F	/	/	/	/	3.23	/	/
	p	/	/	/	/	.050	/	/
VTA-CPu	F	/	/	/	4.998	/	/	/
	p	/	/	/	.011	/	/	/
VTA-MH	F	/	/	23.872	/	/	/	/
	p	/	/	< .001	/	/	/	/
RSP-VTA	F	/	/	/	/	/	/	3.667
	p	/	/	/	/	/	/	.034
RSP-INS	F	/	/	/	/	5.029	/	/
	p	/	/	/	/	.011	/	/
RSP-Acc	F	4.456	/	4.155	3.670	5.609	/	/
	p	.018	/	.048	.034	.007	/	/
RSP-PL	F	6.377	/	/	/	5.019	/	/
	p	.004	/	/	/	.011	/	/
RSP-HP	F	/	7.982	7.268	5.375	/	/	/
	p	/	.007	.010	.008	/	/	/
RSP-CPu	F	/	/	/	/	4.481	/	/
	p	/	/	/	/	.017	/	/
RSP-MH	F	/	/	/	3.539	3.717	10.818	/
	p	/	/	/	.038	.033	.002	/
AMG-RSP	F	3.396	/	/	/	/	/	/
	p	.043	/	/	/	/	/	/
AMG-VTA	F	5.408	/	/	/	6.202	7.227	/
	p	.008	/	/	/	.004	.010	/
AMG-CPu	F	/	7.634	5.570	/	13.212	7.431	/
	p	/	.009	.023	/	< 0.001	.009	/
AMG-MH	F	/	4.565	/	/	/	/	/
	p	/	.039	/	/	/	/	/
INS-Acc	F	/	5.476	/	/	/	/	/
	p	/	.024	/	/	/	/	/
INS-ILA	F	/	/	5.212	/	/	/	/
	p	/	/	.028	/	/	/	/
INS-SN	F	/	/	9.768	/	3.279	/	/
	p	/	/	.003	/	.048	/	/
Acc-ILA	F	/	4.75	/	/	/	/	/
	p	/	.035	/	/	/	/	/
Acc-NAcc	F	/	6.012	/	/	/	/	/
	P	/	.019	/	/	/	/	/
Acc-CPu	F	/	10.824	/	/	/	/	/
	p	/	.002	/	/	/	/	/
ILA-SN	F	/	4.417	/	/	/	/	/
	p	/	.042	/	/	/	/	/

Table 1. Continued

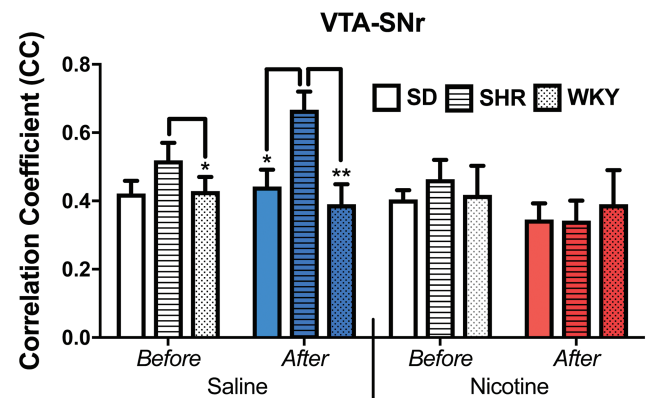
Regional coupling	Strain	Time	Drug	Strain × drug	Strain × time	Drug × time	Strain×drug × time
ILA-NAcc	F	10.821	/	/	4.210	/	/
	<i>p</i>	< .0010	/	/	.022	/	/
ILA-PL	F	5.329	/	/	5.510	/	/
	<i>p</i>	.009	/	/	.008	/	/
NAcc-VTA	F	/	/	/	5.057	/	/
	<i>p</i>	/	/	/	.011	/	/
NAcc-PL	F	5.323	9.22	/	/	/	/
	<i>p</i>	.009	.004	/	/	/	/
NAcc-HP	F	/	/	/	/	4.497	/
	<i>p</i>	/	/	/	/	.040	/
PL-CPu	F	/	5.765	/	/	/	/
	<i>p</i>	/	.021	/	/	/	/
PL-IPN	F	/	4.956	/	/	6.367	16.454
	<i>p</i>	/	.032	/	/	.004	< .0010
PL-MH	F	4.333	/	/	/	/	/
	<i>p</i>	.020	/	/	/	/	/
SN-MH	F	/	/	8.899	/	/	/
	<i>p</i>	/	/	.005	/	/	/
MH-IPN	F	/	/	5.171	/	/	/
	<i>p</i>	/	/	.028	/	/	/

PL = prelimbic area; ILA = infralimbic area; ACC = anterior cingulate cortex; NAcc, nucleus accumbens; VTA = ventral tegmental area; INS = insula, AMG = amygdala; RSP = retrosplenial cortex; HP = hippocampus; CPu = caudoputamen; SNr = substantia nigra; VIS = visual cortex; SI = substantia innominata/ventral pallidum; MH = medial habenula; IPN = interpeduncular nucleus. *F*-value of ANOVA; *p*, statistical probability, unadjusted for multiple testing; /: *p* > .05.



**Figure 1.** Nicotine produced a positive response in RSP-VTA functional connectivity in the SHR, while it had the opposite effect in the SD and WKY. CC = correlation coefficient, RSP-VTA = retrosplenial cortex-ventral tegmental area, SD = Sprague-Dawley, SHR = spontaneously hypertensive rat, WKY = Wistar-Kyoto; \**p* < .05, \*\**p* < .01.

in animal sensitization studies because it produces robust effects and reflects human cotinine plasma levels after smoking,<sup>38</sup> while higher doses tend to produce toxicity.<sup>58</sup> Nevertheless, a higher dose might have produced more perceptible effects on rsFC for these regions. Another consideration could be the differential impact of repeated exposure. We measured rsFC in response to a single dose of nicotine administered to naïve animals (Table 1). It is known that rats become sensitized to the effects of nicotine such that neural



**Figure 2.** VTA-SNr functional connectivity enhancement that was present in the SHR following a saline injection was absent after nicotine administration. CC = correlation coefficient, SD = Sprague-Dawley, SHR = spontaneously hypertensive rat, VTA-SNr = ventral tegmental area-substantia nigra, WKY = Wistar-Kyoto; \**p* < .05, \*\**p* < .01.

activation is much more widely distributed across brain regions after repeated doses as compared to the first exposure.<sup>46</sup> It may be that changes in rsFC in some circuits appear only after repeated exposures to nicotine have triggered neuroplastic changes. Future studies could examine the effects of repeated exposures to test this hypothesis.

Human neuroimaging studies suggest a role for the RSC in craving and smoking-cue responsivity.<sup>59,60</sup> In humans, the RSC exhibits



the highest nicotinic receptor binding among the cingulate subregions.<sup>61</sup> In animals, it responds to nicotinic exposure with increased blood flow.<sup>43,46,62,63</sup> Following chronic nicotine exposure, rsFC between the RSC and VTA remains sensitized beyond 12 hours.<sup>48</sup> Given the role of the RSC in smoking cue responsivity, our data suggest that ADHD may be associated with increased susceptibility to developing incentive-salience.

In relation to the VTA-SNr functional circuitry, differences between the SHR and control strains were evident in the absence of nicotine and disappeared when nicotine was administered (Figure 2), opposite to previously discussed RSP-VTA connectivity for which differences emerged, rather than disappeared, upon nicotine exposure (Figure 1). Compared to the control strains, the SHR showed increased rsFC in response to a saline injection. This suggests that the SHR is more sensitive to stressful stimuli. However, when the injection delivered nicotine instead of saline, there was no difference in rsFC between the SHR and controls. This suggests that nicotine may have normalized the stress response in the SHR. Smokers commonly report that they smoke to cope with stress.<sup>64</sup> Our data suggest that individuals with ADHD might find nicotine to be more effective in this role.

Nicotinic receptor modulators appear to present positive cognitive and attentional effects in ADHD.<sup>65,66</sup> The theory that adolescents with ADHD self-medicate with nicotine to improve their symptoms is widely asserted<sup>11</sup> but sparsely researched,<sup>67</sup> with mixed results.<sup>68,69</sup> The data presented in Figure 2 suggest that it is plausible that nicotine might help to normalize some brain functioning in individuals with ADHD.

We found that the SHR differed from both control strains in its initial reaction to nicotine. Humans also differ in their initial reactions to nicotine. While only a minority of individuals experience relaxation with their first exposure to nicotine, those who do are more likely to become addicted smokers.<sup>70–72</sup> While socially derived expectations might influence initial reactions to nicotine in humans, expectations cannot explain the differences in initial reactions observed in this experiment.

Genetic factors influence susceptibility to nicotine addiction.<sup>9,73</sup> Our data show differences in brain reactivity to the initial dose of nicotine in related, but genetically distinct strains. As the process of becoming addicted can be initiated by the first cigarette,<sup>74</sup> differences in the initial reaction to nicotine may be a manifestation of genetic susceptibility. ADHD has been associated with nicotinic receptor subunit single nucleotide polymorphisms.<sup>75–77</sup> Gene variants of subunits  $\alpha 3$ ,  $\alpha 6$ , and  $\beta 3$  predict the level of cigarette consumption with an interaction with ADHD symptoms.<sup>37</sup> Furthermore, a genetic variation of the  $\alpha 3$  subunit decreased the likelihood of smoking in the general population but increased the risk in individuals with ADHD.<sup>78</sup> This is notable as the SHR/NHsd strain used in the current study also carries a variation in the  $\alpha 3$  receptor.<sup>36</sup> To the degree that the SHR is a useful model of human ADHD, our findings suggest that the brains of adolescents with ADHD may react differently to nicotine than the brains of unaffected adolescents.

Among the limitations of this study is that only one dose and dosage of nicotine were evaluated. Another concern could be the elevated blood pressure that typically appears in SHR animals older than those used in this study.<sup>12,13</sup> While elevated blood pressure could affect brain blood flow, such effects should be global and not limited to a few circuits and would not be expected to influence coordination of regional brain activity. Strengths of this study included the use of two control strains and our requirement that the SHR differ from both control strains.

In conclusion, we identified significant differences in brain responses to the initial exposure to nicotine in an adolescent animal model of ADHD as compared to control strains. Brain activity was altered in regions with known involvement in nicotine reward, aversion, and addiction. These data suggest that genetically determined individual differences may alter how individuals respond to nicotine and such differences may contribute to the increased risk of nicotine addiction in adolescents with ADHD.

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## Declaration of Interests

None declared.

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GLP and WH contributed equally to this work.

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