BRAIN COMMUNICATIONS

Adolescent nicotine administration increases nicotinic acetylcholine receptor binding and functional connectivity in specific cortico-striatal-thalamic circuits

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Nicotine exposure is associated with regional changes in brain nicotinic acetylcholine receptors subtype expression patterns as a function of dose and age at the time of exposure. Moreover, nicotine dependence is associated with changes in brain circuit functional connectivity, but the relationship between such connectivity and concomitant regional distribution changes in nicotinic acetylcholine receptor subtypes following nicotine exposure is not understood. Although smoking typically begins in adolescence, developmental changes in brain circuits and nicotinic acetylcholine receptors following chronic nicotine exposure remain minimally investigated. Here, we combined in vitro nicotinic acetylcholine receptor autoradiography with resting state functional magnetic resonance imaging to measure changes in [^{3}H]nicotine binding and α4ß2 subtype nicotinic acetylcholine receptor binding and circuit connectivity across the brain in adolescent (postnatal Day 33) and adult (postnatal Day 68) rats exposed to 6 weeks of nicotine administration (0, 1.2 and 4.8 mg/kg/day). Chronic nicotine exposure increased nicotinic acetylcholine receptor levels and induced discrete, developmental stage changes in regional nicotinic acetylcholine receptor subtype distribution. These effects were most pronounced in striatal, thalamic and cortical regions when nicotine was administered during adolescence but not in adults. Using these regional receptor changes as seeds, resting state functional magnetic resonance imaging identified dysregulations in cortico-striatal-thalamic-cortical circuits that were also dysregulated following adolescent nicotine exposure. Thus, nicotine-induced increases in cortical, striatal and thalamic nicotinic acetylcholine receptors during adolescence modifies processing and brain circuits within cortico-striatal-thalamic-cortical loops, which are known to be crucial for multisensory integration, action selection and motor output, and may alter the developmental trajectory of the adolescent brain. This unique multimodal study significantly advances our understanding of nicotine dependence and its effects on the adolescent brain.

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Abbreviations: ACF = autocorrelation function; AFNI = analysis of functional neuroimaging; Au1 = primary auditory cortex; AuD = secondary auditory cortex, dorsal area; AuV = secondary auditory cortex, ventral area; BOLD = blood oxygen level dependent; CSTC = cortico-striatal-thalamic-cortical; dHP = dorsal hippocampus; Ect = ectorhinal cortex; FSL = FMRIB Software

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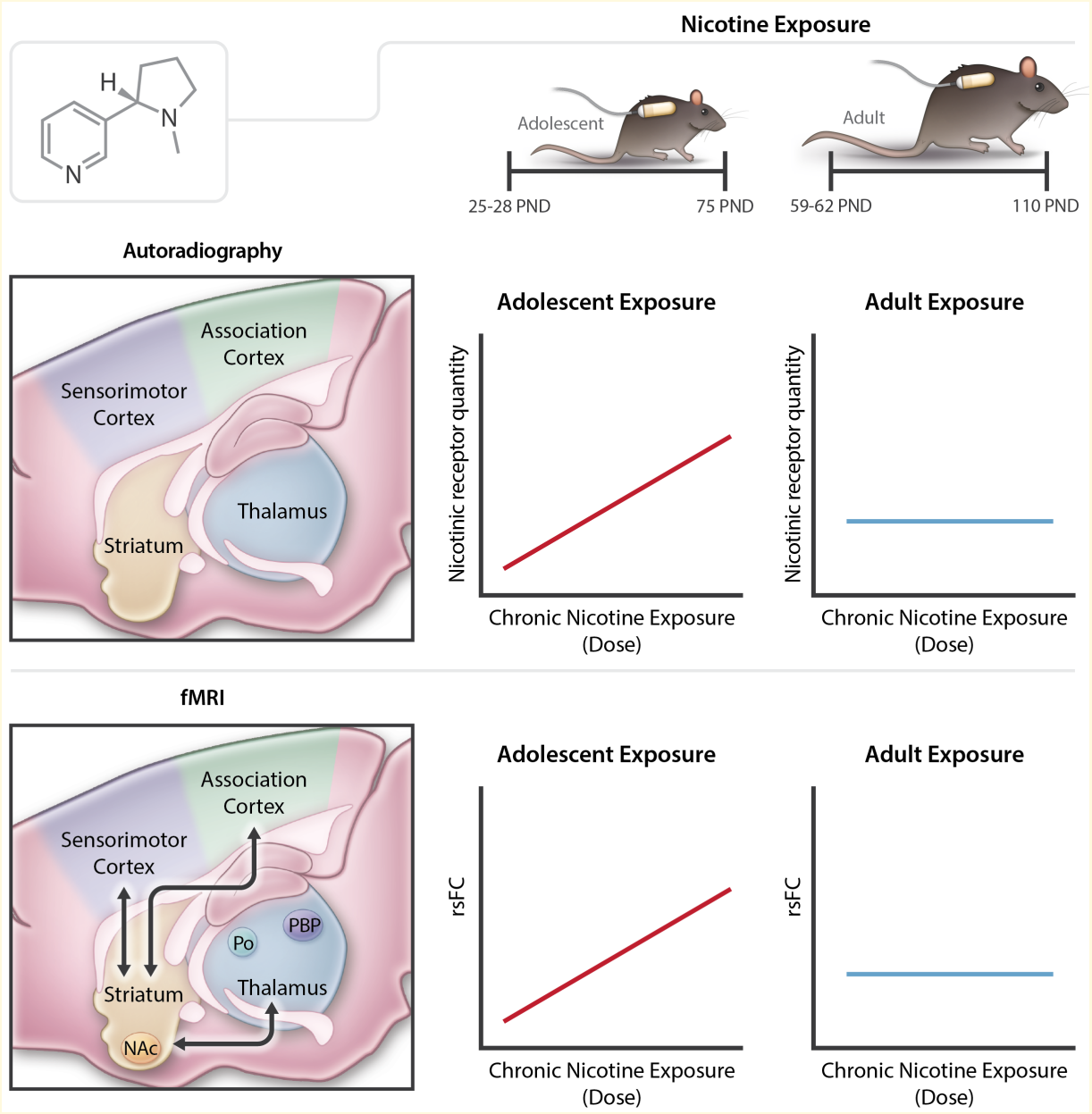
Library; FWHM = full width half maximum; HN = high 4.8 mg/kg/d; ICA = independent component analysis; LN = low 1.2 mg/kg/d nicotine; LP = lateral posterior nucleus of the thalamus; MG = medial geniculate nucleus of the thalamus; NAc = nucleus accumbens; nAChR = nicotinic acetylcholine receptor; *P* = postnatal day; PBP = parabrachial nucleus; PET = positron emission tomography; PF = parafasicular nucleus group of the thalamus; Po = posterior nucleus group of the thalamus; PP = peripeduncular nucleus of the thalamus; PtPR = parietal cortex, postural rostral part; ROI = region of interest; rsFC = resting state functional connectivity; rsfMRI = resting state functional magnetic resonance imaging; RSGD = retrosplenial cortex; S1 = primary somatosensory cortex; SAL = saline; s.c. = subcutaneous; tSNR = temporal signal to noise ratio; vHP = ventral hippocampus

Graphical Abstract



Nucleus accumbens (NAc)
Parabrachial nucleus (PBP)
Postnatal day (PND)
Posterior nucleus group of the thalamus (Po)
Resting state functional connectivity (rsFC)

Introduction

Cigarette smoking and subsequent nicotine dependence is the leading cause of preventable death in the USA, with 90% of smokers starting to smoke during adolescence.[1] With the recent swell of e-cigarette use among teenagers,[2] and evidence that nicotine vaping increases the likelihood of subsequent cigarette smoking,[3] understanding nicotine use and abuse in adolescence is critical to understanding, preventing and treating nicotine dependence and addiction.

Adolescence is an evolutionarily conserved developmental period[4] characterized by anatomical and functional alterations of cortical, striatal, thalamic and other brain circuits,[5,6] many of which overlap with those implicated in addiction.[7] Changes in these circuits in adolescence may underlie increases in vulnerability to substance use disorders.[8] This developmental vulnerability has been observed in both humans and in rodent laboratory models of nicotine exposure, where adolescents generally exhibit greater sensitivity to nicotine's rewarding effects but less sensitivity to its aversive consequences, including the nicotine withdrawal syndrome.[9–11]

Nicotine binds to endogenous nicotinic acetylcholine receptors (nAChRs),[4] pentameric ligand-gated ion channels[12] whose unique channel subunit composition determines ligand affinity, ion selectivity and sensitivity. The major nAChR subtype in the mammalian brain is the α4β2, which are localized throughout the brain.[13] They differ from other subtypes in terms of their sensitivity,[14–17] and regulation following agonist exposure.[18–27] Behaviourally, β2 subunits are critical for nicotine-induced reinforcement[28,29] and nicotine withdrawal.[30] However, a mechanistic understanding of how differences in nAChR regulation patterns following chronic nicotine exposure are involved in the developmental sensitivity to nicotine, and to nicotine dependence liability, remains limited due to the need to use radioactivity to measure receptors in humans using positron emission tomography (PET)[14] or research design limitations inherent to preclinical models.[31] Linking these regional changes in receptor density to circuit-level functional changes, and subsequently to their roles in mediating behaviours related to nicotine dependence may require a different experimental approach.

Resting state functional connectivity (rsFC) has been widely applied as a measure of functional circuit coherent activity.[32] Due to its non-invasive measurement, rsFC represents a unique tool for bridging the gap between human and rodent studies, as the identical blood oxygen level dependent (BOLD) MRI signal is measurable in the same way across species.[33] For example, across both humans and rodents, the severity of nicotine dependence has been shown to be negatively associated with the connectivity strength between the striatum and cingulate cortex,[34–37] whereas rsFC strength of an insular-frontal module is associated with an increased risk to develop nicotine dependence.[38] Nevertheless, how changes in nAChR densities as a function of chronic nicotine exposure relate to these and other circuit-level changes, especially with respect to the increased vulnerability of adolescents, remain to be studied.

Here, using a rat model of chronic nicotine exposure, we investigated changes in nAChR density throughout the brain following nicotine administration and subsequently how these effects relate to functional changes in associated brain circuits, providing a unique, integrative approach from receptors to circuits. Using a combined, within subject multimodal experimental design, we administered one of three doses of nicotine for 6 weeks to two aged cohorts (spanning the entirety of adolescent development and adulthood) and measured [^{3}H]nicotine to label the majority of nAChR subtypes and the specific α4β2 nAChR subtype using in vitro receptor autoradiography and MRI BOLD signal to quantify rsFC. Identified changes in regional nAChR receptor subtype binding density specific to adolescent exposure to nicotine (i.e. a significant interaction effect) were subsequently used as 'seeds' in a whole brain rsFC analytical framework to identify the functional circuit correlates of these receptor changes.

Materials and methods

For additional details, please see the supplementary material.

Subjects

Male Sprague-Dawley rats (Charles River Laboratories, Kingston, NY, USA) arrived at the National Institute on Drug Abuse Intramural Research Program (NIDA-IRP) between the ages of postnatal day (p) 25–28 for the adolescent and p59–62 for the adult cohorts. All procedures were conducted during the light phase and in accordance to approved protocols by the Animal Care and Use Committee of the NIDA-IRP.

Surgery

Rats were weighed daily, and their weights and relative weight gains across the study can be found in Supplementary Fig. 1. Using aseptic techniques, rats were implanted with subcutaneous osmotic minipumps (Model #2002, Alzet, Cupertino, CA, USA) preloaded with either saline (SAL) or nicotine hydrogen bitartrate salt [dissolved in saline (pH ~ 7.4) calculated as the free base to deliver either 1.2 mg/kg/d (low nicotine; LN) or 4.8 mg/kg/d (high nicotine; HN)]. These nicotine doses were chosen as they produce nicotine withdrawal symptoms in adult rats[39,40] and are comparable to doses that produce blood nicotine concentrations similar to those observed in human smokers.[31,41–45] The first implantation occurred at p33 and p68 for the adolescent and adult cohort, respectively. Pumps were replaced every 14 days for a total of 42 days of nicotine exposure in each cohort; in an attempt to maintain a relatively constant dosage, nicotine concentrations were adjusted prior to each surgery to account for weight gain. There were six experimental groups (total rats: $n = 66$): SAL ($n = 9$), LN ($n = 10$)

and HN ($n = 10$) starting in the adolescent period (p33), and SAL ($n = 13$), LN ($n = 12$) and HN ($n = 12$) in the adult period (p68)—all animals were assigned to groups in a quasi-random fashion.

Functional magnetic resonance imaging acquisition

Longitudinal neuroimaging measures were collected starting in adolescence at p33 and adulthood at p68, and rats were scanned every 14 days; only imaging data collected on the final scan (p75 for rats beginning in adolescence and p110 for rats beginning in adulthood) were analysed here to match the timing of the terminal autoradiography measurements (see supplementary material for details).

Brain collection and processing for autoradiography

After 42 days of nicotine administration, and immediately after the last imaging data acquisition, rats were anaesthetized with 5% isoflurane, rapidly decapitated and brains immediately harvested, flash frozen on dry ice and stored at −80°C. Brains were subsequently sectioned coronally (1:3; 20 µm) using a cryostat (−17°C) and mounted onto Superfrost Plus slides (Fisher Scientific, Newark, DE, USA), which were stored at −80°C.

Frozen sections were thawed for 1–2 h at room temperature prior to ligand binding procedures using [^{3}H]nicotine (specific activity: 79.8 Ci/mmol, Perkin Elmer, Waltham, USA) as a marker for total nicotinic receptors and [^{3}H]A-85380 (specific activity: 18 Ci/mmol, Novandi Chemistry, Sweden) for α4ß2 receptor specific subtype, which were adapted from previous protocols.[46,47]

Autoradiography quantification

Autoradiograms were analysed using ImageJ-Fiji.[48] Binding quantification was determined by comparing greyscale values from anatomically defined regions of interest (ROIs) to standard curves constructed from ^{14}C standards specific to each phosphor screen. A wire frame rat atlas including 91 *a priori* ROIs was created from a standard rat stereotaxic atlas,[49] imported as overlays into ImageJ-Fiji and fit onto corresponding brain sections in the autoradiograms. Median grey scale values from each region were averaged across two to three sections spanning most of the anterior–posterior plane. To calculate nicotine and α4ß2 nAChR specific binding, non-specific binding was subtracted from total binding values, and this value was averaged across sections for each ROI. Binding levels are expressed in fmol/g by dividing the calibrated activity values (nCi/g) by the specific activity of the radioligand (Ci/mmol), which was corrected for radioactive decay and estimated chemical degradation (calculated using the data on the technical data supplied by the manufacturer).

Statistical analysis

Autoradiography statistical analysis—using RStudio 1.2.5033,[50] individual two-way ANOVAs (nicotine dose × exposure age) were conducted for each ROI, followed by a pairwise *t*-test with Bonferroni correction for multiple comparisons. Significance was set to $p_{corrected} < 0.05$ throughout.

Functional magnetic resonance imaging data analysis—functional MRI data were processed using analysis of functional neuro-images (AFNI),[51,52] and the FMRIB software library (FSL) package.[53] Functional images were preprocessed using a standard pipeline, which included skull stripping, temporal SNR (tSNR) estimation, motion correction, registration, ICA denoizing, slice timing correction, band pass filtering (0.01–0.1 Hz), regression of average signals from cerebral spinal fluid and white matter and spatial blurring (FWHM of smooth kernel = 0.6 mm).[54]

Brain regions identified to have significant changes in nAChR autoradiography as a function of adolescent exposure to nicotine were used as seeds to identify the functional circuit consequences of changes in nAChR binding. The same atlas[49] was used to anatomically define seed regions. Seed-based, whole brain rsFC analyses between the mean beta weight values in the seed ROI and all other brain voxels were performed using 3dMVM to test for the interaction between age (adolescent versus adult) and nicotine dose (0, 1.2 and 4.8 mg/kg/d). Spatial autocorrelation function (ACF), an indicator of smoothness, was estimated using the 3dFWHMx function in AFNI and the average ACF across rats was used as a smoothing parameter in the 3dClustSim function to estimate the probability of false positive clusters. The significance threshold was set to $p_{corrected} < 0.05$ ($p_{uncorrected} < 0.01$, cluster size > 7 voxels).

Data availability

Raw data are available upon request to the corresponding author.

Results

Autoradiography: nicotine dose × age interaction

Representative autoradiograms of [^{3}H]nicotine total and [^{3}H]A-85380 α4ß2 binding from all experimental groups and their non-specific binding (lower panels) can be found in Supplementary Fig. 2. Since the nicotine dose (SAL, LN and HN) × age (adult, adolescent) interaction was the main contrast of interest herein, we address that below and then briefly describe the main effects of nicotine and main effects of age analyses at the end of Results section. Full details are given in Supplementary Tables 1–4 and results section.

[^{3}H]nicotine nAChR binding

Subregions of the thalamus [lateral posterior nucleus (LP), medial geniculate nucleus (MG), parafascicular nucleus

(PF) and posterior nucleus group (Po)], the parabrachial nucleus (PBP), association [ectorhinal cortex (Ect)] and sensorimotor cortices [secondary auditory cortex, ventral area (AuV); Fig. 1] demonstrated a significant nicotine dose × age interaction, such that increased [^{3}H]nicotine nAChR binding density was seen in the adolescent cohort, regardless of nicotine dose; this effect was not observed among adult rats (all $p_{corrected} < 0.05$; Fig. 2A–G; Supplementary Table 5). Further, HN administration induced greater binding across all these regions in adolescents when compared with adults. Of these regions, only AuV and Ect displayed dose-dependent increases in binding in adolescent but not adult-exposed rats. Furthermore, all identified thalamic subregions demonstrated increased binding in both the LN and HN exposed adolescents when compared with their adult counterparts.

α4β2 nAChR binding

Across multiple regions, there was a significant nicotine dose × age interaction such that in adolescents, nicotine administration changed [^{3}H]A-85380 binding across subregions of the sensorimotor [primary auditory cortex (Au1), secondary auditory cortex, dorsal area (AuD), AuV and primary somatosensory cortex (S1)] and association (parietal cortex, postural rostral part (PtPR) and retrosplenial cortex (RSGD) cortices, PBP, dorsal (dHP) and ventral hippocampus (vHP), and thalamus (LP and peripeduncular nucleus (PP) (Fig. 3; Supplementary Table 6). α4β2 receptor binding was elevated in the HN- when compared with their SAL-exposed adolescent counterparts in Au1, AuD, AuV and all measured subregions of the association cortex and the thalamus ($p_{corrected} < 0.05$; Fig. 4A–B, D–E, H–K). Finally, in all these regions except dHP, rats exposed to HN during adolescence had increased receptor density when compared with their HN adult exposed counterparts ($p_{corrected} < 0.05$; Fig. 2A–F, H–K). dHP displayed a unique pattern of α4β2 binding, with elevated receptor density observed only in adults exposed to LN when compared with the age matched SAL exposed counterparts (Fig. 2G).

Functional connectivity

We next asked whether circuits anchored in the regions highlighted above that showed significant nAChR density age × dose interactions also demonstrated modified functional circuit strength. Using these 16 regions as 'seeds' (the DLEnt region was not included in this analysis due to poor temporal signal to noise ratio), whole brain rsFC analyses revealed only two regions whose connectivity changed as a function of adolescent nicotine exposure: PBP (Fig. 5A), which shows a significant age × dose interaction for both [^{3}H]nicotine and α4ß2 nAChR binding, and Po (Fig. 6A), which shows a significant age × dose interaction only for [^{3}H]nicotine nAChR binding. In these areas, functional connectivity strength between the PBP and portions of the nucleus accumbens (NAc) showed greater connectivity in adolescent nicotine-exposed rats, whereas rats exposed to nicotine as adults showed decreased connectivity (Fig. 5B; note that bar graphs are for visual presentation and do not represent statistical contrasts). Notably, the change in connectivity between PBP and portions of the NAc with adolescent nicotine exposure paralleled the observed changes in [^{3}H]nicotine and α4ß2 nAChR binding, i.e. as the dose of nicotine increased among adolescents, both binding and connectivity increased; no such parallel was observed in adults, regardless of exposure to nicotine. In an exploratory follow-up analysis, we next placed a seed in this FC identified NAc region but observed no significant differences as a function of the age × dose interaction. Functional connectivity using the Po as a seed, whose [^{3}H]nicotine nAChR binding similarly changed as a function of age × dose, identified a Po-NAc circuit (Fig. 6A) with the same pattern of connectivity changes as the PBP seed. Compared with the adult cohort, adolescent exposure to nicotine increased connectivity between these two regions whereas adult exposure to nicotine blunted connectivity versus the adolescent group (Fig. 6B). Once again, these FC findings in adolescents parallel the effects on [^{3}H]nicotine nAChR binding; i.e. as the dose of nicotine increased among adolescents, [^{3}H]nicotine nAChR binding increased. Finally, in an exploratory analysis, seeding this NAc region revealed changes in connectivity (Fig. 7A) with multiple regions of the sensorimotor cortex (Fig. 7B and C) and thalamus (Fig. 7D and E). Most regions followed a similar pattern, such that nicotine administration in adolescence increased circuit strength between striatal and thalamic and thalamic and cortical regions whereas connectivity was decreased in adult rats exposed equally to nicotine (Fig. 7F). These data suggest that chronic nicotine-induced binding changes had profound, but spatially limited effects on functional circuits in adolescent exposure to nicotine.

Autoradiography: main effect of nicotine and age

Since the nicotine dose × age interaction on rsFC was the a priori focus of this experiment, the receptor binding main effects of nicotine dose and age were analysed (and reported below for completeness), but they did not subsequently serve as functional connectivity seeds.

nAChR binding

A dose-dependent increase in nAChR density was observed in most subregions of the frontal, sensorimotor and association cortices and all insular subregions, such that rats exposed to HN had greater [^{3}H]nicotine binding than those exposed to LN who in turn, had greater binding than the SAL group (Supplementary Fig. 3A and Table 1; $p_{corrected} < 0.05$). Effects for [^{3}H]nicotine binding were widely spatially distributed, with 32 of 91 ROIs showing significantly higher nAChR density in adolescent compared with adults rats (all $p_{corrected} < 0.05$; Supplementary Fig. 3C and Table 3), notably in regions such as the cingulate cortex, claustrum, auditory cortex, and select association cortices,

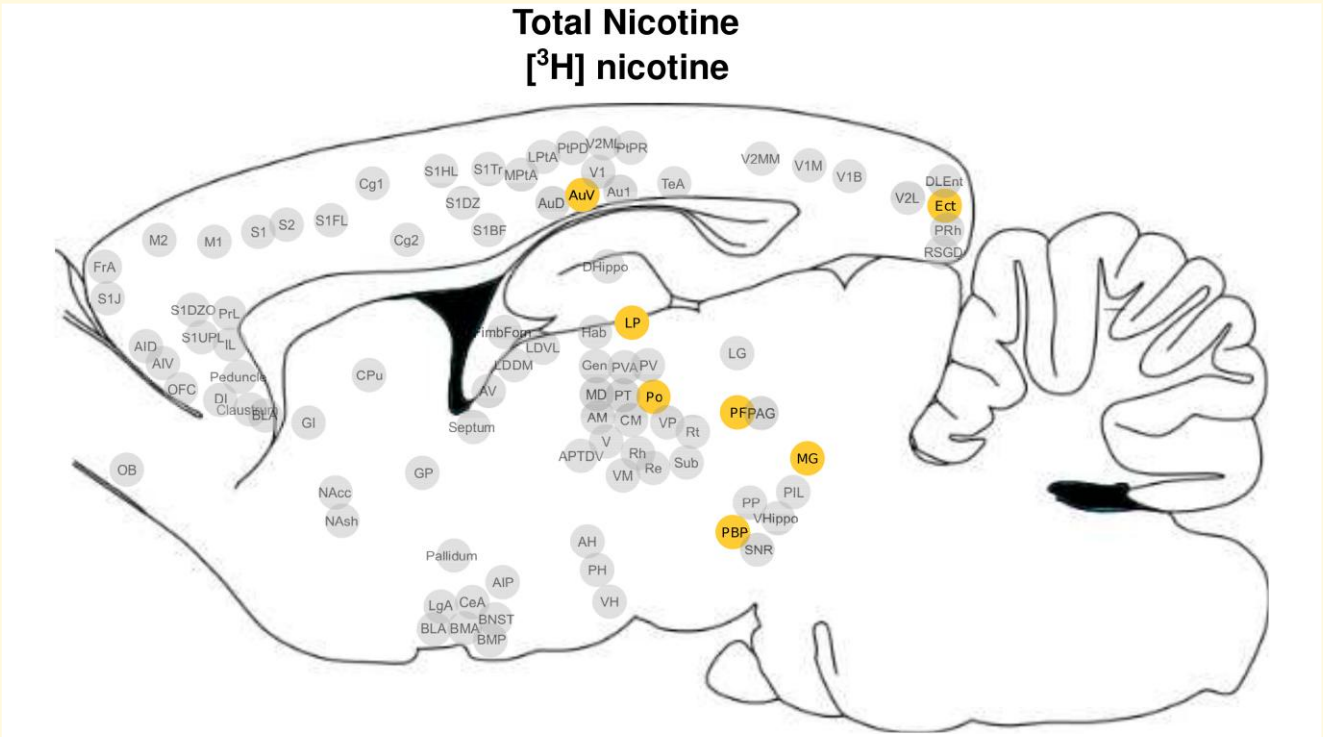


Figure 1 Brain regions with a significant nicotine dose × age interaction in [^{3}H]nicotine receptor binding. Pictorial representation of the seven ROIs that demonstrated significant nicotine dose × exposure age interaction effects for [^{3}H]nicotine receptor binding. A list of abbreviations used in this figure can be found in the Supplementary Material.

striatal, hippocampal, amygdala, extended amygdala and thalamic regions.

α4β2 nAChR binding

In all frontal, amygdalar, association and sensorimotor subregions measured and specifically in the ventral pallidum, the majority of the insula and select medial and posterior thalamic subregions, rats exposed to nicotine had significantly greater α4β2 density than those exposed to SAL (Supplementary Fig. 3B and Table 2; $p_{corrected} < 0.05$). In contrast to [^{3}H]nicotine receptor binding, a smaller subset of ROIs displayed significantly elevated α4β2 nAChR binding in adolescent rats, including select striatal and thalamic regions as well as the peduncle (all $p_{corrected} < 0.05$; Supplementary Fig. 3D and Table 4).

Discussion

Interaction analyses between nicotine dose and drug exposure age revealed that high doses of nicotine administered to adolescents (but not adults) caused elevated [^{3}H]nicotine and α4ß2 nAChR binding in portions of the association and sensorimotor cortices and the thalamus and elevated α4ß2 binding in the ventral hippocampus. These dose × age differences in [^{3}H]nicotine and α4ß2 nAChR density were accompanied by changes in select striatal and thalamic regional rsFC circuits, such that circuit connectivity strength between striatal-thalamic and striatal-cortical brain regions increased as a function of adolescent, but not adult exposure to nicotine.

Adolescent exposure to a high nicotine dose increases [^{3}H]nicotine and α4ß2 nAChR upregulation in the cortex, striatum and thalamus and increases striatal-thalamic-cortical connectivity

Adolescent-specific increases in nAChR as a result of nicotine exposure has been observed previously, along with concomitant longer lasting effects upon the removal of nicotine.[55] Notably, adolescents can also tolerate greater doses of nicotine than adults.[56,57] Here, we observed binding and functional connectivity changes within and between the cortex, striatum and thalamus, suggesting that nicotine modulates cortico-striatal-thalamic-cortical (CSTC) circuit loops. In CSTC circuits, neural activity is transmitted from multiple cortical regions through the striatum and converges within the thalamus, which then projects back onto cortical subregions to close the loop,[58,59] nAChRs can as regulators at CSTC loops.[60–66] Acquisition, integration and execution of context-appropriate behavioural responses requires synthesis of emotional, cognitive and motor functions, which are mediated by functionally and anatomically segregated

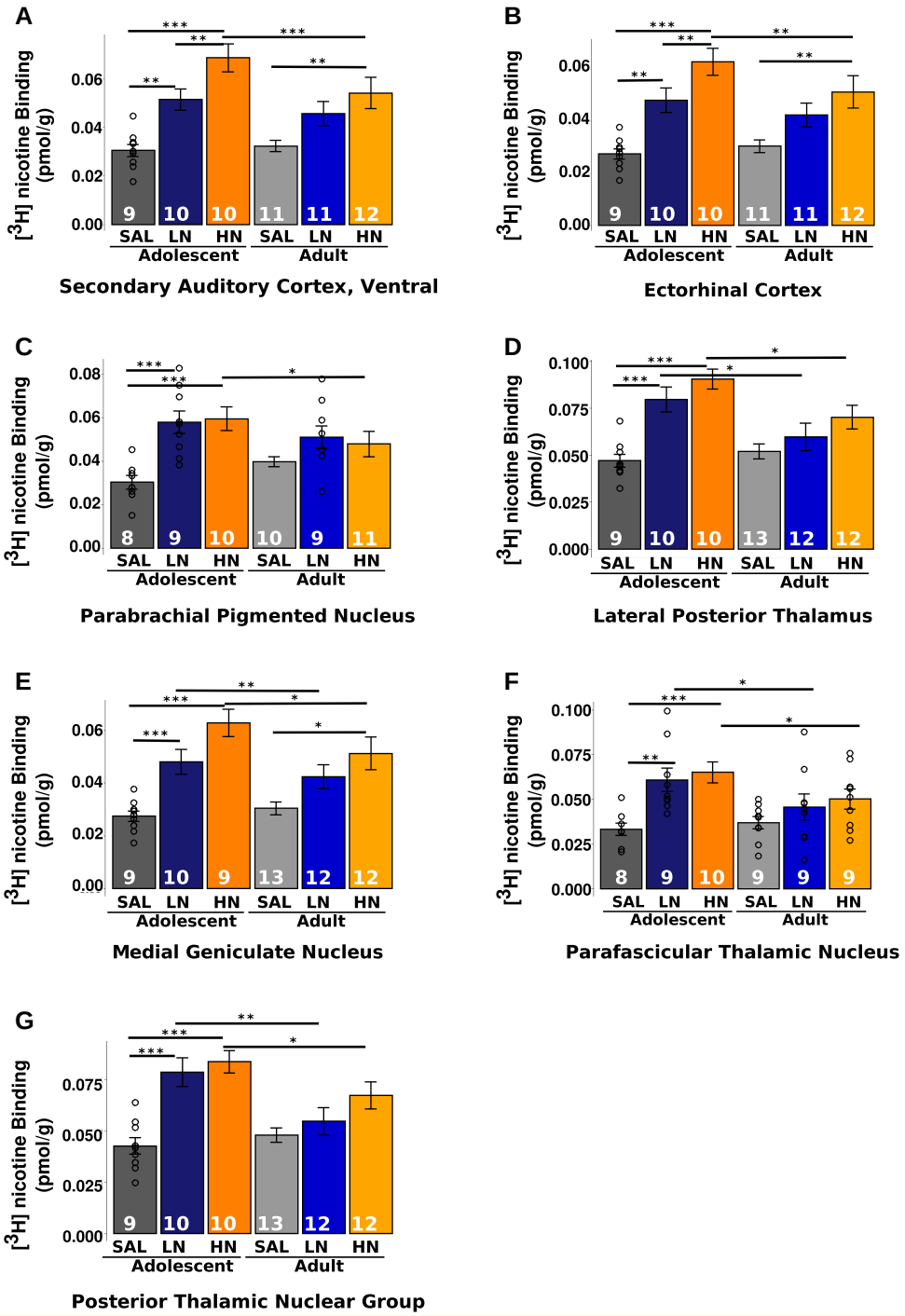


Figure 2 Graphs of [^{3}H]nicotine receptor binding in ROIs with a significant nicotine dose × age interaction. (**A–G**) Graphical depiction of the regional dose × age interactions shown in Fig. 1. Individual two-way ANOVAs were conducted followed by pairwise *t*-tests with Bonferroni correction for multiple comparisons. Bars represent mean ± SEM, and individual data points represent receptor binding of a brain region for an individual rat. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

parallel CSTC pathways, such that integration occurs within each of these anatomically distinct brain regions.[67–69] CTSC circuit strength is at least partially developmental in origin.[70,71] For example, anatomical tracts connecting the cortex and thalamus undergo synaptic pruning during adolescence,[72] and cholinergic modulation within the thalamus plays a crucial role in cortical plasticity, sensory processing and arousal.[73,74] Notably, CSTC circuit structure, function and connectivity are altered in multiple neuropsychiatric diseases,[75] including substance use disorder[76–79]

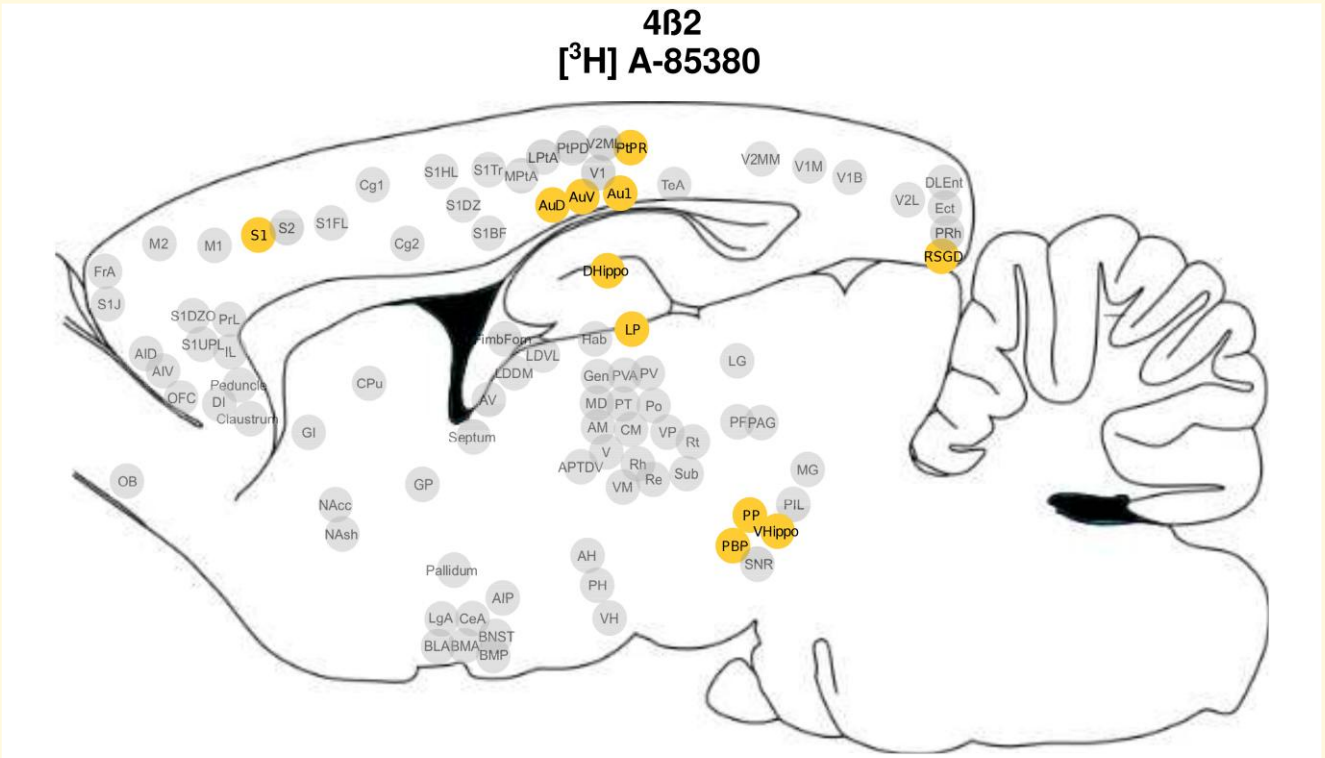


Figure 3 Brain regions with a significant nicotine dose × age interaction in [^{3}H]A-85380 binding. Pictorial representation of the 11 regions of interest that demonstrated significant nicotine dose × exposure age interaction effects for [^{3}H]A-85380 binding, reflecting the α4ß2 nAChR subtype. A list of abbreviations used in this figure can be found in the Supplementary Material.

Here, we propose that adolescent nicotine exposure modifies cholinergic processing, partially mediated and expressed by alterations in nAChRs, primarily α4ß2 nAChRs, which concomitantly results in changes in functional connectivity between CSTC circuit nodes. Nicotine-induced increases in cortical, striatal and thalamic nAChRs during adolescence are associated with an imbalance in CSTC loops, which are crucial for multisensory processing, action selection and motor output, and may fundamentally alter the developmental trajectory of the adolescent brain with potentially important behavioural consequences. Indeed, changes in thalamic function and connectivity with the striatum and cortex may modulate the processing of learned smoking-related cues, which are known to induce craving and, via negative reinforcement, repeated drug use.[80,81] As such, the changes in observed functional connectivity strength may mediate multiple elements of smoking behaviour. For example, connectivity between the ventral striatum and cingulate cortex has been shown to be inversely related to the severity of nicotine dependence in both humans and rodents.[34–37] Critically, the observed effects of nicotine on CSTC circuits are likely directly due to nicotine exposure and not resultant from any individual differences in predispositions to develop nicotine dependence, since we observed no overlap between the CSTC circuits identified here and previously identified insular circuits in rats, measured before nicotine exposure, that predict nicotine dependence severity.[38] Further research is needed to examine potential behavioural effects associated with these nicotine-induced receptor and circuit changes.

Deficits in the expression of goal-directed behaviours emerge from damage to a combination of nodes within CSTC circuits.[82] With the distinct roles of these CSTC loops in behaviours such as reward, cognition and motor processes related to dependence and addiction,[83] discrete targeting of CSTC loops that mediate each of these behavioural outputs may help reveal the complex aetiology of drug dependence and addiction. For example, direct manipulation of the identified Po-NAc-sensorimotor cortical circuits using targeted chemogenetic or optogenetic techniques in animal models of adolescent nicotine dependence and their relationship to nicotine dependence behaviour may elucidate the role of these circuits in mediating nicotine dependence and potentially provide insight into circuits to be targeted for treatment. Disentangling the distinct, parallel and combinatorial roles of the CSTC circuits in addiction-related behaviours requires a careful consideration of the anatomical connectivity between these brain regions and their association with functional connectivity strength. Further, we highlight here effects that are specific to adolescent nicotine exposure (i.e. those effects observed solely in adolescents exposed to nicotine, identified through the dose × age interaction effects) and not the pharmacological effects of nicotine administration (i.e. those

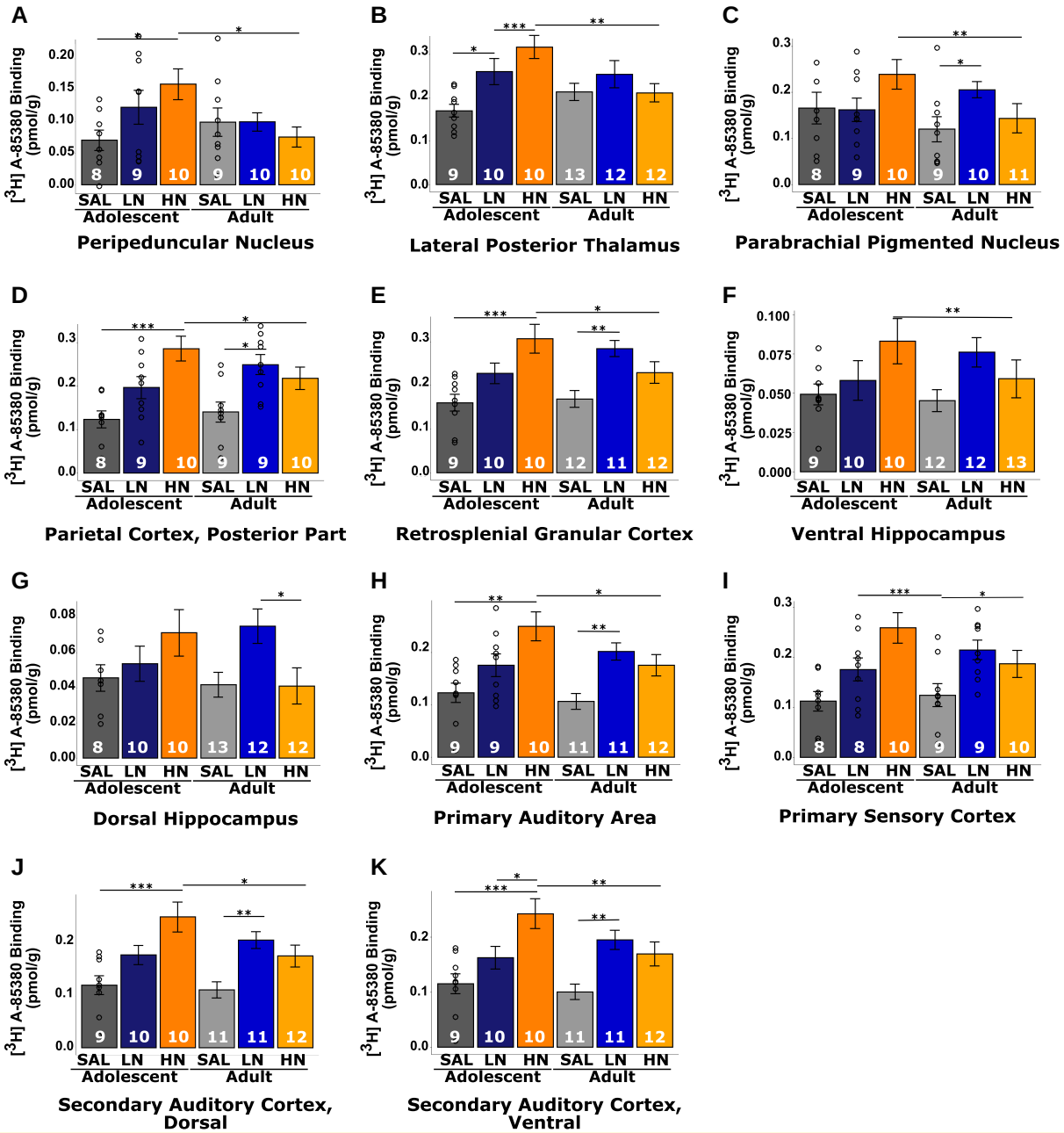


Figure 4 Graphs of [3H]A-85380 binding in ROIs with a significant nicotine dose × age interaction. (**A–K**) Graphical depiction of the regional dose × age interactions shown in Fig. 3. Individual two-way ANOVAs were conducted followed by pairwise *t*-tests with Bonferroni correction for multiple comparisons. Bars represent mean ± SEM, and individual data points represent receptor binding of a brain region for an individual rat. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

effects observed regardless of the age of exposure, identified through the main effects of nicotine dose) or developmental changes (i.e. those effects observed dependent on the age of the rat, identified through the main effects of age) in nAChRs per se. It is critical to note that the identified changes are in the number of binding sites, which does not necessarily reflect functional susceptibility. Given that the vast majority of smokers begin smoking during the adolescent period,[1] we provide insight into those regions and circuits that are susceptible to nicotine only in adolescence and not simply global, widespread changes that occur with nicotine exposure.

Chronic nicotine administration increases nAChR binding

Central cholinergic transmission predominantly acts by altering neuronal excitability, changing presynaptic release of neurotransmitters and synchronizing the activity of

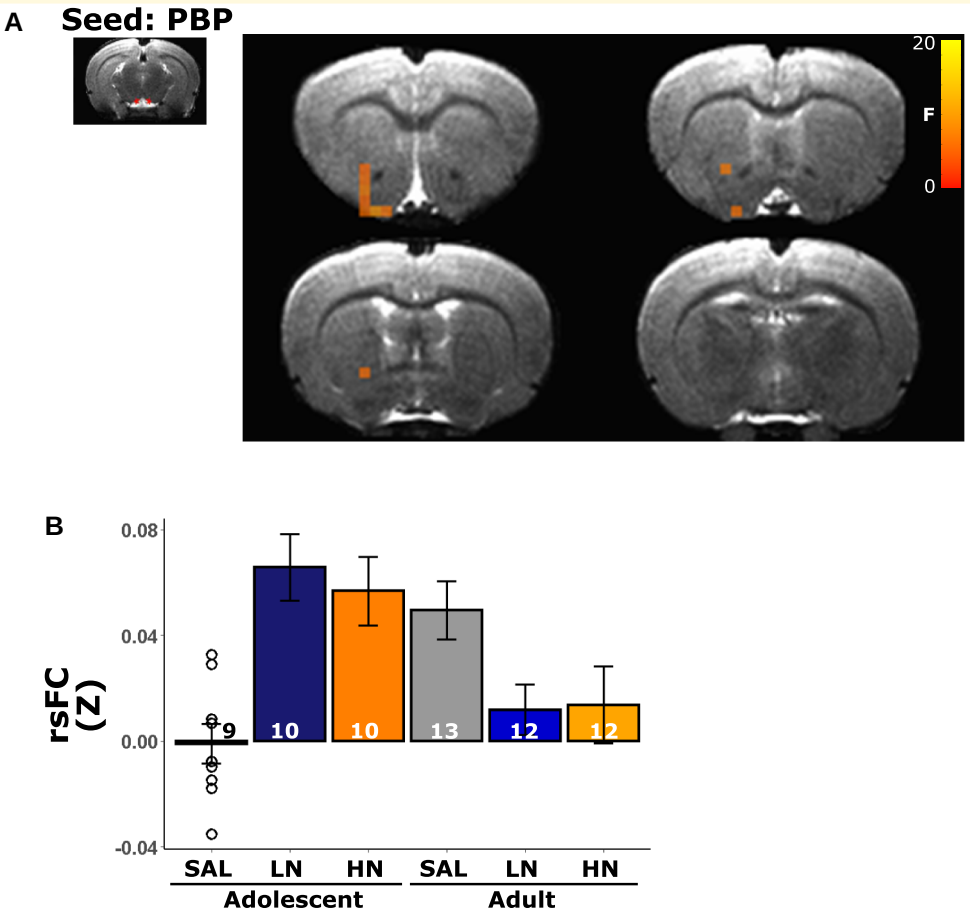


Figure 5 Parabrachial nucleus (PBP) seed identified the functional circuit consequences of changes in nAChR binding. (**A**) Using the PBP as a 'seed', we identified a cluster within the NAc whose connectivity changed as a function of the exposure age × dose interaction. (**B**) Extracted rsFC Z values reveal higher PBP-NAc connectivity in adolescent nicotine exposed rats, comparable to adult saline exposed rats. The significance threshold was set to $p_{corrected} < 0.05$ ($p_{uncorrected} < 0.01$, cluster size > 7 voxels). Note that extracted data are for illustrative purposes only and no statistical comparisons were performed between groups. Bars represent mean ± SEM, and individual data points represent rsFC of a brain region for an individual rat.

neurons; in essence, CNS acetylcholine acts predominantly as a neuromodulator of synaptic activity.[84] It bears noting that the global effect of increased nAChR binding as a function of chronic nicotine exposure observed here is consistent with the extant literature[85,86] and was likely driven by changes in the α4ß2 subtypes, since they comprise the majority of nAChR in the mammalian brain.[87] The consequences of global upregulation of nAChR as a result of nicotine administration have been discussed extensively elsewhere (for example,[88,89]) and here we highlight some overlapping changes in ROI known to be implicated in nicotine dependence and addiction.

Increased [^{3}H]nicotine and α4ß2 nAChR binding was observed in insular and cingulate cortices. Significant increases in α4ß2 nAChR binding in the cingulate (and many other brain regions) were described previously by Doura et al.,[31] using a comparable dose of nicotine; the insula was not measured in this study. However, Cano et al.[90] did not find any significant differences in α4ß2 nAChR binding in the brain of adult or adolescent rats that were exposed to chronic nicotine, although they used a single low dose of nicotine (1.5 mg/kg/day) and drug exposure was limited to only 10 days. Taken together with our findings, the results of these prior studies suggest that chronic nicotine exposure leads to widespread increases in α4ß2 nAChR binding in the brain in both adolescent and adult rats and that the extent of such changes depends on both the dose of nicotine and the duration of exposure.

The insula, together with cingulate cortices, are key components of the salience network (SN).[91,92] This large scale network is thought to help shift an individual's attention to homeostatically relevant stimuli,[93] is engaged during smoking cue processing[94] and is functionally and structurally altered in smokers.[95–102] Insular, prefrontal and cingulate cortex grey matter density are decreased in smokers compared with non-smokers, and the extent of this decrease

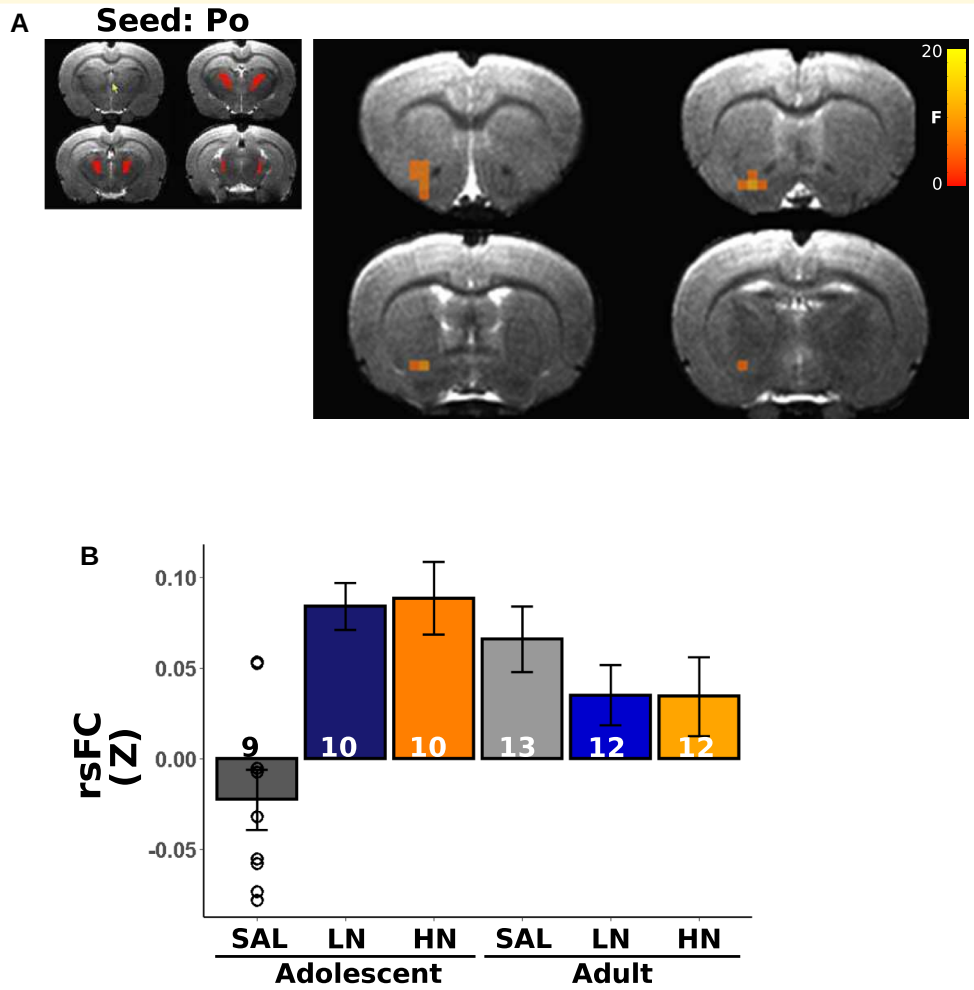


Figure 6 Posterior nucleus group of the thalamus (Po) seed identified the functional circuit consequences of changes in nAChR binding. (**A**) Using the Po as a 'seed', we identified a cluster within the NAc whose connectivity changed as a function of the exposure age × dose interaction. (**B**) Extracted rsFC Z values reveal higher Po-NAc connectivity in adolescent nicotine exposed rats, comparable to that observed in adult saline exposed rats. The significance threshold was set to $p_{corrected} < 0.05$ ($p_{uncorrected} < 0.01$, cluster size > 7 voxels). Note that extracted data are for illustrative purposes only and no statistical comparisons were performed between groups. Bars represent mean ± SEM, and individual data points represent rsFC of a brain region for an individual rat.

correlates with lifetime cigarette exposure.[102] Functional connectivity with these SN hubs are associated with nicotine dependence severity in humans[34,36] and can predict the severity of nicotine dependence in rats.[37,38] Results from human imaging studies in combination with the increases in receptor binding in key cortical areas implicated in smoking observed herein suggest that nicotine-induced nAChR upregulation and selective grey matter volume decreases may be especially linked in regional components of the SN. We did not explicitly investigate changes in insular functional connectivity herein, since there was no significant exposure age × dose interaction, the main thrust of this study. That said, nicotine dependence is often co-morbid with other neuropsychiatric diseases,[103] which are developmentally dependent,[104,105] and disruption in SN processing and function have been proposed as a transdiagnostic marker of neuropsychiatric disease.[106]

Limitations

Although this multimodal study significantly advances our understanding of the effects of chronic nicotine exposure specifically in the adolescent brain, there are a number of limitations that should be mentioned. Since nicotine dependence related behaviours and somatic withdrawal signs are not consistently observed in adolescent rats,[11] this absence of behavioural correlates of dependence precludes linking changes in receptor binding with overt behavioural signs of dependence severity. Furthermore, we examined only a subset of nAChR subunits, used a narrow range of nicotine doses, and our measurement period occurred after 6 weeks of nicotine exposure, such that we only captured the chronic effects of nicotine and not those effects that may have changed rapidly following initial nicotine exposure prior to tolerance formation and receptor re-regulation. To this point, polymorphisms in the α5 nAChR

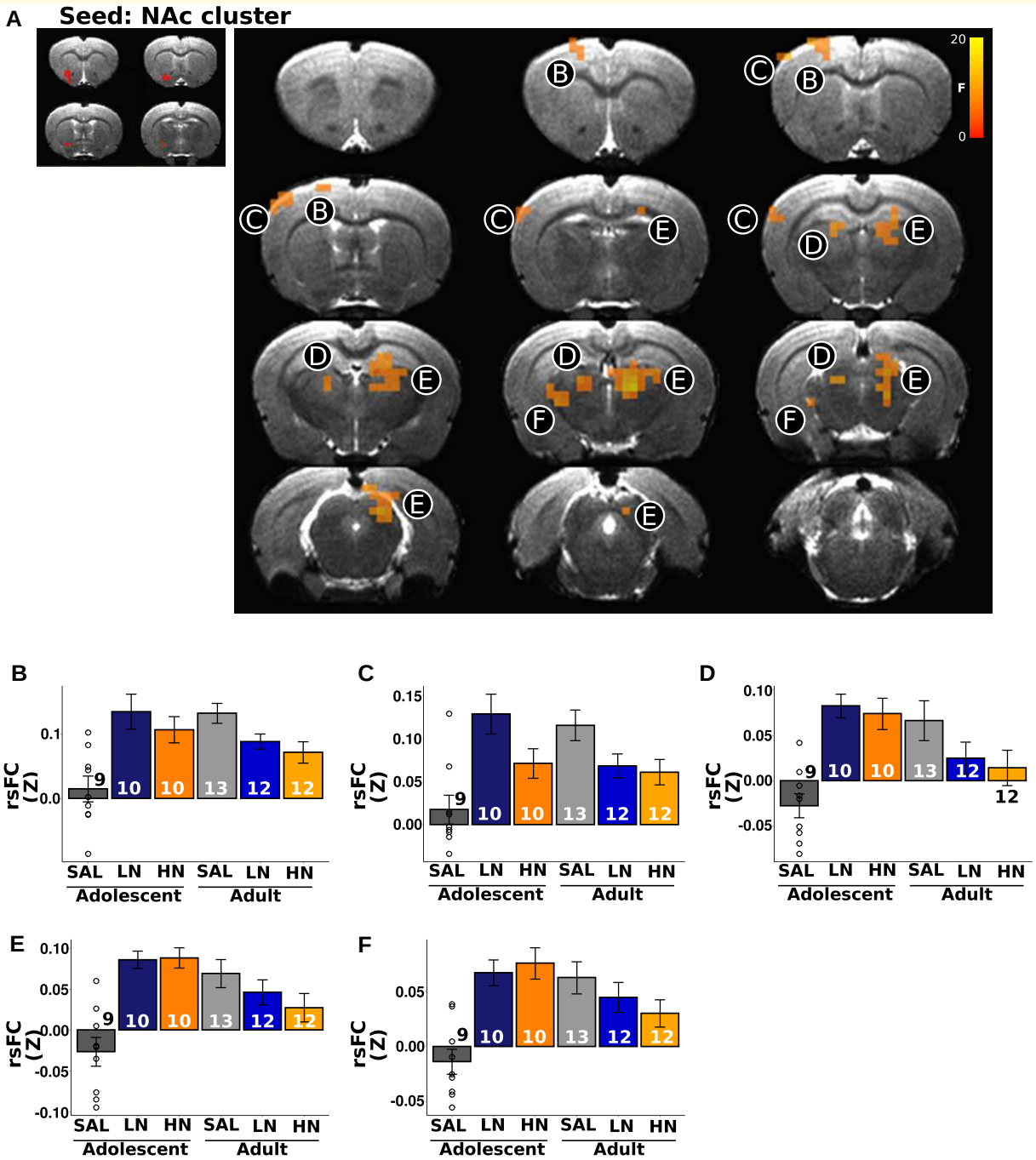


Figure 7 The identified NAc cluster seed identified the functional circuit consequences of changes in nAChR binding. (**A**) In an exploratory analysis using the NAc identified in Fig. 6A as a 'seed', we observed changes in connectivity as a function of the exposure age × dose interaction across the sensorimotor cortex (**B+C**) and thalamus (**D**, **E** and **F**). All regions showed heightened connectivity with adolescent exposure to nicotine, comparable to that observed in adult saline exposed rats. The significance threshold was set to $p_{corrected} < 0.05$ ($p_{uncorrected} < 0.01$, cluster size > 7 voxels). Note that extracted data are for illustrative purposes only, and no statistical comparisons were performed between groups. Bars represent mean ± SEM, and individual data points represent rsFC of a brain region for an individual rat.

gene have been shown to modulate cingulate based circuits in humans.[35] In addition, our nicotine administration method was not ecologically valid, since the drug was administered passively and continuously, which differs substantially from normal nicotine administration in humans,[107] and our experiments included only male rats, limiting any translational interpretations to only male smokers. Further, given the substantial weight gain observed in the first 2 weeks of adolescence, our dosage of nicotine was likely lower than what was calculated. Notably, we only examined changes in connectivity associated with age × dose interaction differences in receptor binding to minimize the number of comparisons for statistical

significance correction. As such, additional functional circuit changes likely exist in the absence of testing herein. It is important to point out that we do not fully understand the nAChR binding selectivity profiles of [^{3}H]nicotine and [^{3}H]A-85380 in rats. [^{3}H]nicotine is thought to also bind, although at low levels, to heteromeric subunits containing α3 and α6.[108] In contrast, [^{3}H]A85380 is expected to label only α4β2.[109] Accordingly, we cannot be completely certain the extent to which they will bind to different receptor populations in the rat brain. Finally, our connectivity measures were collected in anaesthetized animals, which might not reflect the awake, unanaesthetized state.

Conclusions

Using a model of adolescent nicotine exposure, we observed dose, developmental stage and regional nicotine-dependent changes to specific nAChR subtype expression, especially for the α4ß2 subtype, as well as changes in functional connectivity in CSTC loop regions. These observations can help distinguish those circuit changes observed cross-sectionally in human studies that are resultant from nicotine exposure and not predispositional in nature. Most current smoking pharmacotherapies attempt to modulate nAChR function, so it remains imperative to understand the effects of nicotine on this receptor system for the purpose of understanding, preventing and treating smoking addiction and nicotine dependence.

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Competing interests

M.M. has received research funding from AstraZeneca, Redpin Therapeutics and Attune Neurosciences for work unrelated to this study. The authors have declared no other conflict of interests.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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