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Chronic Exposure to Cigarette Smoke Extract Upregulates Nicotinic Receptor Binding in Adult and Adolescent Rats

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Abstract

Heavy smokers display increased radioligand binding of nicotinic acetylcholine receptors (nAChRs). This "upregulation" is thought to be a contributing factor to tobacco dependence. Although cigarette smoke contains thousands of constituents that can contribute to nicotine dependence, it is not well understood whether non-nicotine constituents contribute to nAChR upregulation. In this study, we used an aqueous cigarette smoke extract (CSE), which contains nicotine and soluble constituents of cigarette smoke, to induce nAChR upregulation in adult and adolescent rats. To do this, male rats were exposed to nicotine or CSE (1.5 mg/kg/day nicotine equivalent, intravenously) daily for ten days. This experimental procedure produces equivalent levels of brain and plasma nicotine in nicotine- and CSE-treated animals. We then assessed nAChR upregulation using quantitative autoradiography to measure changes in three nAChR types. Adolescents were found to have consistently greater α4β2 nAChR binding than adults in many brain regions. Chronic nicotine exposure did not significantly increase nAChR binding in any brain region at either age. Chronic CSE exposure selectively increased α4β2 nAChR binding in adolescent medial amygdala and α7 binding in adolescent central amygdala and lateral hypothalamus. CSE also increased α3β4 nAChR binding in the medial habenula and interpeduncular nucleus, and α7 binding in the medial amygdala, independent of age. Overall, this work provides evidence that cigarette smoke constituents influence nAChR upregulation in an age-, nAChR type- and region-dependent manner.

Keywords

nicotine; tobacco constituents; receptor upregulation; age

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AUTHOR CONTRIBUTION

D.D.R and F.M.L designed the study; D.D.R and M.C. performed all experiments; D.D.R. and M.C. analyzed data and created figures; D.D.R wrote early drafts of the manuscript and MC later drafts; S.E.L. consulted on neuroanatomy; J.D.B. and F.M.L. consulted on statistical analysis; F.M.L wrote and edited final version of the manuscript.

CONFLICT OF INTEREST

None.

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1. INTRODUCTION

Despite a recent decline in tobacco use, there are an estimated 34 million smokers in the United States, with 480,000 tobacco-related deaths each year (CDC, 2019). Furthermore, there has recently been a large increase in teen use of nicotine through e-cigarettes (Miech et al., 2019). Nicotine, the primary psychoactive component of cigarette smoke, targets nicotinic acetylcholine receptors (nAChRs) in the brain. These receptors are pentameric ligand-gated ion channels that consist of different combinations of α and β subunits, creating both heteromeric and homomeric nAChR types (Dani, 2015).

Heavy smokers show increased radioligand binding to nAChRs (Brody et al., 2013; Schwartz & Kellar, 1983). This "upregulation" of receptor binding has been implicated as a possible mechanism underlying the addictive potential of nicotine (Feduccia et al., 2012; Ngolab et al., 2015). Increased radioligand binding is also observed in preclinical models of *in vitro* (Perry et al., 2002) and *in vivo* (Doura et al., 2008; Fasoli et al., 2016; Govind et al., 2009) chronic nicotine exposure, and has been shown to be dependent on nAChR subtype, brain area, nicotine dose, and treatment paradigm. Upregulation of α4β2 and α7 nAChRs after chronic nicotine is seen in a multitude of brain areas in adults, but is more limited in adolescents (Doura et al., 2008). This resistance to receptor upregulation may explain why adolescent rodents display less nicotine withdrawal than adults (Keeley et al., 2019; O'Dell et al., 2006).

Importantly, there is a discrepancy between these preclinical findings and clinical data which show that teenagers are more sensitive than adults to tobacco-related withdrawal (Prokhorov et al., 2001; Zhan et al., 2012). One possible reason is that most preclinical studies expose animals to nicotine alone, and not tobacco smoke. Tobacco smoke contains over 7000 constituents including acetaldehyde and nicotine alkaloids that may augment nicotine's effects (Clemens et al., 2009; USDHHS, 2014). In one prior study adolescent rats exposed to cigarette smoke display increased withdrawal symptoms compared to adult rats (De la Pena et al., 2016), indicating that non-nicotine tobacco constituents may influence dependence. Furthermore, rats exposed to whole tobacco smoke have been shown to have increased nAChR binding in the cortex, striatum, and cerebellum (Yates et al., 1995) and increased α7 nAChR density in the hippocampal subregions, CA 2/3 and striatum oriens (Small et al.,2009).

In the present study we have evaluated the effects of chronic exposure to tobacco smoke constituents on nAChR binding using aqueous cigarette smoke extract (CSE), made by bubbling cigarette smoke through saline (Gellner et al., 2016a). We have previously shown that CSE is readily self-administered in both adult and adolescent rats (Costello et al., 2014; Gellner et al., 2016b). Furthermore, adult rats that self-administered CSE were more sensitive to stress- or drug-induced reinstatement than animals that self-administered nicotine alone, and exhibited differences in nAChR pharmacology (Costello et al., 2014; Cross et al., 2020). In this study we have analyzed differences in receptor binding levels after chronic exposure to cigarette smoke extract (CSE) or nicotine in adolescent and adult rats. With evidence to suggest non-nicotine constituents may contribute to nicotine's addictive properties, we hypothesized that the change in nAChR binding after chronic CSE

treatment would be greater in both adult and adolescent rats as compared to nicotine- or saline-treated controls.

2. MATERIALS AND METHODS

2.1. Animals

Male Sprague Dawley rats (Charles River Labs, Hollister CA) arrived at postnatal day (P) 17 with dam (n = 45), or at P81 (n = 36), and were housed 2 per cage (after weaning at P21 for adolescents) in an AALAC-accredited vivarium on a 12-h light/dark cycle. All experimental procedures were done during the light cycle. After one day of acclimation, animals were handled for two days prior to catheterization surgery, in order to reduce surgical stress. Animals were kept at a 95% free-feeding weight throughout the study. All procedures were in compliance with NIH guidelines and were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine.

2.2. Drugs

Nicotine hydrogen tartrate (Sigma, St. Louis, MO) was dissolved in sterile saline and adjusted to pH 7.2–7.4. All nicotine doses were calculated as free base amounts. CSE was made daily by bubbling the smoke from eight unfiltered commercial cigarettes (Camel unfiltered, R.J. Reynolds Co.; 1.7 mg nicotine per cigarette; Ouyang et al., 2000) through 35 ml of sterile saline solution with a 50 ml glass syringe (35ml puffs over 2 s, repeated every 30 s) and the final solution was adjusted to pH 7.2–7.4, as described previously (Costello et al., 2014; Gellner et al., 2016a). The desired nicotine concentration of CSE is 150 μg/ml (Gellner et al., 2016a). Methyllycaconitine (MLA) (Sigma, St. Louis, MO) and cytisine (Sigma, St. Louis, MO) were dissolved in sterile H_2O.

2.3. Surgery

Adult (P85–87) and adolescent (P26–28) animals were anesthetized with equithesin (0.0035 ml/g body weight) and implanted with indwelling jugular vein catheters into the right vein using previously published methods (Belluzzi et al, 2005). During the 2–3 day recovery period, and for the remainder of the study, animals were flushed daily with heparinized saline solution (1 ml of 1000 units/ml heparin into 30 ml bacteriostatic saline). Catheter patency was verified by infusing 0.1 ml of propofol (Abbott Laboratories, Chicago, IL) for rapid anesthesia 24 hrs before the last infusion. If catheter patency failed animals were excluded from analysis.

2.4. Drug Treatment

Following recovery from surgery, adult (P89–91) and adolescent rats (P30–32) were weighed daily and given passive intravenous injections of saline, nicotine or CSE in an operant chamber programmed to deliver one injection per minute for 15 minutes (15 infusions per session), to yield a total of 0.5 mg/kg nicotine (free base) or CSE nicotine content per session (33.33 μg/kg nicotine content/infusion ; 70μl/infusion in 3.92 sec for adults and adolescents 35 μl/infusion in 1.96 sec). Rats received three daily sessions (9am, 12pm, 3pm) totaling 1.5mg/kg/day of nicotine content for 10 consecutive days. The 1.5 mg/kg/ day dose produces blood nicotine levels equivalent to humans who smoke a pack of

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cigarettes a day (Murrin et al., 1987). We used an intermittent paradigm of exposure instead of the more commonly used osmotic pump to ensure stability of the cigarette smoke constituents. Additionally, by preparing the drug solution daily we could compensate for adolescent animals' growth. We have previously shown that this experimental method produces equivalent levels of brain and plasma nicotine in nicotine- and CSE-treated animals (Costello et al., 2014).

2.5. Autoradiography

Rat brains were extracted one hour after the last drug infusion and flash-frozen in 2-methylbutane at −20°C for 30 sec before being stored at −80°C until processing. Twenty μm sections were cut in a cryostat and thaw-mounted onto 4°C positively charged slides (Fisher Scientific, Waltham, MA). Mounted slides were dried and stored at −20°C with desiccant until processing the next day. Receptor binding was measured in brains using ^{125}I-epibatidine or ^{125}I-α-bungarotoxin (Perkin-Elmer, Waltham, MA). For ^{125}I-epibatidine, slides were removed from the freezer and allowed to thaw at room temperature, then pre-incubated for 10 min in room temperature buffer (50 mM Tris, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2, pH 7.4). Binding conditions were varied to selectively label different nAChR types (Costello et al., 2014; Perry et al., 2002). In the α4β2 nAChR binding condition, slides were incubated with 0.08 nM ^{125}I-epibatidine. Since ^{125}I-epibatidine also has affinity for other nAChR types, α4β2 nAChR binding was analyzed in brain areas shown to contain at least 85% expression of α4β2 (Perry, et al. 2002). For α3β4 nAChRs, the binding conditions were identical except that 200 nM cytisine was added to the incubation solution to block binding to α4β2 nAChRs. In both conditions, nonspecific binding was determined in the presence of 300μM nicotine. For ^{125}I-α-bungarotoxin binding to label α7 nAChRs, similar conditions were used, except the buffer was 50 mM Tris HCl with 120 mM NaCl at pH 7.4. Slides were pre-incubated for 15 min in room temperature buffer, then incubated for 2 hrs with 5 nM ^{125}I-α-bungarotoxin (Perkin-Elmer, Waltham, MA). Nonspecific binding was determined in the presence of 10 μM MLA (Ospina, et al., 1998). All slides were then washed twice for 10 min in ice-cold buffer, dipped briefly in ice-cold water, and blown dry. The dried slides were placed in light-tight cassettes with ^{14}C standards of known radioactivity and exposed to Kodak BioMax MR film (Sigma, St. Louis, MO) for 6–18 hrs for the ^{125}I-epibatidine-treated slides, or 30 hrs for the ^{125}I-α-bungarotoxin-treated slides. Autoradiograms were quantified using a MicroComputer Imaging Device (MCID) computer-based imaging system (Imaging Research, St. Catherine, Ontario) based on the standards exposed with the slides. Non-specific binding in an adjacent section was subtracted from the total binding in the equivalent anatomical section to calculate specific binding. Data were not collected from brain tissue that was damaged during collection or processing.

Brain areas were chosen based on an *a priori* hypothesis that they may regulate negative emotional and other aversive states associated with nicotine dependence, with the focus on areas that contained high populations of the specific nAChR type being studied, according to previous reports (Doura et al., 2008; Perry et al., 2002). These areas included subregions of the striatum, the limbic system, and the medial habenula and interpeduncular nucleus circuit. Both β2* (* indicates the possible presence of other nAChR subunits) and α7 nAChR

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subunits are highly expressed throughout the brain, including the striatum and limbic system (Doura et al., 2008; Klink et al., 2001; Perry et al., 2002; Wada et al., 1989). For α4β2 nAChRs, binding was analyzed in the nucleus accumbens core and shell (AcbC, AcbSh), cingulate cortex (Cg), caudate-putamen (CPu), bed nucleus of the stria terminalis (BNST), substantia nigra (SN), and raphe magnus (MnR). For both α4β2 and α7 nAChRs, binding was analyzed in amygdala nuclei, including basolateral (BLA), central (CeA), and medial (MeA), and in lateral hypothalamus (LH). β4* nAChRs are highly expressed in the habenula-interpeduncular pathway (Hb-IPN), with no mRNA observed in the LHb by *in situ* hybridization techniques (Gotti et al., 2009; Grady et al., 2009; Quik et al., 2000). Co-distribution of the β4 nAChR subunit with the α3 nAChR subunit has been shown in abundance (Winzer-Serhan & Leslie, 1997). Thus for α3β4, binding was analyzed in the medial habenula (MHb), and interpeduncular nucleus (IPN). Brain regions were defined by the "The Rat Atlas" (Paxinos & Watson, 1997).

2.6. Data Analysis

Means for weight by drug treatment were determined for animals chronically treated with saline, nicotine, and CSE, and were analyzed with a three-way ANOVA for Age, Drug, and Day with repeated measures on days. Means for regional binding to each nAChR type were determined for animals chronically treated with saline, nicotine, and CSE, and were analyzed with a two-way ANOVA for Age and Drug. Post hoc analyses were conducted when there were significant main effects (Wei et al., 2011). Age comparisons were analyzed further with unpaired t-test. Drug comparisons were analyzed further with Bonferroni-corrected paired t-test. Receptor upregulation was defined as a significant increase in binding from saline treated controls. Exclusion criterion for statistical outliers was determined as two standard deviations from the mean.

3. RESULTS

3.1. CSE drug treatment does not alter weight in adolescent and adult rats

Adolescent and adult rats did not display differences in weight with chronic CSE treatment compared to nicotine or saline treated animals (Figure 1). There was an overall main effect of Age ($F_{1,70} = 3568.27$, $p = 0.0001$) and Day ($F_{9,630} = 82.97$, $p=0.0001$) and a Day x Age interaction ($F_{9,630} = 122.58$, $p=0.0001$), but no Drug effect.

3.2. ^{125}I-epibatidine binding to α4β2 nAChRs is higher in adolescents than adults

Adolescents show higher levels of ^{125}I-epibatidine binding to α4β2 nAChRs than adults in a majority of areas analyzed (Table 1). The following areas showed significant Age, but not Drug, effects (Table 1): AcbSh ($F_{1,54} = 18.317$, $p < 0.0001$), AcbC ($F_{1,54} = 16.557$, $p < 0.0001$), Cg ($F_{1,45} = 14.877$, $p < 0.0001$), CPu ($F_{1,54}= 12.242$, $p = 0.001$), BLA ($F_{1,52} = 18.764$, $p < 0.0001$), CeA ($F_{1,52} = 7.820$, $p = 0.007$), LH ($F_{1,53} = 9.204$, $p = 0.004$), and MnR ($F_{1,47} = 11.215$, $p = 0.002$). There were no overall Age or Drug effects for ^{125}I-epibatidine binding to α4β2 nAChRs in the BNST.

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3.3. CSE-induced increase in α4β2 nAChR binding in adolescent MeA

Whereas the MeA and SN did not show significant age differences in binding to saline-treated controls, there was a significant effect of CSE treatment on binding to α4β2 nAChRs in the MeA (Figure 2). In the MeA, there was an overall effect of Age ($F_{1,51} = 14.483$, $p < 0.0001$), Drug ($F_{2, 51} = 7.09$, $p = 0.002$), and an Age x Drug interaction ($F_{2, 51} = 3.880$, $p = 0.027$). Adolescents treated with CSE or nicotine showed higher binding than adults ($t(18) = 3.0429$, $p = 0.007$ and $t(16) = 2.7245$, $p = 0.015$, respectively). Furthermore, adolescents, but not adults, showed an overall effect of Drug ($F_{2,23} = 5.294$, $p = 0.013$), with those treated with CSE showing significantly higher binding than saline-treated controls ($t(15) = 2.9467$, $p = 0.01$).

In the SN, there were overall main effects of Age ($F_{1,52} = 22.012$, $p < 0.0001$) and Drug ($F_{2,52} = 3.489$, $p = 0.038$). Adolescents treated with CSE and nicotine showed significantly higher binding than adults ($t(18) = 2.9272$, $p = 0.009$ and $t(16) = 3.252$, $p = 0.005$, respectively). However, there were no significant differences in binding after CSE or nicotine treatment when ages were analyzed separately.

3.4. CSE-induced upregulation of α3β4 nAChRs in the MHb and IPN independent of age

CSE treatment resulted in significant upregulation of α3β4 nAChR binding in the MHb and IPN (Figure 3). In the MHb, there was an overall effect of Drug ($F_{2,72} = 3.853$, $p = 0.026$), but not Age and no Age*Drug interaction. CSE treatment resulted in higher binding than saline-treated controls, in both age groups combined ($t(50) = 2.4692$, $p = 0.017$). In the IPN, there were overall effects of Age ($F_{1,68} = 5.420$, $p = 0.023$) and Drug ($F_{2, 68} = 5.657$, $p = 0.005$). In adolescents, there was a significant Drug effect ($F_{2,38} = 3.242$, $p = 0.05$), where CSE treatment resulted in higher binding compared to controls ($t(26) = 2.1061$, $p = 0.045$). There was also a significant Drug effect in adults ($F_{2, 30} = 3.644$, $p = 0.038$) where CSE treatment upregulated binding as compared to controls ($t(20) = 2.1276$, $p = 0.046$).

3.5. CSE-induced increase in α7 nAChR binding in hypothalamus and amygdala

In general, adolescents showed higher levels of ^{125}I-α-bungarotoxin binding than adults in a treatment-specific manner in the majority of the areas analyzed (Figure 4). In the LH, there were overall effects of Age ($F_{1,60} = 4.594$, $p = 0.036$) and Drug ($F_{2, 60} = 6.951$, $p = 0.002$). In adolescents, there was an overall Drug effect ($F_{2,24} = 5.080$, $p = 0.014$), where CSE treatment resulted in higher binding than nicotine-treated ($t(15) = 2.5106$, $p = 0.024$) or control ($t(17) = 2.1308$, $p = 0.048$) animals. Adolescents treated with CSE also showed higher binding than adults treated with CSE ($t(20) = 2.6314$, $p = 0.016$). There were no Drug effects in adult LH.

Whereas an age-specific effect of CSE treatment was seen in the LH, those in amygdaloid nuclei were more complex. In the BLA, there were main effects of Age ($F_{1,65} = 23.748$, $p < 0.0001$) and Drug ($F_{2, 65} = 4.077$, $p = 0.021$), with adolescents showing higher binding than adults across all treatment groups (CSE $t(22) = 2.7383$, $p = 0.012$, nicotine $t(21) = 2.1894$, $p = 0.040$, saline $t(22) = 3.505$, $p = 0.002$). Adults showed a significant Drug effect ($F_{2,36} = 4.157$, $p = 0.024$), where CSE treatment induced significantly upregulated binding as compared to controls ($t(24) = 2.4491$, $p = 0.022$). There were no significant drug effects in

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adolescents. In the CeA, there was an overall effect of Age ($F_{1,64} = 15.632$, $p < 0.0001$) and Drug ($F_{2, 64} = 6.563$, $p = 0.003$). Adolescents showed higher binding than adults across all treatments (CSE $t(22) = 2.7027$, $p = 0.013$, nicotine $t(20) = 2.1169$, $p = 0.047$, saline $t(22) = 2.5823$, $p = 0.017$). In adolescents, there was also an overall Drug effect ($F_{2, 28} = 4.158$, $p = 0.026$), with CSE treatment resulting in higher binding than controls ($t(20) = 2.2482$, $p = 0.036$). No significant drug effect was seen in adult CeA. In the MeA, there was an overall effect of Age ($F_{1,65} = 23.990$, $p < 0.0001$) and Drug ($F_{2, 65} = 4.516$, $p = 0.015$). Adolescents showed higher binding than adults across all treatments (CSE $t(22) = 4.7361$, $p < 0.0001$, nicotine $t(21) = 2.6493$, $p = 0.015$, saline $t(22) = 2.0838$, $p = 0.049$). No individual Drug differences were observed when adults and adolescents were analyzed separately. When ages were combined, animals treated with CSE showed higher binding than those treated with nicotine ($t(45) = 2.4554$, $p = 0.018$).

4. DISCUSSION

The present study is the first to show that cigarette smoke constituents enhance nicotine-induced upregulation of nAChR radioligand binding in both adult and adolescent rodents. Although prior studies in both adolescent and adult rats have shown nicotine to reduce weight gain (Trauth et al., 1999; Winders & Grunberg, 1990), we found that adolescent rat growth was not impacted by either nicotine or CSE and that adult animals maintained steady weight independent of drug treatment. In agreement with earlier studies (Counotte et al., 2012, Doura et al., 2008, Trauth et al., 1999), age differences in radioligand binding were apparent in saline-treated control animals, with adolescents showing higher binding than their adult counterparts in many regions. However, in contrast to earlier findings in which adolescents demonstrated a limited nAChR upregulation as compared to adults (Doura et al., 2008), here we demonstrate that adolescents chronically treated with CSE show higher upregulation in many regions than their adult counterparts. This is consistent with a prior finding that tobacco smoke exposure increased nAChR expression in adolescent mouse brain even at low levels of nicotine (Abreu-Villaça et al., 2016). This suggests that nAChR pharmacology differs between adult and adolescents, and may differentially influence networks that mediate drug-associated behaviors.

In contrast to other studies (Govind et al., 2009; Doura et al., 2008), chronic nicotine did not induce significant upregulation of nAChRs. This discrepancy may be due to different drug exposure paradigms. Typically, studies of chronic nicotine exposure use osmotic pumps, but here we use intermittent intravenous injections. This method allowed us to prepare CSE daily which contains various constituents of unknown stability, and permitted control for nicotine dose in growing adolescent animals. It has been shown that the method of exposure, as well as nicotine dose, can influence the rate and level of receptor upregulation (Semenova et al., 2018). For instance, transient exposure to high dose nicotine seems to favor $\alpha 6\beta 2$ nAChR upregulation rather than $\alpha 4\beta 2$ upregulation, which is induced by prolonged exposure to low dose nicotine (Nashmi et al., 2007). Since our animals received a moderately high dose (1.5 mg/kg nicotine content per day) in three daily intravenous sessions for 10 days, it is possible that not all nAChRs were responsive to the effects of nicotine using this schedule of drug exposure. Nevertheless, the inclusion of cigarette smoke constituents resulted in increased nAChR upregulation using this exposure method, although

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in most regions CSE-induced binding was significantly different from saline-treated, not nicotine-treated, animals. Exceptions were found in the adolescent LH and MeA of both ages, where α7 nAChR binding in CSE-treated animals was significantly higher than in those treated with nicotine.

An important goal of this study was to get a deeper understanding of the neuropharmacological adaptive mechanisms within circuits that mediate the transition from initial tobacco use to dependence. Thus, the focus was to examine nicotine- and CSE-induced upregulation of nAChR binding in brain areas involved in addiction and negative emotional states. In summary, CSE-induced upregulation was observed in the amygdala, LH, MHb, and IPN. The anatomical connections between these areas and how they influence the withdrawal syndrome, as well as our findings, are summarized in Figure 5 and are discussed further below.

The change from positive to negative reinforcing effects of drugs of abuse are mediated by neurotransmitter systems in the striatum, either directly or via indirect actions in the ventral tegmental area (VTA) and SN (Koob & Volkow, 2010; Koob, 2008; Koob & Le Moal, 2008). Drug-induced upregulation of nAChR binding, however, was not observed in the Acb or CPu, as is consistent with an earlier study using a non-contingent, intermittent exposure paradigm (Semenova et al., 2018). Additionally, methodological limitations did not allow for the analysis of nAChR binding in the VTA, and significant drug-induced upregulation of α4β2 nAChRs was not observed in SN at either age. Several other areas modulate the negative aversive state of nicotine dependence, including the amygdala, LH, and MB-IPN circuit (Kenny & Markou, 2001; Natividad et al., 2010; Zhang et al., 2012; Antolin-Fontes et al., 2015). In these areas, drug-induced binding upregulation was observed in a nAChR subtype- and age-specific manner. Adolescents, but not adults, displayed CSE-induced upregulation of α4β2 nAChRs in the MeA and α7 nAChRs in the CeA and LH, areas that mediate the shift to negative reinforcement and the negative emotional state of withdrawal that is a key factor in dependence (Antolin-Fontes et al., 2015; Koob & Volkow, 2010; Narita, 2006). Adults, but not adolescents, displayed a CSE-induced upregulation of α7 nAChRs in the BLA which is involved in craving, a component of withdrawal that often leads to relapse (Koob & Volkow, 2010). Lastly, CSE-induced upregulation of α3β4 nAChRs was seen in both age groups in the MHb and IPN, two regions that have been critically implicated in nicotine withdrawal (Salas et al., 2009; Shih et al., 2015).

There are many mechanistic possibilities as to how the non-nicotine constituents in CSE may enhance nicotine-induced upregulation of nAChRs. The term “upregulation” may explain phenomena beyond just receptor number. Increased binding may be due to increases in affinity resulting from changes in conformation or stoichiometry of the receptor, reduction in turnover rate, or an increase in trafficking of nAChR proteins intracellularly, or a combination of these (Govind et al., 2012; Henderson & Lester, 2015; Nelson et al., 2003). Since there are many constituents in CSE, it is possible that they may be acting both directly on the receptor or intracellularly. Further studies will be needed to address this issue. Future studies should also investigate sex differences in the effects of CSE and nicotine on nAChR upregulation since clinical and preclinical studies have shown females are more susceptible to nicotine/tobacco craving and withdrawal (Conti et al., 2020; Torres et al., 2013).

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Furthermore, denicotinized tobacco produces greater alleviation of withdrawal symptoms in women than men, implicating an important sex-dependent role of non-nicotine tobacco constituents (Barrett, 2010).

There are a few limitations in these experiments. One of the major challenges in studying smoking in animals is using a model that best mimics smoking in humans. CSE is no exception. The composition of the CSE solution does not allow for the use of the more commonly used osmotic minipump, thus preventing us from fully comparing our findings with other studies, as the route of administration largely differs. Nevertheless, passive intravenous administration of CSE is a valid model of smoking, as the time it takes for the drug to reach the brain is comparable to that of a smoker (Benowitz et al., 2009). Another limitation is the composition of CSE. Due to the fact that it is made in saline, an aqueous solvent, CSE contains only the aqueous constituents of cigarette smoke, hence we are not accounting for the other ~ 60% of non-aqueous constituents of cigarette smoke (Schumacher et al., 1977). The extracts commonly used in tobacco research are prepared in an organic solvent in order to dissolve the tar phase of the smoke (Ambrose et al., 2007; Brennan et al., 2014; Danielson et al., 2014). However, given that our experimental paradigm requires intravenous infusion of CSE, an organic solvent was not practical. In spite of these limitations, aqueous CSE is a reliable and improved tool to model tobacco dependence in rodents, and can produce upregulation of nAChR binding in both adult and adolescent rats. Together, the current report supports the increased validity of using CSE over nicotine alone in preclinical models of tobacco dependence.

5. CONCLUSION

In summary, chronic treatment of CSE results in an upregulation of $\alpha4\beta2$, $\alpha7$, and $\alpha3\beta4$ nAChRs binding and, in some cases, to a significantly greater extent than chronic nicotine treatment. We have also confirmed prior reports that nAChR binding is higher in adolescent than adult rats (Counotte et al., 2012, Doura et al., 2008, Trauth et al., 1999). However, both age groups were susceptible to upregulation of nAChR binding after chronic CSE treatment, although changes were age-, subtype- and region-dependent. Overall, upregulation was observed in amygdala nuclei, LH, and the MHb-IPN; brain areas critical for mediating the aversive aspects of nicotine. The regions of upregulation suggest an increased drug dependence potential of cigarette smoke compared to nicotine alone (Morean et al., 2018; Shiffman & Sembower, 2020). In conclusion, these results provide evidence that the non-nicotinic constituents in CSE can uniquely influence nAChR pharmacology across development. A better understanding of how these constituents enhance nAChR upregulation will assist in the development of nAChR-based pharmacotherapies for smoking cessation, and potentially improve efficacy and specificity for different age groups.

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Highlights

- Adolescents display higher nAChR binding compared to adults in many of the same regions
- Cigarette smoke extract upregulates nAChR binding in an age-, subtype- and region-dependent manner
- Chronic cigarette smoke extract produces greater changes to nAChRs compared to chronic nicotine alone

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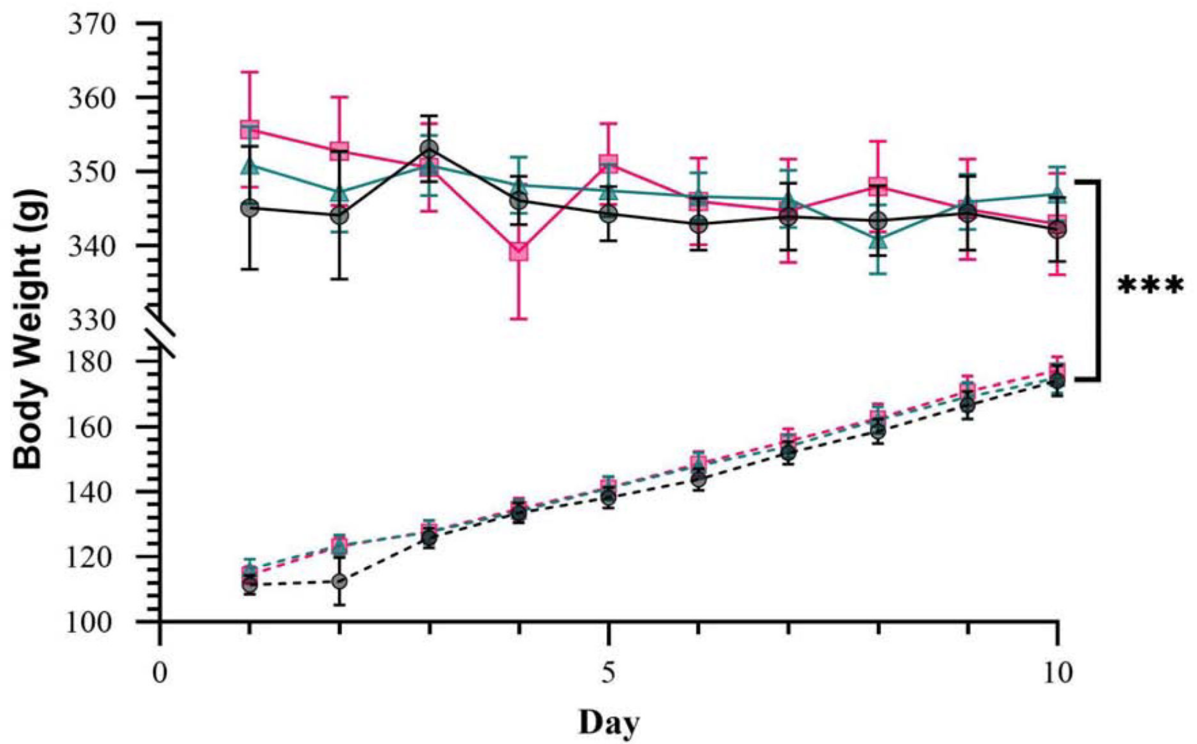

Figure 1.

Mean body weight +/− SEM throughout ten day drug exposure. Neither adolescent nor adult weights were impacted by drug treatment. Dashed lines represent adolescent data; Solid lines represent adult data. black circles = saline; blue triangles = nicotine; pink squares = CSE; *** = p 0.001 vs adolescent group. n = 11 – 13 per adult group. n = 15 per adolescent group.

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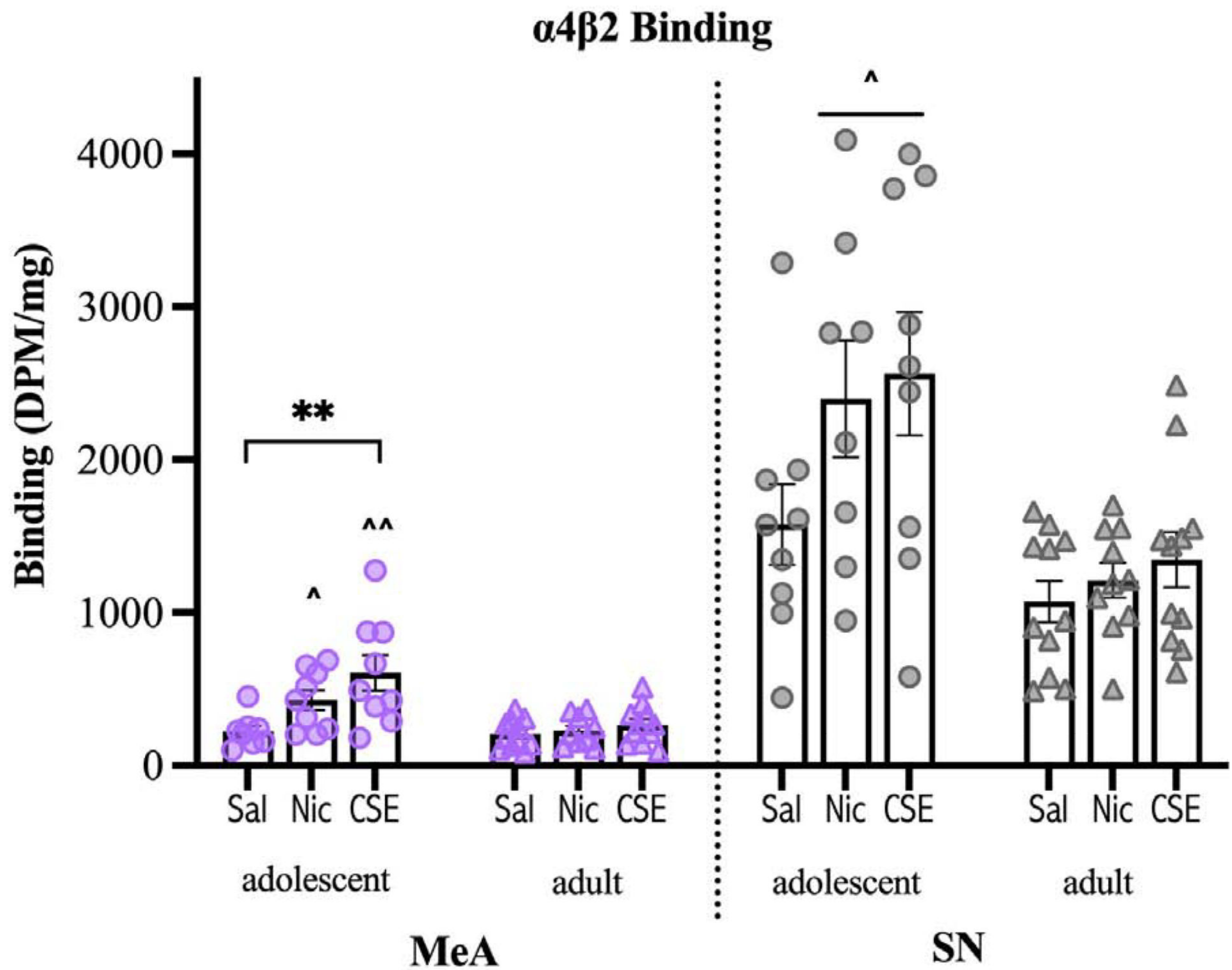

Figure 2.

α4β2 nAChR binding in the MeA and SN. In the MeA, adolescents display a significant CSE-induced upregulation. CSE and nicotine treated adolescent animals display significantly higher binding than adults in the MeA and SN. Circles represent adolescent data; triangles represent adult data. MeA = medial amygdala, SN = substantia nigra. ** = p 0.05 vs saline group. ^ = p 0.05, ^^ = p 0.01 vs. adult group. n = 8 – 11 per group.

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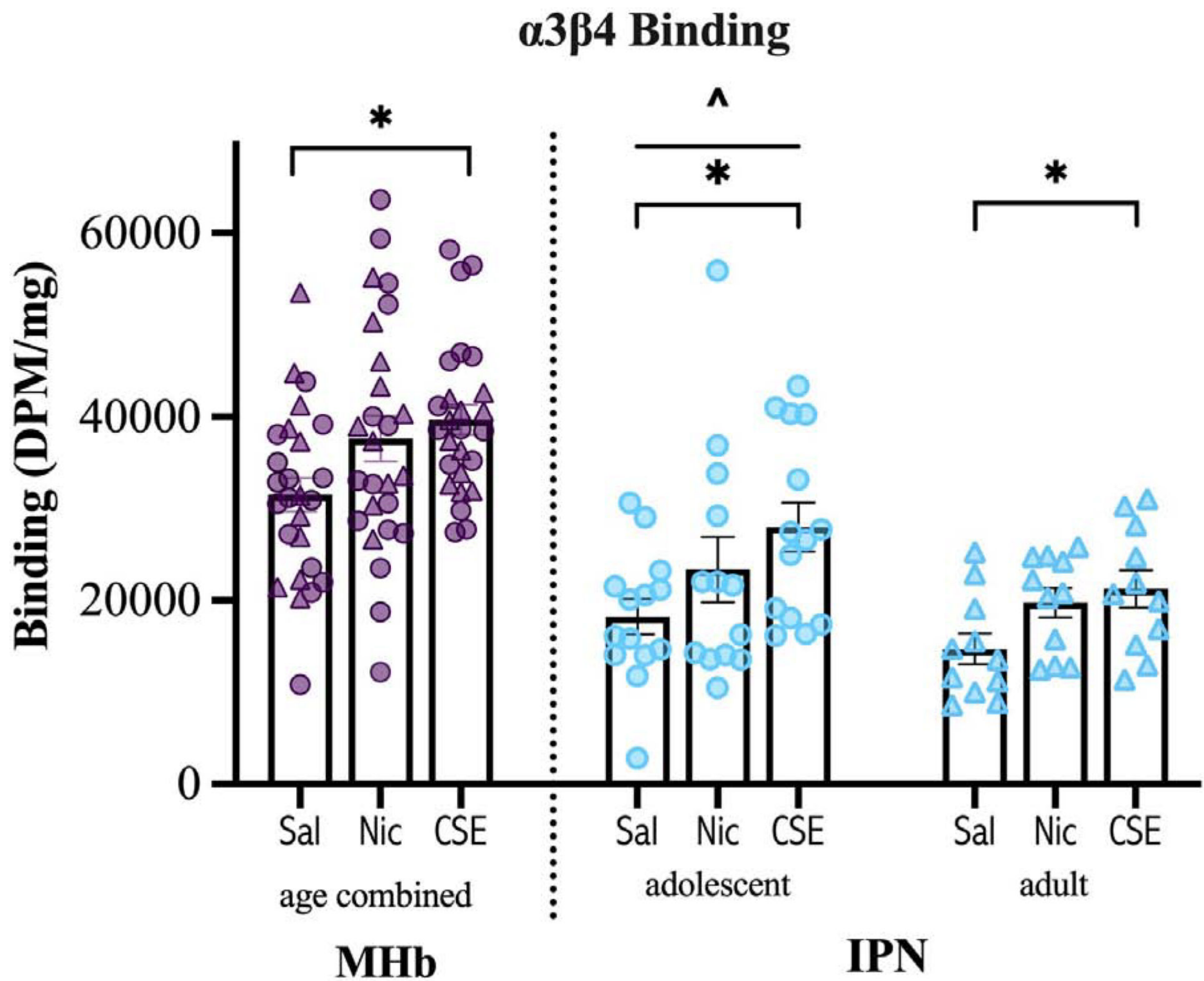

Figure 3.

CSE-induced upregulation of α3β4 nAChR in the MHb and IPN. CSE treated animals show significantly higher binding compared to saline controls in MHb and IPN. In the IPN, adolescents show higher overall binding compared to adults. Circles represent adolescent data; triangles represent adult data. MHb = medial habenula, IPN = Interpeduncular nucleus. * = p 0.05 vs. saline group. ^ = p 0.05 vs. adult group. MHb n = 26 per group. IPN n = 11 – 14 per group.

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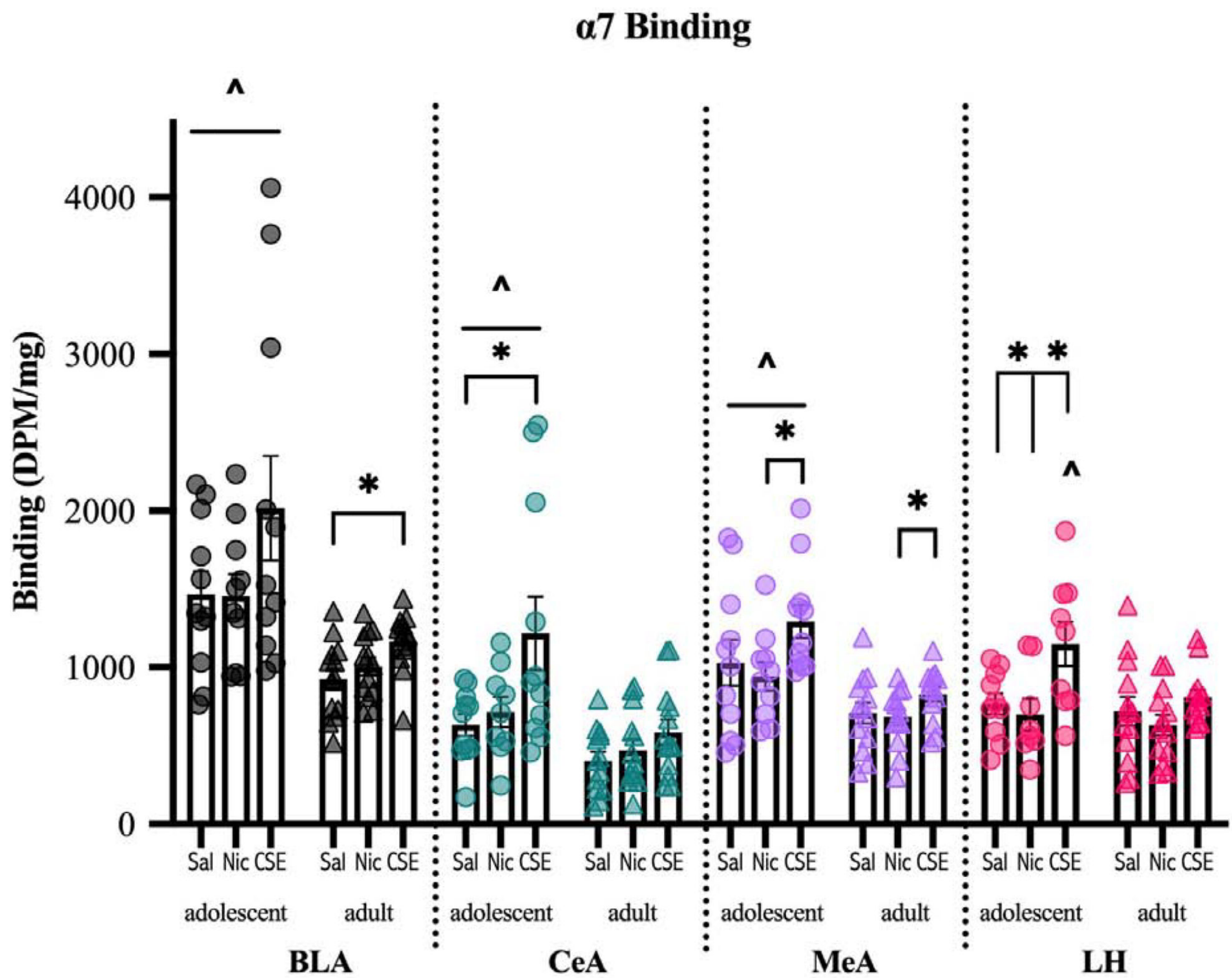
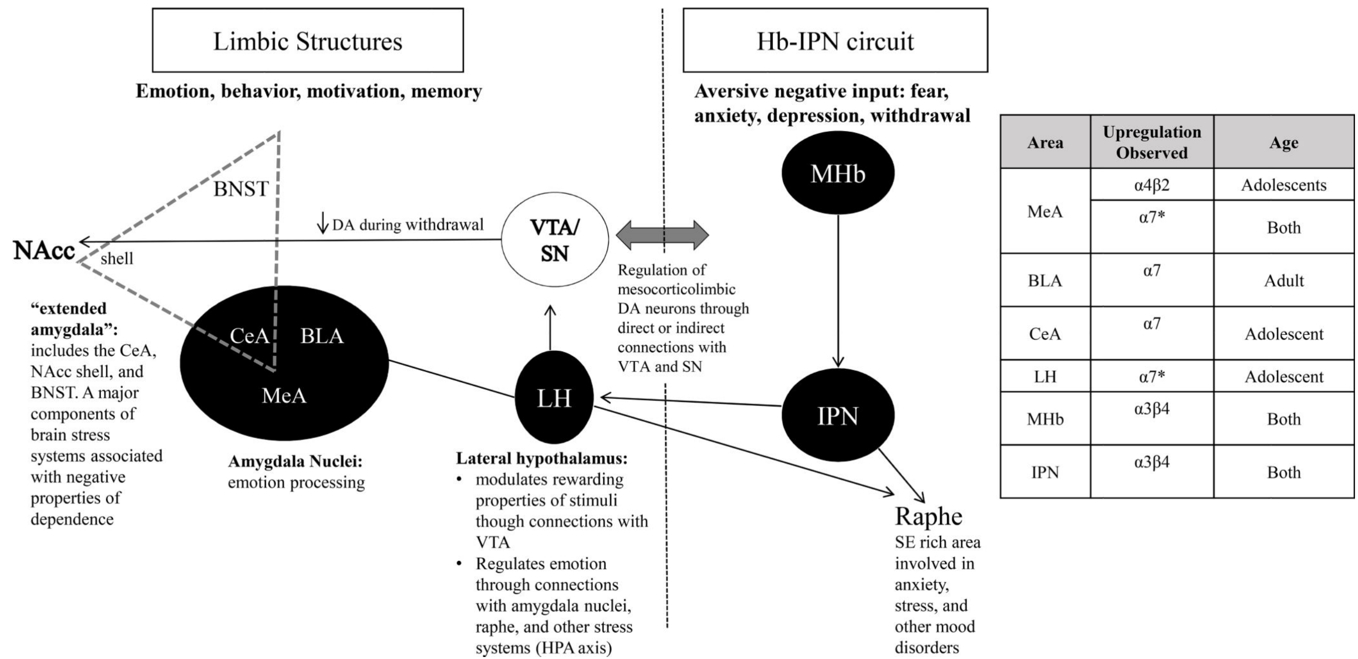


Figure 4.

α7 nAChR binding in the hypothalamus and amygdala. Overall adolescents have higher binding than adults in the amygdala. In the BLA, CSE treated adults show higher binding than saline treated controls. In the CeA, adolescent CSE treated animals displayed higher nAChR binding compared to saline controls. Adolescent CSE treated animals show increased binding in the LH compared to nicotine and saline treated animals. Circles represent adolescent data, triangles represent adult data. BLA = basolateral amygdala, CeA = central amygdala, MeA = medial amygdala, LH = lateral hypothalamus * = p 0.05 vs. saline group. ^ = p 0.05 vs. adult group. n = 8 – 13 per group.



Area	Upregulation Observed	Age
MeA	α4β2	Adolescents
	α7*	Both
BLA	α7	Adult
CeA	α7	Adolescent
LH	α7*	Adolescent
MHb	α3β4	Both
IPN	α3β4	Both

Figure 5.

Anatomical connections within the limbic system and HB-IPN circuit that mediate withdrawal. Black circles represent brain areas where nAChR upregulation was observed. The table summarizes areas of increased binding to nAChRs seen after chronic CSE exposure. * denotes a significant increase from nicotine treated rats.

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Table 1.

Binding to α4β2 nAChRs. Overall there was a significant age effect, where adolescents had higher binding in α4β2-rich regions independent of drug treatment, denoted by the gray boxes. AcbSh = accumbens shell, AcbC = accumbens core, Cg= cingulate cortex, CPu = caudate putamen (striatum), BNST= bed nucleus of the stria terminalis, BLA = basolateral amygdala, CeA = central amygdala, LH = lateral hypothalamus, MnR = median raphe. Gray boxes = p 0.01 vs. adult group. n = 8 – 11 per group.

Brain Region	**Adults**			**Adolescents**		
	Saline	**Nicotine**	**CSE**	**Saline**	**Nicotine**	**CSE**
	Mean ± SEM (DPM/mg)			**Mean ± SEM (DPM/mg)**		
Binding of [125I]epibatidine to α4β2						
AcbC	600.29 ± 83.66	606.74 ± 79.93	693.20 ± 99.06	987.48 ± 142.14	1049.70 ± 154.48	1022.01 ± 144.49
AcbSh	462.18 ± 69.87	539.78 ± 93.38	557.34 ± 92.49	815.50 ± 113.69	967.65 ± 127.79	992.68 ± 192.03
Cg	630.14 ± 99.76	609.00 ± 92.33	801.94 ± 86.97	968.55 ± 139.17	1290.76 ± 208.38	1376.50 ± 260.37
Cpu	650.30 ± 84.30	755.31 ± 87.54	746.82 ± 90.51	1133.87 ± 198.75	1040.19 ± 128.70	1131.51 ± 208.03
BNST	457.98 ± 75.24	581.90 ± 107.79	648.13 ± 101.26	440.10 ± 60.89	511.96 ± 90.20	574.05 ± 93.76
BLA	396.70 ± 81.47	523.68 ± 104.09	530.09 ± 85.55	748.17 ± 143.61	936.25 ± 87.58	939.56 ± 160.27
CeA	354.75 ± 65.65	345.08 ± 69.14	381.39 ± 60.82	480.08 ± 90.73	619.03 ± 92.72	542.20 ± 112.75
LH	270.46 ± 49.67	324.71 ± 47.94	399.06 ± 55.08	481.05 ± 120.96	548.81 ± 83.84	529.77 ± 104.48
MnR	573.15 ± 87.82	653.84 ± 83.99	644.40 ± 130.36	884.58 ± 256.82	1515.39 ± 356.61	1116.36 ± 282.41