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Long-term nicotine treatment downregulates $\alpha 6\beta^*$ nicotinic receptor expression and function in nucleus accumbens

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Abstract

Long-term nicotine exposure induces alterations in dopamine transmission in nucleus accumbens (NAcc) that sustain the reinforcing effects of smoking. One approach to understand the adaptive changes that arise involves measurement of endogenous dopamine release using voltammetry. We therefore treated rats for 2-3 months with nicotine and examined alterations in nAChR subtype expression and electrically-evoked dopamine release in rat NAcc shell, a region key in addiction. Long-term nicotine treatment selectively decreased stimulated $\alpha 6\beta^*$ nAChR-mediated dopamine release compared to vehicle-treated rats. It also reduced $\alpha 6\beta^*$ nAChRs, suggesting the receptor decline may contribute to the functional loss. This decreased response in release after chronic nicotine treatment was still partially sensitive to the agonist nicotine. Studies with an acetylcholinesterase inhibitor demonstrated that the response was also sensitive to increased endogenous acetylcholine. However, unlike the agonists, nAChR antagonists decreased dopamine release only in vehicle- but not nicotine-treated rats. Since antagonists function by blocking the action of acetylcholine, their ineffectiveness suggests that reduced acetylcholine levels partly underlie the dampened $\alpha 6\beta^*$ nAChR-mediated function in nicotine-treated rats. Since long-term nicotine modifies dopamine release by decreasing $\alpha 6\beta^*$ nAChRs and their function, these data suggest that interventions that target this subtype may be useful for treating nicotine dependence.

Keywords

addiction; nicotine; nicotinic receptors; nucleus accumbens; voltammetry

Nicotine addiction is the most common form of chemical dependence in the United States, with ~25% of the adult population using tobacco products despite their well known adverse health effects (Benowitz 2010). Although many smokers would like to quit, the vast majority are back to smoking within a week of cessation (Zhu *et al.* 2012). These are disturbing statistics in view of the significant health and economic burdens of smoking. Several smoking cessation medications exist; however, the best one-year abstinence rates are still only ~25% (Dhalaria *et al.* 2012, Mills *et al.* 2012, Zhu *et al.* 2012, Keating & Lyseng-Williamson 2010). Identification of the nAChRs and neurobiological processes that maintain nicotine dependence are thus important as such work will help in the development of more successful smoking cessation strategies.

It is well known that nicotine is the main addictive component of tobacco products. Like other drugs of abuse, nicotine enhances dopamine transmission in the mesolimbic dopamine pathway, which is thought to play a critical role in the reinforcing effects that initiate and maintain nicotine dependence (Tuesta *et al.* 2011, Corrigan *et al.* 1992, Balfour 2009, De Biasi & Dani 2011, Di Chiara *et al.* 2004). Extensive studies using genetically modified mice show that nicotine's effects are mediated through an interaction at neuronal nicotinic receptors (nAChRs) of which there are multiple subtypes (Changeux 2010). Converging evidence suggests that both $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs in the mesolimbic dopaminergic pathway mediate numerous aspects related to nicotine reward, as well as withdrawal (Brunzell 2012, Epping-Jordan *et al.* 1999, Gotti *et al.* 2010, Jackson *et al.* 2009, Picciotto *et al.* 1998, Pons *et al.* 2008, Tapper *et al.* 2004). It was initially somewhat difficult to reconcile these research findings with voltammetry data which suggested that $\alpha 6\beta 2^*$ nAChRs are the prominent regulators of dopamine release in NAcc (Exley *et al.* 2008, Perez *et al.* 2012). However, recent reports suggest that the $\alpha 6\alpha 4\beta 2^*$ nAChR population may be the one important for nicotine's effects on dopamine transmission. Support for this idea comes from studies showing that $\alpha 6\alpha 4\beta 2^*$ nAChRs primarily modulate dopamine neuron firing in the ventral tegmental area (VTA) and endogenous dopamine release in the NAcc (Exley *et al.* 2011, Liu *et al.* 2012, Zhao-Shea *et al.* 2011). In addition, long-term nicotine exposure decreases $\alpha 6\alpha 4\beta 2^*$ nAChR expression (Perez *et al.* 2008a) providing further evidence for a role of this receptor subtype in nicotine dependence.

The purpose of this study was to further understand how long-term nicotine treatment modifies mesolimbic dopaminergic function by examining adaptive changes in dopamine release using cyclic voltammetry. We focused on the accumbens shell since dopamine release in this region has been linked to the rewarding or reinforcing properties of nicotine while release in the accumbens core and striatum is more highly associated to the presentation of a conditioned stimulus (Balfour 2009, Changeux 2010, Sellings *et al.* 2008, Wise 2009). We also measured nAChR subtype levels using receptor autoradiography. The results show that long-term nicotine exposure modulates electrically-evoked dopamine release in the NAcc by reducing $\alpha 6\beta 2^*$ nAChR levels and their function. These data suggest that drugs that stimulate $\alpha 6\beta 2^*$ nAChRs may be of benefit in treating nicotine addiction.

Materials and methods

Animal treatment

Adult male Sprague-Dawley rats (220-250 g) purchased from Charles River Laboratories (Gilroy, CA) were placed in a temperature-controlled room with a 12 h dark/light cycle and housed 3-4 per cage. All animals had free access to food and water. After several days of acclimation, rats were given drinking water containing 1% saccharin (Sigma Chemical Co., St. Louis, MO), which was used as a vehicle to mask the bitter taste of nicotine. They were then randomly divided into two treatment groups 3 d later. One group was maintained on saccharin only. Nicotine was added to the saccharin-containing solution of the other group at a concentration of 25 $\mu\text{g/ml}$ nicotine (free base). Nicotine treatment was given for 2-3 months, as other studies have shown that the changes that arise during such a time period may model those in long-term smokers (Zhang *et al.* 2011). Weights were monitored twice a week, with no significant differences between the two treatment groups. The rats were killed by decapitation using a guillotine. All procedures conform to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of SRI International.

Plasma Cotinine levels

Blood was drawn from the lateral saphenous vein under isofluorane anesthesia 2-3 wk after the rats were on nicotine treatment. Plasma cotinine was determined using an EIA kit (Orasure Technologies, Bethlehem, PA). Cotinine is a long-lasting nicotine metabolite used as an index of nicotine intake. The plasma cotinine levels in nicotine-treated rats were 358 ± 29 ng/ml ($n=10$), which are comparable to those in the plasma of smokers (Matta *et al.* 2007). For some animals, plasma was also collected after 2 months of treatment, with cotinine values similar to samples from the first blood draw. No rats were excluded due to low cotinine levels. There was no detectable plasma cotinine in saccharin-treated rats.

Tissue preparation

The brain was quickly removed and chilled in ice-cold, pre-oxygenated (95% O₂/5% CO₂) buffer containing: 30 mM NaCl, 4.5 mM KCl, 1.2 mM NaH₂PO₄, 1.0 mM MgCl₂, 10 mM glucose, 26 mM NaHCO₃, and 18 mM sucrose (pH 7.4). Coronal slices containing the NAcc (350 μ m thick) were cut using a vibratome (Leica VT1000S) in the same buffer. Slices were then incubated for at least 1 hour in oxygenated physiological buffer containing: 125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH₂PO₄, 2.4 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 26 mM NaHCO₃ (pH 7.4) at room temperature. Each slice was transferred to a submersion-recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at 1 ml/min with 30° C, oxygenated buffer, and allowed to equilibrate for 30 min.

Some brains were immediately quick frozen in isopentane on dry ice and stored at -80° C. Sections (8 μ m) were prepared using a cryostat (Leica Microsystems, Inc., Deerfield, IL) cooled to -20° C. Frozen sections were thaw-mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA), air-dried and stored at -80° C for nAChR autoradiography.

Cyclic Voltammetry

Carbon fiber microelectrodes (7 μ m in diameter; tip length ~100 μ m) were constructed as previously described (Perez *et al.* 2008b). The electrode was positioned below the surface of the slice and its potential linearly scanned every 100 ms from 0 to -400 to 1000 to -400 to 0 mV versus an Ag/AgCl reference electrode at a scan rate of 300 mV/ms. Only the carbon fiber was inserted into the slice to avoid tissue damage by the glass. Current was recorded and digitized at a frequency of 50 kHz with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Triangular wave generation and data acquisition were controlled by pClamp 9.0 software (Molecular Devices, Sunnyvale, CA). Background current was digitally subtracted to obtain the voltammograms used for the identification of dopamine (confirmed by an oxidation peak ~500-600 mV and a reduction peak around -200 mV). Peak oxidation currents were converted into concentrations after post-experimental calibration of the electrode with fresh solutions of 0.5-1.0 μ M dopamine. Dopamine release was measured as the maximal peak response obtained after electrical stimulation.

Electrically-evoked dopamine release was measured in the dorsal half of NAcc shell. Electrical stimulation was applied using a bipolar stimulating electrode (Plastics One, Roanoke, VA) connected to a linear stimulus isolator (WPI, Sarasota, FL) and triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). The stimulating electrode was consistently placed so that it just touched the surface of the slice and the carbon fiber electrode was positioned ~100 μ m away. Evoked release was elicited by either a single electrical pulse or a train of 4 pulses (1-4 ms in duration) at 30 Hz applied every 2.5 min. This stimulation paradigm was based on our previous studies showing similar drug effects with a 30 Hz and a 100 Hz stimulation frequency (Perez *et al.* 2010). In addition, previous reports show that burst firing of rat dopamine neurons *in vivo* occurs at approximately 20 Hz (Zhang *et al.* 2009a). Control evoked release was assessed in physiological buffer. NACHR-

modulated release was assessed in the presence of 100 nM α -conotoxinMII (α -CtxMII) to antagonize $\alpha 6\beta 2^*$ nAChRs followed by 100 μ M mecamylamine (Mec) or 100 nM dihydro- β -erythroidine (DH β E) to block all nAChR subtypes. The effects of Mec or DH β E alone are similar to that of either antagonist plus α -CtxMII (data not shown). Superfusion of the slice with α -CtxMII maximally decreased release within ~15 min and responses were recorded over a 1 h period. Responses in the presence of Mec and DH β E were recorded over at least a 2 h period. Slices were also exposed to nicotine (300 nM) and signals recorded over at least a 1 h period to determine how release was altered under this treatment condition. To determine the effects of acetylcholinesterase inhibition, slices were superfused with ambenonium dichloride at increasing concentrations (0.1, 1, 10, 40, 80, 100 and 1000 nM). All drug concentration used have been shown to yield a maximal effect (Exley et al. 2008, Zhou *et al.* 2001). The changes in release observed with all drugs were reversible upon prolonged wash (1-2 h). The reported effects represent the average of those signals obtained once a stable maximal response was established.

¹²⁵I-Epipatidine Autoradiography

Binding of ¹²⁵I-epibatidine (2200 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) was done as previously reported (Quik *et al.* 2000). Slides were pre-incubated at 22° C for 15 min in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, and 1.0 mM MgCl₂. They were incubated for 40 min with 0.015 nM ¹²⁵I-epibatidine in the presence or absence of α -CtxMII (100 nM). They were then washed, dried and exposed to Kodak MR film with ¹²⁵I-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for several days. Nonspecific binding was assessed in the presence of 100 μ M nicotine and was similar to the film blank.

¹²⁵I- α -CtxMII Autoradiography

Binding of ¹²⁵I- α -CtxMII (specific activity, 2200 Ci/mmol) was done as reported previously (Quik *et al.* 2001). Striatal sections were preincubated at room temperature for 15 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2mM CaCl₂ 1mM MgSO₄, 20mM HEPES and 0.1% bovine serum albumin, pH 7.5) plus 1 mM PMSF (phenylmethylsulfonyl fluoride). This was followed by 1 h incubation at room temperature in binding buffer containing 0.5% bovine serum albumin, 5 mM EDTA, 5 mM EGTA and 10 μ g/ml each of aprotinin, leupeptin and pepstatin A plus 0.5 nM ¹²⁵I- α -CtxMII. The assay was terminated by washing the slides for 10 min at room temperature, 10 min in ice cold binding buffer, twice for 10 min in 0.1X buffer at 0° C and two final 5 s washes in ice cold deionized water. The striatal sections were air-dried and exposed to Kodak MR for 2 to 5 days together with ¹²⁵I-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nicotine (100 μ M) was used to determine nonspecific binding.

Dopamine Transporter Autoradiography

Binding to the dopamine transporter was measured using ¹²⁵I-RTI-121 (2200 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA), as previously described (Quik et al. 2001). Thawed sections were pre-incubated twice for 15 min each at room temperature in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl, and then incubated for 2 h in buffer with 0.025% BSA, 1 μ M fluoxetine and 50 pM ¹²⁵I-RTI-121. Sections were washed at 0° C for 4 x 15 min each in buffer and once in ice-cold water, air dried, and exposed for 2 d to Kodak MR film with ¹²⁵I-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nomifensine (100 μ M) was used to define nonspecific binding.

Data Analyses

The ImageQuant program from GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK was used to determine the optical density values from autoradiographic films. Background tissue values were subtracted from total tissue binding to evaluate specific binding of the radioligands. Specific binding values were then converted to fmol/mg tissue using standard curves determined from ^{125}I standards. Care was taken to ensure that sample optical density readings were within the linear range.

All statistics and curve fittings were conducted using GraphPad Prism (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using unpaired t-test analysis, one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparisons test or two-way ANOVA followed by Bonferroni post hoc test. A value of $p < 0.05$ was considered significant. All values are expressed as the mean \pm SEM of the indicated number of animals, with release values for each animal representing the average of 6-15 signals from 1-2 slices.

Results

Long-term nicotine treatment decreases single pulse and four pulse-stimulated dopamine release in rat NAcc shell

The present results show that long-term nicotine treatment significantly decreased dopamine release in rat NAcc shell, in agreement with our previous studies in monkeys (Perez et al. 2012). Single pulse and low frequency stimulation are commonly used to mimic tonic signaling while stimulus trains may be more closely associated with burst activity related to reward (Grace 2000). Therefore, release was measured using a single pulse, as well as a four-pulse stimulus train at 30 Hz as previous studies have shown that differential results may arise with varying firing frequency (Exley et al. 2008, Exley et al. 2011, Perez et al. 2008a, Rice & Cragg 2004, Zhang & Sulzer 2004, Zhang et al. 2009a). Both single pulse and four pulse-evoked dopamine release were reduced by ~50% compared to the vehicle-treated group (Fig. 1).

nAChR-mediated dopamine release in rat NAcc is primarily mediated via $\alpha 6\beta 2^*$ nAChRs

We next assessed the effect of nAChR antagonists on dopamine release in vehicle- and nicotine-treated rats. We first examined the nAChR subtypes that mediate dopamine release in vehicle-treated rats as previous studies have only been done in mouse and monkey NAcc (Exley et al. 2008, Exley et al. 2011, Perez et al. 2012). In vehicle-treated rats, the $\alpha 6\beta 2^*$ nAChR antagonist α -conotoxinMII (α -CtxMII) decreased single pulse-stimulated dopamine release by ~45% (Fig. 2). No further decrease was observed upon application of the general nAChR antagonist mecamylamine (Mec) or the $\beta 2^*$ nAChR antagonist dihydro- β -erythroidine (DH β E) (Fig. 2). These data show that $\alpha 6\beta 2^*$ nAChRs regulate nAChR-mediated dopamine release in NAcc, consistent with work in mice and monkeys (Exley et al. 2008, Perez et al. 2012, Exley et al. 2011).

In nicotine-treated rats, there was a significant 50% decrease in control single pulse-stimulated dopamine release compared to the vehicle-treated group (Fig. 2). However, in contrast to the results in vehicle-treated rats, exposure of the slices to α -CtxMII, Mec or DH β E did not significantly decrease single pulse-stimulated release (Fig. 2). These data suggest that long-term nicotine treatment primarily affects $\alpha 6\beta 2^*$ nAChR-regulated dopamine release in rat accumbens.

Long-term nicotine treatment increases $\alpha 4(\text{non } \alpha 6)\beta 2^*$ nAChRs and decreases $\alpha 6\beta 2^*$ nAChR expression

To investigate how the current long-term nicotine treatment modulated nAChR subtype expression in the NAcc, we measured $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs. $\alpha 4\beta 2^*$ nAChR binding was assessed using ^{125}I -epibatidine autoradiography in the presence of the $\alpha 6\beta 2^*$ nAChR antagonist α -CtxMII (Fig. 3). $\alpha 4(\text{non } \alpha 6)\beta 2^*$ nAChRs were significantly increased ($p < 0.01$) in the NAcc shell of nicotine-treated animals. We also assessed changes in $\alpha 6\beta 2^*$ nAChRs using ^{125}I - α -CtxMII binding (Fig. 3). This receptor subtype was significantly decreased ($p < 0.05$) with nicotine treatment. Thus, the decline in dopamine release with long-term nicotine treatment could be linked to decreases in this receptor subtype.

Bath-applied nicotine decreases single pulse-stimulated dopamine release in slices of vehicle- and nicotine-treated rats

We next tested whether dopamine release was still sensitive to agonists after chronic nicotine treatment. To determine this, the exogenous agonist nicotine (300 nM) was bath-applied to slices from vehicle- and nicotine-treated animals and the effects on electrically-evoked dopamine release recorded. Bath-applied nicotine significantly decreased ($p < 0.001$) single pulse-stimulated dopamine release by ~52% in vehicle-treated rats (Fig. 4), in agreement with previous findings in rodents (Exley et al. 2008, Rice & Cragg 2004, Zhang & Sulzer 2004, Zhang *et al.* 2009b). A significant reduction ($p < 0.01$) in dopamine release (~49%) was also observed in slices from nicotine-treated rats (Fig. 4). These data indicate that the residual nAChRs are still functional and modulate dopamine release.

Acetylcholinesterase inhibition leads to similar effects on single pulse-stimulated release as bath-applied nicotine

To further investigate the effect of agonists, we tested the acetylcholinesterase inhibitor ambenonium, which increases endogenous acetylcholine levels. The results show that ambenonium (0.1-1000 nM) attenuated dopamine release in slices of vehicle-treated animals up to 56% ($p < 0.001$), consistent with earlier work (Exley et al. 2008, Zhang *et al.* 2004). Acetylcholinesterase inhibition also affected release in nicotine-treated rats with a maximal 47% decline ($p < 0.05$). Dose-response curves show that ambenonium attenuated single-pulse stimulated dopamine release in a concentration-dependent manner in both treatment groups (Fig. 5). Notably, a significantly lower ($p < 0.001$) dose of ambenonium was required to induce a half-maximal inhibition of dopamine release in slices from nicotine-treated ($\text{IC}_{50} = 4.81 \pm 2.04$ nM) versus vehicle-treated animals ($\text{IC}_{50} = 24.1 \pm 0.73$ nM).

Long-term nicotine treatment does not affect dopamine transporter expression or dopamine uptake

Dopamine transporter levels were measured to determine whether the decline in release observed with long-term nicotine treatment might be due to a reduction in neurotransmitter transport. No differences in dopamine transporter levels were found between vehicle- (1.86 ± 0.10 fmol/mg tissue) and nicotine-treated animals (1.75 ± 0.05 fmol/mg tissue).

We also determined whether long-term nicotine treatment modified the kinetic constants describing dopamine reuptake following release. The decaying portion of each dopamine peak was fitted to a one-phase exponential decay at points where the amount of release matched in vehicle- and nicotine-treated animals as previously described (Cragg *et al.* 2001, John *et al.* 2006, Wightman & Zimmerman 1990, Yorgason *et al.* 2011). Nicotine treatment did not modify dopamine uptake rate constants (5.12 ± 0.26 and 4.46 ± 0.24 s⁻¹ for vehicle- and nicotine-treated, respectively). Similarly, neither α -CtxMII, Mec, DH β E or frequency of

stimulation had a significant effect on uptake rates in either treatment group (data not shown).

Effect of nAChR drugs and ambenonium on four pulse-stimulated dopamine release in slices from vehicle- and nicotine-treated animals

We also evaluated how long-term nicotine treatment altered dopamine release evoked via a four-pulse stimulus train at 30 Hz. Neither nAChR blockade, bath-applied nicotine nor ambenonium modified four pulse-stimulated release in vehicle-treated rats (Fig. 6). These findings agree with previous studies showing that nAChR antagonism or desensitization facilitates or does not change electrically-evoked dopamine release stimulated by high-frequencies (Exley & Cragg 2008, Exley et al. 2011, Perez et al. 2008a, Zhang & Sulzer 2004, Zhang et al. 2009a).

Discussion

Enhanced dopamine release within the NAcc is a key event in nicotine reinforcement and reward (Balfour 2009, Corrigall et al. 1992, De Biasi & Dani 2011). β_2 , α_4 and α_6 nAChR subunits expressed in the mesolimbic dopaminergic pathway are involved in these functional and behavioral effects of nicotine, with extensive studies providing evidence for a role for $\alpha_4\beta_2^*$ and $\alpha_6\beta_2^*$ nAChRs (Changeux 2010, Tapper et al. 2004, Brunzell 2012, Epping-Jordan et al. 1999, Picciotto et al. 1998, Pons et al. 2008). Interestingly, converging work suggests that the $\alpha_6\alpha_4\beta_2^*$ nAChR may be the subtype which plays a key regulatory role in endogenous dopamine neurotransmission both in the VTA and NAcc (Exley et al. 2011, Liu et al. 2012, Zhao-Shea et al. 2011), thus reconciling findings that suggest that only the $\alpha_4\beta_2^*$ or $\alpha_6\beta_2^*$ nAChR populations were involved. In view of the importance of nAChRs in regulating dopamine transmission and nicotine dependence, we initiated the present studies in slices containing the nucleus accumbens to further understand how long-term nicotine treatment modulated dopamine release.

Cyclic voltammetry studies showed that long-term nicotine treatment (2-3 months) decreased electrically-evoked dopamine release in the rat NAcc shell as it had in the monkeys (Perez et al. 2012). Thus, long-term nicotine exposure decreases dopamine release in response to both single and four pulse stimulation across species. To understand the nAChR subtypes that underlie the reduction in dopamine release, we tested the effect of the $\alpha_6\beta_2^*$ nAChR blocker α -conotoxinMIII, the β_2^* -selective antagonist dihydro- β -erythroidine and the general nAChR antagonist mecamylamine. The results in vehicle-treated rat NAcc show that similar declines were observed in single-pulse stimulated dopamine release with all the blockers. These data indicate that the $\alpha_6\beta_2^*$ nAChR is the primary regulator of nAChR-mediated dopamine release, consistent with previous studies in mice and monkeys (Exley et al. 2008, Perez et al. 2012). The present data also showed a loss in nAChR-mediated dopamine release in nicotine-treated animals suggesting that alterations in nAChR expression and function contribute to the decrease in overall dopamine release, with the remaining release possibly reflecting mechanisms governed by direct electrically-evoked depolarization unaffected by nicotine treatment. Since the $\alpha_6\beta_2^*$ nAChR is the major subtype that controls release in NAcc, function mediated by this nAChR subtype would thus be affected by long-term nicotine treatment.

As an alternate, possibly more direct approach to evaluate the effect of the current nicotine treatment regimen on nAChR levels, we did receptor autoradiography. A ~50% decrease was observed in NAcc $\alpha_6\beta_2^*$ nAChR binding, consistent with the decline in dopamine release. The question remains which $\alpha_6\beta_2^*$ nAChR subtype might be involved, that is, the $\alpha_6\alpha_4\beta_2^*$ or $\alpha_6\beta_2^*$ receptors. Previous work had shown that $\alpha_6\alpha_4\beta_2^*$ nAChRs were decreased in rodent striatum after long-term nicotine treatment, with some increase in the

$\alpha 6\beta 2^*$ subtype (Perez et al. 2008a). These findings suggest that the decline in nAChR-modulated dopamine release with nicotine treatment may be partly due to a loss of $\alpha 6\alpha 4\beta 2^*$ nAChRs, the primary regulator of nAChR-mediated release in NAcc. As well, the increase in $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs, which are predominantly located post-synaptically on inhibitory GABAergic terminals and interneurons (Quik & Wonnacott 2011) could also play a role. It should be noted however that there are limitations of the binding assays when comparing the effects of nicotine on $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs. Unlike epibatidine which binds to desensitized receptors and may recognize immature complexes, α -conotoxin may only bind to mature $\alpha 6\beta 2^*$ receptors. Moreover, epibatidine is an agonist whereas α -conotoxinMIII is an antagonist. Thus caution is warranted with respect to interpretation of the nAChR autoradiography.

An important issue was whether the evoked dopamine release component remaining after long-term nicotine dosing was sensitive to the desensitizing effects of nicotine on dopamine release, that is, was the residual release still regulated by nAChRs. To test this, we used a nicotine concentration of 300 nM, which is similar to that reported in the plasma of smokers (Jarvik *et al.* 2000). Dopamine release was still responsive to bath-applied nicotine, suggesting that release was still regulated by nAChRs in NAcc of nicotine-treated rats. Similar results were obtained with the acetylcholinesterase inhibitor ambenonium, which sustains enhanced endogenous acetylcholine levels, hence leading to desensitization. These combined findings support the idea that although nAChR desensitization most likely contributes to the decrease in nAChR-mediated release, there are receptors that are still functional after long-term nicotine dosing. This conclusion would agree with recent work showing that nicotine desensitizes $\alpha 6\beta 2^*$ nAChRs to a lesser extent than other nAChR subtypes such that only 25% of their function is lost (Grady *et al.* 2012, Kuryatov & Lindstrom 2011). As a result, these receptors could still partially respond to the desensitizing effects of prolonged agonist exposure. Such an effect may provide a rationale as to why cigarettes are still rewarding to and craved by chronic smokers.

The data showing that dopamine release evoked by a train of pulses (4 pulses) at 30Hz was similar in the absence or presence of agonists and antagonists is consistent with previous studies (Meyer *et al.* 2008, Perez *et al.* 2009, Zhang & Sulzer 2004, Zhang et al. 2009a, Zhang et al. 2009b). This lack of effect of nAChR antagonism or desensitization on burst-induced release is due to a relief of short-term depression. Tonically active cholinergic interneurons provide a constant source of acetylcholine in the striatum and nucleus accumbens both *in vivo* and *in vitro* (Exley & Cragg 2008, Rice *et al.* 2011). This endogenous acetylcholine promotes dopamine release by interacting at nAChRs (Cragg 2003, De Biasi & Dani 2011). With a burst of action potentials, however, the initial enhanced dopamine release limits release with subsequent pulses – a phenomenon known as short-term depression (Cragg 2003). In the presence of nAChR antagonists or with desensitization, dopamine release is partially inhibited so that the first pulse induces a smaller amount of dopamine release due to receptor blockade. As a result, there is less short-term depression. Subsequent pulses within a burst cause more dopamine release such that overall release with burst firing is similar or enhanced in the presence of antagonists or with desensitization. These effects of nicotinic receptor blockade/desensitization may mimic the pause in cholinergic interneuron activity observed *in vivo* upon the presentation of reward-related events while dopamine neurons switch into bursting mode (Exley & Cragg 2008, Rice et al. 2011).

The finding that agonists but not antagonists affect dopamine release in the NAcc of nicotine-treated rats may have two implications. First, nicotine treatment may modify acetylcholine tone. Since antagonists generally act by blocking the action of endogenous acetylcholine at nAChRs, a possible explanation for their lack of effect may be that long-

term nicotine treatment reduces acetylcholine levels. This idea is consistent with findings showing that antagonist-dependent changes in electrically-evoked release require an interaction of endogenous acetylcholine with nAChRs (Exley et al. 2008, Zhang & Sulzer 2004, Zhou et al. 2001). Recent work also shows that endogenous acetylcholine release arising from the synchronized activity of cholinergic interneurons directly influences dopamine release regardless of the extent of dopaminergic activity (Cachope *et al.* 2012, Threlfell *et al.* 2012). Alternately, the ability of desensitizing concentrations of nicotine or acetylcholinesterase inhibition to further decrease dopamine release may be independent of $\alpha 6\beta 2^*$ and $\alpha 4\beta 2^*$ nAChRs. For instance, nAChR agonists may stimulate $\alpha 7$ nAChRs on glutamatergic terminals and indirectly modulate release from dopaminergic terminals. Studies to determine whether such a mechanisms contribute to the effects of agonists after long-term nicotine treatment remain to be done.

A question that arises concerns reconciliation of the observed decrease in nAChR receptor number and function following prolonged nicotine administration via drinking water with *in vivo* studies showing a sensitized response to nicotine after chronic nicotine exposure. One factor that may influence these differential effects of nicotine on dopamine signaling is route of administration. Indeed, studies show that sensitization is observed following daily nicotine injections but not chronic nicotine infusions (Benwell & Balfour 1997). Our drinking water model may replicate the sustained nicotine levels experienced with smoking and provide information about the functional effects of this long-term exposure. Conversely, measurements during an *in vivo* injection after nicotine exposure may better mimic the changes in dopamine release that occur during the nicotine peaks deemed essential for reward. An additional factor is that the brain slice preparation used in our studies is an isolated system devoid of the circuitry normally influencing the nucleus accumbens. Thus, it does not yield information on how changes in the expression and function of $\alpha 6\beta 2^*$ nAChRs in the VTA contribute to dopamine release in the accumbens shell *in vivo* after long-term nicotine exposure. Therefore, bath-application of nicotine may not fully replicate the *in vivo* effects of an acute nicotine exposure. Nevertheless, nAChRs at the terminal level are known to play an important role in directly modulating dopamine release and it is important to determine how nicotine exposure modifies their function as such local alterations may directly or indirectly affect signaling in the entire reward pathway.

In summary, the present results suggest that long-term nicotine decreases NAcc dopamine release via a downregulation of $\alpha 6\beta 2^*$ nAChR levels and a decline in their function, possibly due to reduced extracellular acetylcholine levels. These findings, coupled with observations that the smoking habit is thought to arise from the need to preserve nicotine-induced changes (Paolini & De Biasi 2011), support two possible therapeutic strategies to treat nicotine withdrawal. First, therapies that enhance the action of acetylcholine may help alleviate the urge to smoke by mimicking the functional effects of nicotine. Indeed, the use of acetylcholinesterase inhibitors is being explored as a potential smoking cessation therapy, with the advantage that such drugs could also reverse withdrawal-induced cognitive impairments (Hopkins *et al.* 2012, Sofuoglu & Mooney 2009). Second, our results suggest that $\alpha 6\beta 2^*$ nAChR agonists may represent a useful approach to combat nicotine dependence because of their prominent modulatory control of accumbal dopaminergic signaling.

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Abbreviations used

nAChRs	nicotinic acetylcholine receptors
α-CtxMII	α -conotoxinMII
Mec	Mecamylamine
DHβE	dihydro- β -erythroidine
RTI-121	3 β -(4-iodophenyl)tropane-2 β -carboxylic acid
*	the asterisk indicates the possible presence of other nicotinic subunits in the receptor complex, with α 4 β 2* nAChRs including α 4 β 2 and α 4 α 5 β 2 subtypes, and α 6 β 2* including α 6 α 4 β 2 β 3, α 6 β 2 β 3 and α 6 β 2 subtypes

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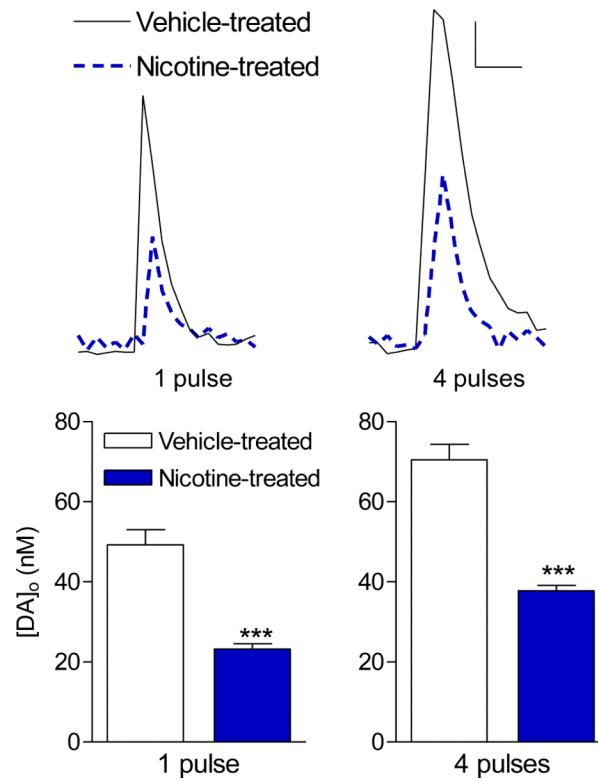


Fig. 1. Long-term nicotine treatment decreases single pulse and 4 pulse-stimulated dopamine release in rat NAcc shell. Dopamine release $[DA]_0$ was measured in the dorsal portion of the accumbens shell in response to a single pulse or a four-pulse stimulus train delivered at 30 Hz. Representative traces of $[DA]_0$ are shown for vehicle- and nicotine-treated animals. Scale bar represents 10 nM and 0.5 s. Quantitative analyses show that long-term nicotine treatment significantly decreased $[DA]_0$ under both stimulation conditions. The values represent the mean \pm SEM of 3-13 rats. Significance of difference from vehicle-treated rats, *** $p < 0.001$.

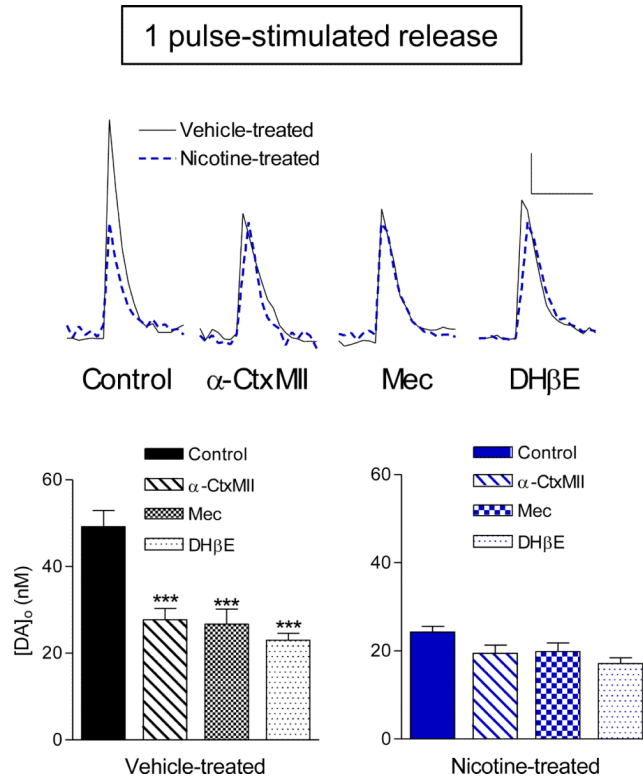


Fig. 2. Long-term nicotine treatment abolishes the nAChR antagonist-induced decrease on single pulse-stimulated dopamine release. Dopamine release [DA]_o was measured in the dorsal portion of the accumbens shell in response to single pulse electrical stimulation. Representative traces of [DA]_o in the absence (control) and presence of the α 6 β 2* antagonist α -CtxMII (100 nM), the general nAChR blocker Mec (100 μ M), and the β 2* nAChR antagonist DH β E (100 nM) are shown for vehicle- and nicotine-treated animals. Scale bar represents 10 nM and 0.5 s. Quantitative analyses show that α -CtxMII, Mec, and DH β E similarly affect single pulse-stimulated release in vehicle-treated animals, indicating that nAChR-modulated [DA]_o occurs through α 6 β 2* nAChRs. Single pulse-stimulated release was not further decreased in the presence of any of the nAChR antagonists after nicotine treatment. The values represent the mean \pm SEM of 3-13 rats. Significance of difference from control release, *** p < 0.001.

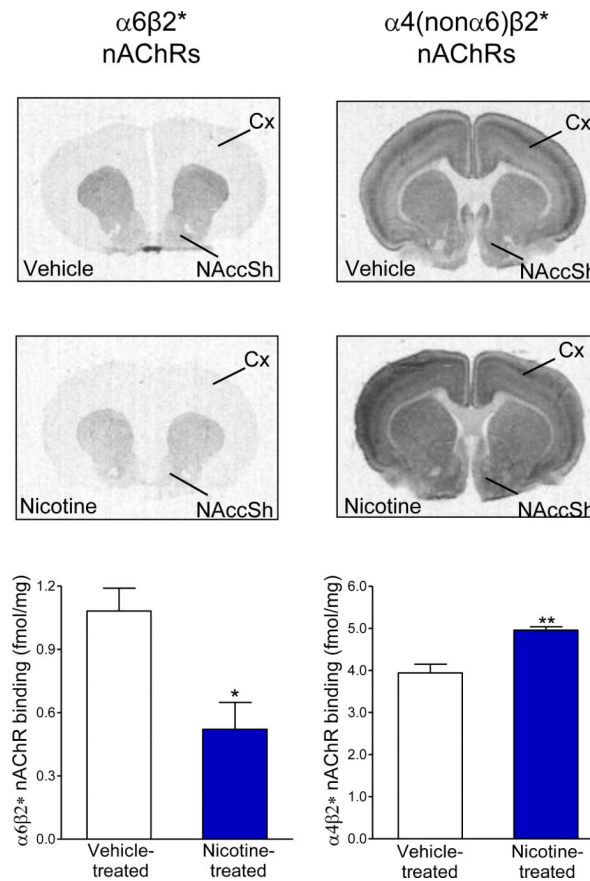


Fig. 3. Long-term nicotine treatment decreases $\alpha 6\beta 2^*$ nAChR expression and increases $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs in rat NAcc. Representative autoradiograms of $\alpha 6\beta 2^*$ nAChR binding using ^{125}I - α -CtxMII and $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR binding using ^{125}I -Epibatidine binding in the presence of α -CtxMII are shown for each treatment group. NAccSh, NAcc shell; Cx, Cortex. Quantitative analyses show a significant decrease in $\alpha 6\beta 2^*$ nAChRs with an increase in $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs after long-term nicotine treatment. Each value represents the mean \pm SEM of 4 animals per group. Significance of difference from vehicle-treated rats, ** $p < 0.01$, * $p < 0.05$.

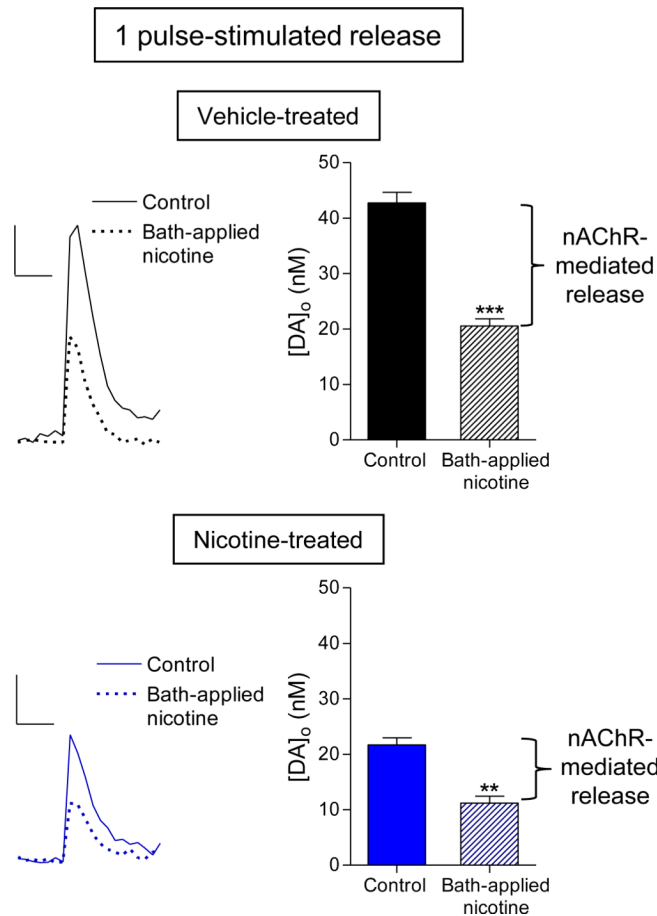


Fig. 4. Decrease in single pulse-stimulated dopamine release in slices of vehicle- and nicotine-treated rats with bath-applied nicotine. Dopamine release $[DA]_0$ was determined in brain slices from vehicle-treated (top panel) and nicotine-treated (bottom panel) animals in the absence (control) and presence of bath-applied nicotine (300 nM), as shown in the representative traces. Scale bar represents 10 nM and 0.5 s. Quantitative analyses show that single pulse-evoked $[DA]_0$ was significantly decreased in the presence of bath-applied nicotine in both vehicle- and nicotine-treated animals, indicating that nAChRs in NAcc are responsive after long-term nicotine treatment. The values represent the mean \pm SEM of 3-10 rats. Significance of difference from control, *** $p < 0.001$, ** $p < 0.01$.

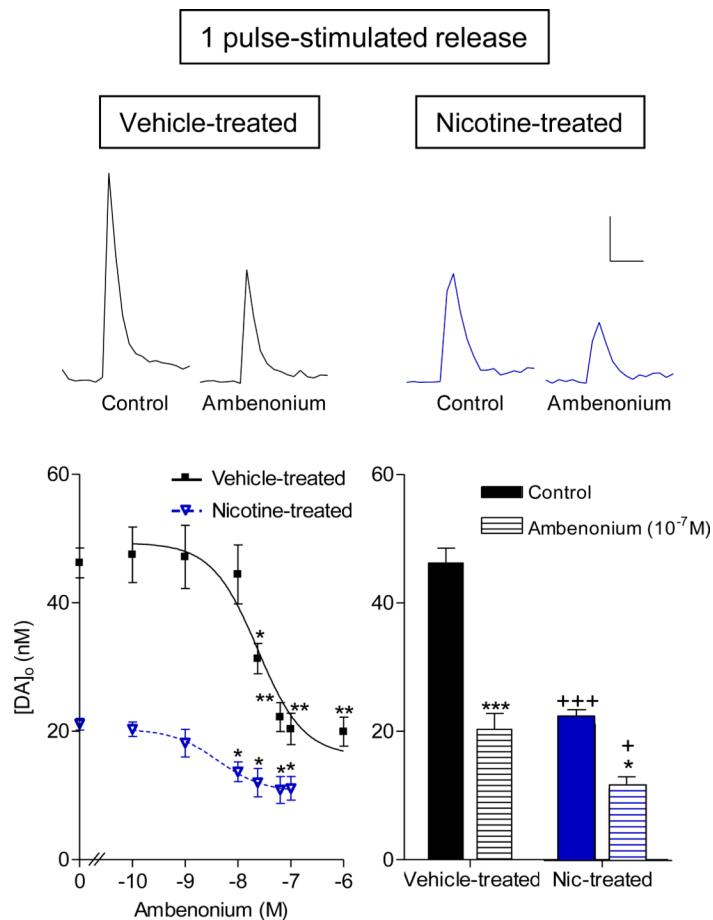


Fig. 5. Decrease in single pulse-stimulated dopamine release in slices of vehicle- and nicotine-treated rats with the acetylcholinesterase inhibitor ambenonium. Dopamine release $[DA]_o$ was determined in the absence (control) and presence of ambenonium (0.1-1000 nM). Representative traces show that ambenonium decreases single pulse-stimulated $[DA]_o$ in slices from vehicle- and nicotine-treated rats. Scale bar represents 10 nM and 0.5 s. Dose-response curves are shown for vehicle- and nicotine-treated animals (Curve fits were sigmoidal with $R = 0.8-0.9$). Quantitative analyses show that ambenonium decreases release by approximately 50% at the maximal effective dose in both treatment groups (1000 nM and 100 nM for vehicle- and nicotine-treated respectively). The values represent the mean \pm SEM of 3-4 rats. Significance of difference from control (zero ambenonium), *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Significance of difference from vehicle-treated rats under each given condition, +++ $p < 0.001$, + $p < 0.05$.

