



Published in final edited form as:

Mol Pharmacol. 2008 September ; 74(3): 844–853. doi:10.1124/mol.108.048843.

Chronic nicotine treatment differentially regulates striatal $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR expression and function

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Abstract

Nicotine treatment has long been associated with alterations in $\alpha 4\beta 2^*$ nicotinic acetylcholine receptor (nAChR) expression that modify dopaminergic function. However, the influence of chronic nicotine treatment on the $\alpha 6\beta 2^*$ nAChR, a subtype specifically localized on dopaminergic neurons, is less clear. Here we used voltammetry, as well as receptor binding studies, to identify the effects of nicotine on striatal $\alpha 6\beta 2^*$ nAChR function and expression. Chronic nicotine via drinking water enhanced non-burst and burst endogenous dopamine release from rat striatal slices. In control animals, $\alpha 6\beta 2^*$ nAChR blockade with α -conotoxinMII (α -CtxMII) decreased release with non-burst stimulation but not with burst firing. These data in control animals suggest that varying stimulus frequencies differentially regulate $\alpha 6\beta 2^*$ nAChR-evoked dopamine release. In contrast, in nicotine-treated rats, $\alpha 6\beta 2^*$ nAChR blockade elicited a similar pattern of dopamine release with non-burst and burst firing. To elucidate the $\alpha 6\beta 2^*$ nAChR subtypes altered with chronic nicotine treatment, we used the novel α -CtxMII analogue E11A, in combination with $\alpha 4$ nAChR knockout mice. ^{125}I - α -CtxMII competition studies in striatum of knockout mice showed that nicotine treatment decreased the $\alpha 6\alpha 4\beta 2^*$ subtype, but increased the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR population. These data indicate that $\alpha 6\beta 2^*$ nAChR-evoked dopamine release in nicotine-treated rats is mediated by the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR subtype, and suggest that the $\alpha 6\alpha 4\beta 2^*$ nAChR and/or $\alpha 4\beta 2^*$ nAChR contribute to the differential effect of higher frequency stimulation on dopamine release under control conditions. Thus, $\alpha 6\beta 2^*$ nAChR subtypes may represent important targets for smoking cessation therapies and neurological disorders involving these receptors such as Parkinson's disease.

Introduction

Nicotine, the primary reinforcing component in tobacco, exerts its effects in large part by acting on dopamine neurons (Dani and Bertrand, 2007; Wonnacott et al., 2005). This includes the mesolimbic and nigrostriatal dopaminergic systems that originate in the ventral tegmental area and substantia nigra and project to the nucleus accumbens and striatum, respectively. Dopaminergic nerve terminals synapse on multiple targets in these regions including cholinergic interneurons. These neurons are tonically active and continually release acetylcholine, which subsequently affects dopaminergic function (Zhou et al., 2002). The mesolimbic dopamine system is primarily involved in reward and reinforcement (Dani and Bertrand, 2007; Wonnacott et al., 2005), while nigrostriatal dopamine neurons are involved in addictive behaviors and the control of movement (Janhunen and Ahtee, 2007; Singh et al., 2007).

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Nicotine exerts its effects on dopaminergic function by interacting with nicotinic acetylcholine receptors (nAChRs) of which there are multiple subtypes (Dani and Bertrand, 2007; Wonnacott et al., 2005). One of these is the $\alpha 4\beta 2^*$ nAChR population which consists of $\alpha 4\beta 2$ and $\alpha 4\alpha 5\beta 2$ subtypes, as well as other minor subtypes (Gotti et al., 2007; Grady et al., 2007; Quik et al., 2007). Interestingly, chronic nicotine treatment differentially influences $\alpha 4\beta 2^*$ nAChR subtype expression, with an increase in $\alpha 4\beta 2$ sites and no change in $\alpha 4\alpha 5\beta 2$ nAChRs (Mao et al., 2008). These alterations in $\alpha 4\beta 2^*$ nAChR expression most likely influence reward-related behaviors (Maskos et al., 2005; Tapper et al., 2004) and the locomotor effects of nicotine (Marubio et al., 2003; Tapper et al., 2007).

The involvement of $\alpha 6\beta 2^*$ nAChRs in behavior is less well studied than that for $\alpha 4\beta 2^*$ nAChRs; however, accumulating evidence suggests this subtype also plays an important role. Several major $\alpha 6\beta 2^*$ nAChRs have been identified in striatum including the $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6$ (non $\alpha 4$) $\beta 2^*$ subtypes (Gotti et al., 2007; Grady et al., 2007; Quik et al., 2007; Salminen et al., 2004). These receptors are selectively localized to catecholaminergic regions and represents 20-45% of nAChRs in the mesolimbic and nigrostriatal systems, depending on species (Quik and McIntosh, 2006). In addition, ^3H -dopamine release studies show that $\alpha 6\beta 2^*$ nAChRs mediate 30-75% of the response in striatum and nucleus accumbens (Grady et al., 2007; Quik and McIntosh, 2006), while voltammetry studies indicate that $\alpha 6\beta 2^*$ nAChRs are responsible for the greater majority of dopamine release in the nucleus accumbens (Exley et al., 2007). These data suggest that $\alpha 6\beta 2^*$ nAChRs may also mediate effects of nicotine on behaviors linked to dopamine function, including addiction and motor control. Indeed, it has been shown that $\alpha 6\beta 2^*$ nAChRs are regulated by nicotine treatment, but in a manner distinct from $\alpha 4\beta 2^*$ nAChRs. Chronic nicotine treatment leads to a downregulation or no change in $\alpha 6\beta 2^*$ nAChR expression, although studies to determine selective effects on the $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6$ (non $\alpha 4$) $\beta 2^*$ subtypes remain to be evaluated.

Because nicotine exposure in humans via smoking or drug treatment is generally long term, we investigated changes in striatal endogenous dopamine release and $\alpha 6\beta 2^*$ nAChR subtype expression after chronic nicotine administration in rodents. Our results show that nicotine treatment results in an upregulation of the $\alpha 6$ (non $\alpha 4$) $\beta 2^*$ subtype but a loss of $\alpha 6\alpha 4\beta 2^*$ nAChRs, suggesting that the $\alpha 6$ (non $\alpha 4$) $\beta 2^*$ subtype primarily modulates $\alpha 6\beta 2^*$ nAChR-mediated dopamine release after chronic nicotine. These results have implications for the development of subtype selective nicotinic agonists for smoking cessation and the treatment of neurological disorders.

Materials and Methods

Mouse studies

Mice engineered to contain a null mutation in $\alpha 4$ nAChR subunit gene obtained from the laboratory of Dr. John Drago (Ross et al., 2000) were maintained and bred at the Institute for Behavioral Genetics, University of Colorado (Boulder, CO). A 12 h light/dark cycle was used with room temperature at 22°C during which they had free access to food and water. Mice weaned at 25 days of age were housed with same-sex littermates. The genotype was determined by PCR (Salminen et al., 2004) using isolated tail DNA at 40 days of age. Mice used for the present study were age matched littermates of mixed genetic background; wildtype and $\alpha 4$ (-/-). Nicotine was added to the saccharin-containing solution starting at an initial concentration of 25 $\mu\text{g}/\text{ml}$. This dose was gradually increased to a final dose of 300 $\mu\text{g}/\text{ml}$ over a period of 10 days. The animals were maintained on the final dose of nicotine for 14 days, and then killed by cervical dislocation. All procedures were in accordance with guidelines and approval of the Animal Care and Use Committee of the University of Colorado, Boulder.

The brains were removed and immediately frozen in isopentane on dry ice and stored at -80°C . Sections ($8\ \mu\text{m}$) 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 nicotinic receptor autoradiography.

Rat Studies

Adult male Sprague-Dawley rats (250-300g) were purchased from Charles River Laboratories (Gilroy, CA). Rats were placed in a temperature-controlled room with a 12 h dark/light cycle and were housed 2-3 per cage. All animals had free access to food and water. They were randomly divided into different treatment groups 2 days after arrival. After several days of acclimatization, rats were given drinking water containing 2% saccharin in the initial studies and 1% in subsequent experiments (Sigma Chemical Co., St. Louis, MO), to mask the bitter taste of nicotine. Nicotine was added to the saccharin-containing solution of the treated group, with the nicotine saccharin solution adjusted to pH 7.0 throughout the course of the study. Rats were started at an initial concentration of $25\ \mu\text{g/ml}$ nicotine (free base, Sigma) in the drinking water, which was increased to $50\ \mu\text{g/ml}$ on days 5-6, and to a final dose of $100\ \mu\text{g/ml}$ on days 8-10. The animals were maintained at this final dose for 14 days. Fluid intake and weights were monitored daily, with no significant differences between the two treatment groups. Nicotine was removed from the drinking water 18 h before death to minimize nicotine tissue levels before the functional assays. 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 the Parkinson's Institute.

The brain was quickly removed and chilled in ice-cold, pre-oxygenated (95% O_2 /5% CO_2) physiological buffer containing (in mM): 125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 2.4 mM CaCl_2 , 1.2 mM MgCl_2 , 20 mM HEPES, 11 mM glucose, 25 mM NaHCO_3 (pH 7.4). Corticostriatal slices ($400\ \mu\text{m}$ thick) were cut using a vibratome (Leica VT1000S) and incubated at room temperature in oxygenated buffer. The remaining portion of the brain, which contained the mid to posterior striatum, was quick frozen in isopentane on dry ice immediately after the sections were removed, and stored at -80°C . Sections ($8\ \mu\text{m}$) 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 nicotinic receptor autoradiography.

Electrochemical measurement of dopamine release

For the fast scan cyclic voltammetry experiments, carbon fiber microelectrodes were constructed as previously described (Perez et al., 2008). The electrode potential was linearly scanned from 0 to -400 to 1000 to -400 to 0 mV versus an Ag/AgCl reference electrode at a scan rate of $300\ \text{mV/ms}$ (Perez et al., 2008; Zhou et al., 2001). This triangular wave was repeated every 100 ms at a sampling frequency of 50 Hz. Current was recorded with an Axopatch 200B amplifier (Axon instruments, Foster City, CA). Triangular wave generation and data acquisition were controlled by pClamp 9.0 software (Axon instruments, Foster City, CA). Electrical stimulation was applied using a bipolar tungsten 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). All electrode placements were made in the dorsal striatum with the aid of a stereomicroscope and micromanipulators. Background current was digitally subtracted and the peak oxidation currents were converted into concentration after post-experimental calibration of the carbon fiber electrode with a fresh solution of $1\ \mu\text{M}$ dopamine in experimental buffer.

After at least 2 h of incubation and 30 min before recording, a brain slice was transferred to a submersion recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at $1\ \text{ml/}$

min with 30°C, oxygenated aCSF, and allowed to equilibrate. Dopamine release from dorsal striatum was evoked by either a single, rectangular electrical pulse (4 ms) applied every 2.5 min or by a burst of 4 pulses at 30 Hz applied every 5 min with a stimulus intensity of 8V that achieved 60% maximal release. The recording sites were always restricted to the same area of the dorsal striatum to ensure consistency of the signals across animals. Evoked release by both, a single and a burst of pulses, was first assessed in physiological buffer. α -CtxMII (100nM) prepared in physiological buffer was then perfused through the slice while continuously monitoring release evoked by a single pulse and then through a burst of pulses. This concentration of α -CtxMII maximally blocks $\alpha 6\beta 2^*$ nAChRs without affecting other nAChR subtypes (Exley et al., 2007).

¹²⁵I-Epipatidine autoradiography

Binding of ¹²⁵I-epibatidine (2200 Ci/mmol; Perkin Elmer Life Sciences, Waltham, MA, USA) was done as previously reported (Kulak et al., 2002). 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 (300 nM). They were then washed, dried and exposed to Kodak MR film with ¹²⁵I standards 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

¹²⁵I- α -CtxMII (from J. M. McIntosh) was synthesized and radiolabeled as described (Whiteaker et al., 2000). Binding of ¹²⁵I- α -CtxMII (specific activity, 2200 Ci/mmol) was done as reported previously (Quik et al., 2001). Thawed 8 μ m 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 % BSA (bovine serum albumin), pH 7.5) plus 1 mM phenylmethylsulfonyl fluoride along with different concentrations (1.0 fM-0.1 μ M) of α -CtxMII analog E11A. This was followed by 1-h incubation at room temperature in binding buffer also 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, as well as the competing concentrations of E11A (Bordia et al., 2007). 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 (Perkin Elmer Life Sciences, Boston, MA) 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.

Data analyses

To evaluate optical density values from autoradiographic films, we used the ImageQuant program from Molecular Dynamics, Sunnyvale, CA. To assess specific binding of the radioligands, background tissue levels were first subtracted from total binding to the tissue. The resultant values were converted to fmol/mg tissue using standard curves determined from ¹²⁵I standards. The optical density readings of the samples were always within the linear range of the film. For analyses of striatal values, the ventral striatum (i.e. nucleus accumbens) was always excluded, as there may be differences in the nAChR population between dorsal and ventral striatum.

All curve fittings and statistics were conducted using GraphPad Prism (Graph Pad Software Co., San Diego, CA, USA). Statistical comparisons were performed using either Student's *t*-test or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (GraphPad prism) because this test is conservative and allows for comparison of all pairs of data. A value

of $p < 0.05$ was considered significant. All values are expressed as the mean \pm SEM of the indicated number of animals.

Results

Effect of chronic nicotine treatment via the drinking water on nAChR expression in rat striatum

For the present experiments, we chose a chronic nicotine treatment paradigm via the drinking water since it allows for long-term, intermittent exposure to nicotine (Lai et al., 2005; Pietila and Ahtee, 2000). Experiments were first done to determine the effects of chronic nicotine treatment on overall receptor expression (Fig 1). Quantitative receptor autoradiography using ^{125}I -epibatidine, a ligand that labels multiple nAChR subtypes, was done in the presence of α -CtxMII to identify α -CtxMII-resistant ^{125}I -epibatidine ($\alpha 4\beta 2^*$ nAChRs) and α -CtxMII-sensitive ^{125}I -epibatidine binding sites ($\alpha 6\beta 2^*$ nAChRs). Oral nicotine treatment significantly increased $\alpha 4\beta 2^*$ ($p < 0.05$) and decreased $\alpha 6\beta 2^*$ ($p < 0.05$) nAChRs in the striatum of rats (Fig 1A and 1B) as previously reported in mice and monkeys (Lai et al., 2005; McCallum et al., 2006). To directly measure $\alpha 6\beta 2^*$ nAChR, we performed ^{125}I - α -CtxMII binding studies. Nicotine treatment also significantly decreased ^{125}I - α -CtxMII binding ($p < 0.001$) indicating that ^{125}I - α -CtxMII labeled sites identify the same subset of $\alpha 6\beta 2^*$ nAChRs as α -CtxMII-sensitive ^{125}I -epibatidine binding sites (Fig 1C).

Chronic nicotine treatment via the drinking water increases electrically-evoked dopamine release in rat dorsal striatum

In the present studies, we used voltammetry to evaluate the effect of chronic nicotine treatment on nAChR-mediated dopaminergic function for several reasons. One of these was that it provides a measure of dopamine release from a slice preparation, in which the modulatory influence of other neurotransmitter systems is still partially intact. In addition, voltammetry involves release of endogenous acetylcholine to stimulate nAChRs and subsequently assesses responses in endogenous dopamine release. Voltammetry also offers an excellent temporal resolution (msec) and allows for a determination of dopaminergic activity at varying firing frequency. This is important because nicotine's effect on dopamine release is dependent on the frequency of stimulation of dopaminergic neurons, that is, tonic versus phasic stimulation (Exley et al., 2007; Meyer et al., 2008; Rice and Cragg, 2004; Zhang and Sulzer, 2004).

Electrochemical studies to study alterations in dopamine neurotransmission after nicotine exposure have thus far only investigated dopamine release after acute tissue application (Exley et al., 2007; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001). To evaluate the effects of long-term nicotine treatment on endogenous dopamine release from striatal slices, we first examined the effects of a single pulse (non-burst firing) versus a 4 pulse (burst firing) stimulus on dopamine release in slices from control animals. Single pulse stimulation elicited a rapid release of dopamine as characterized by its typical voltammogram showing an oxidation peak around 500-600 mV and a reduction peak around -200mV (Fig 2 inset). An increase in the number of stimulus pulses (4 pulses at 30 Hz) yielded a similar level of dopamine release as compared to a single pulse stimulus (0.17 ± 0.01 and 0.19 ± 0.01 μM for 1 and 4 pulse stimulation, respectively) (Fig 2). These results support the idea that short term depression in response to burst stimuli decreases the amount of dopamine release with each successive pulse thus tapering total release after a burst of pulses in both control and nicotine-treated rats (Exley et al., 2007; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Zhou et al., 2001)

Chronic nicotine treatment significantly enhanced endogenous dopamine release (Fig 2) with both one and four pulse stimulation as assessed using two-way ANOVA by stimulus pattern and treatment. The increase in dopamine release was similar ($\sim 50\%$) for the nicotine-treated

rats as compared to control with 1-pulse (from 0.17 ± 0.01 to $0.25 \pm 0.02 \mu\text{M}$) and 4-pulse (from 0.19 ± 0.01 to $0.26 \pm 0.02 \mu\text{M}$) stimulation. These findings indicate that burst-stimulated dopamine release is still affected by short-term depression after chronic nicotine treatment.

Chronic nicotine treatment prevents the enhancement of dopamine release with burst firing

To determine the involvement of the $\alpha 6\beta 2^*$ nAChR subtype in the release of endogenous dopamine from striatal slices in control rats, we used the specific $\alpha 6\beta 2^*$ nAChR antagonist α -CtxMII. Release was elicited by either a single pulse or a 4 pulse stimulus to probe the relationship between firing pattern and $\alpha 6\beta 2^*$ nAChR-mediated dopamine function. The identity of the released neurotransmitter was confirmed by the characteristic voltammograms showing an oxidation peak around 500-600 mV and a reduction peak around -200mV (see Fig 3 insets). Addition of α -CtxMII to the perfusion media resulted in a significant decrease ($p < 0.001$) in dopamine release with non-burst stimuli (Fig 3) while α -CtxMII significantly increased ($p < 0.05$) peak dopamine release under burst firing conditions, as analyzed using two-way ANOVA by stimulus pattern and α -CtxMII exposure (Fig 3). These results are in agreement with a recent report showing that blockade of $\alpha 6\beta 2^*$ nAChRs significantly increases the sensitivity of dopamine release to burst versus non-burst stimuli (Exley et al., 2007).

We next used α -CtxMII to assess the effect of chronic nicotine treatment on $\alpha 6\beta 2^*$ nAChR-mediated dopamine release. In nicotine treated animals, addition of α -CtxMII to the perfusion buffer led to a significant 48% decrease ($p < 0.001$) in endogenous dopamine release evoked by a single pulse (Fig 3), similar to results in control rats in which a 53% decline in dopamine release was observed, as analyzed using two-way ANOVA by stimulus pattern and α -CtxMII exposure. These data suggest that there are functional changes in other nAChR subtypes such as the $\alpha 4\beta 2^*$ nAChR with chronic nicotine dosing. In contrast to the effect of $\alpha 6\beta 2^*$ nAChR blockade on burst-evoked dopamine release in the control rats (Fig 3), there was a significant 61% decrease ($p < 0.001$) in dopamine release with burst stimulation in nicotine treated animals (Fig 3). These results suggest that long-term chronic nicotine treatment modifies the $\alpha 6\beta 2^*$ nAChR-mediated regulation of striatal dopaminergic function.

Further analyses were then done to assess the effects of chronic nicotine as compared to the saccharin controls with single pulse and burst stimulation in the presence of α -CtxMII. Two-way ANOVA showed that there was a significant main effect of nicotine treatment ($p < 0.05$) and stimulus pattern ($p < 0.001$). In addition, there was a significant interaction ($p < 0.001$), with an increase in dopamine release with the one pulse stimulus but a decrease in release with the 4-pulse stimulus. These results further highlight the prominent changes that occur in nicotinic subtype-evoked dopamine release with nicotine treatment.

Preferential downregulation of the $\alpha 6\alpha 4\beta 2^*$ or very high affinity $\alpha 6\beta 2^*$ nAChR subtype in striatum of rats treated chronically with nicotine

Our previous study using the α -CtxMII analogue E11A had shown that there are two major $\alpha 6\beta 2^*$ nAChR subtypes in mouse striatum, the $\alpha 6\alpha 4\beta 2^*$ and the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs in agreement with previous findings (Bordia et al., 2007; Salminen et al., 2004). To investigate whether E11A could also distinguish these subtypes in rat striatum, ^{125}I - α -CtxMII competition assays were done with varying concentrations of E11A using serial striatal sections from control rats. E11A inhibition of ^{125}I - α -CtxMII binding yielded a two site inhibition curve (data best fit to two site model) with a ~ 1800 fold difference in affinity between sites (Fig. 4, Table 1). The very high affinity fraction represented 51% of the total E11A sensitive ^{125}I - α -CtxMII binding sites (Table 1). In rats chronically treated with nicotine, competition analysis of E11A inhibition of ^{125}I - α -CtxMII binding yielded a monophasic curve in 4 animals, and a biphasic curve in 2 animals. This diversity between animals could relate to individual variations in outbred rats that influence the effect of nicotine treatment (Fagen et al., 2007). When all the

data were pooled, a biphasic curve was obtained showing a preferential decrease in the very high affinity f1 fraction (control, f1 = 74%: nicotine, f1 = 26%) (Fig. 4, Table 1).

Chronic nicotine treatment differentially regulates $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ subtypes in mouse striatum

The experiments described in the preceding sections were done with rats as one of our long-term objectives is to evaluate effects of nicotine on behavioral measures, which may more readily be performed in this species. However, because the use of knockout mice often provides clearer insight about the specific $\alpha 6\beta 2^*$ nicotinic receptor subtypes ($\alpha 6\alpha 4\beta 2^*$ or/and $\alpha 6(\text{non}\alpha 4)\beta 2^*$) involved in functional effects of nicotine, we next initiated a series of studies in wildtype and $\alpha 4$ nAChR-null mutant mice. Such an approach appeared reasonable since the magnitude of $\alpha 6\beta 2^*$ nAChR expression and function is similar in rat and mouse striatum. The $\alpha 6\beta 2^*$ nAChR subtype represents about 15-20% of the nAChR population in both rat or mouse striatum and is responsible for ~30% of evoked ^3H -dopamine release from striatal synaptosomes in both rat and mouse (Quik and McIntosh, 2006).

^{125}I -Epibatidine binding in the absence and presence of α -CtxMII was used to distinguish between $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ binding sites. Results in Fig. 5, analyzed using two-way ANOVA by genotype and nicotine treatment, show a significant increase ($p < 0.001$) in $\alpha 4\beta 2^*$ nAChRs in wildtype mice with nicotine treatment (Fig. 5A). In contrast, no binding to $\alpha 4\beta 2^*$ nAChRs was observed in striatum of $\alpha 4$ (-/-) mice, as expected since the $\alpha 4$ subunit is not expressed in $\alpha 4$ (-/-) mice. $\alpha 6\beta 2^*$ nAChR sites were significantly decreased ($p < 0.01$) with nicotine treatment in wildtype mice. Surprisingly, there was a significant increase in the α -CtxMII-sensitive ^{125}I -epibatidine binding component in the striatum of $\alpha 4$ (-/-) mice receiving nicotine. Since $\alpha 4$ (-/-) mice do not express $\alpha 6\alpha 4\beta 2^*$ nAChRs, these data suggest there is a selective upregulation of $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs with nicotine treatment.

^{125}I - α -CtxMII binding was used as an alternate measure to study changes in striatal $\alpha 6\beta 2^*$ nAChRs with nicotine treatment in wildtype and $\alpha 4$ (-/-) mice (Fig 5C). Chronic oral nicotine decreased $\alpha 6\beta 2^*$ ($p < 0.01$) nAChRs in wildtype mice. ^{125}I - α -CtxMII binding was also decreased in $\alpha 4$ (-/-) mice indicating a loss in $\alpha 6\alpha 4\beta 2^*$ subtype as predicted in $\alpha 4$ (-/-) mice. Again, with chronic nicotine treatment there was a significant increase ($p < 0.01$) in $\alpha 6\beta 2^*$ binding in $\alpha 4$ (-/-) mice. These data again confirm the upregulation of $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs with nicotine treatment.

To directly determine the effects of nicotine on the $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR subtypes in mouse striatum, we performed ^{125}I - α -CtxMII competition assays using varying concentrations (10^{-15} to 10^{-7} M) of E11A. Competition binding studies in wildtype mice yielded a two site binding curve (Fig. 6), with an IC_{50} of 0.011 pM and 6.5 pM (Table 1). In the striatum of $\alpha 4$ (-/-) mice, E11A inhibited ^{125}I - α -CtxMII binding in a monophasic manner, as expected since $\alpha 4$ (-/-) mice do not express $\alpha 6\alpha 4\beta 2^*$ nAChRs. Interestingly, ^{125}I - α -CtxMII competition curves with E11A were monophasic in wildtype and $\alpha 4$ (-/-) mice chronically treated with nicotine. Moreover, the IC_{50} 's were similar to the lower affinity binding site (2.3 pM and 1.1 pM, for wildtype and $\alpha 4$ (-/-) mice, respectively) (Table 1). These data suggests a preferential downregulation of $\alpha 6\alpha 4\beta 2^*$ and upregulation of the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR in the striatum of mice receiving nicotine.

Discussion

The present results are the first to use cyclic voltammetry to investigate the effects of chronic nicotine treatment on the function of $\alpha 6\beta 2^*$ nAChRs, a subtype that evokes a large fraction of striatal nAChR-mediated dopamine release (Exley et al., 2007; Meyer et al., 2008; Quik and McIntosh, 2006). Our results in control animals show that blockade of $\alpha 6\beta 2^*$ nAChRs leads

to a decrease in dopamine release with single pulse stimulation but an increase in release with burst stimulation in agreement with previous findings (Exley et al., 2007). Interestingly, chronic nicotine treatment prevented this enhanced evoked dopamine release with burst firing. Subsequent receptor binding studies to understand the nAChRs that contribute to this effect showed that chronic nicotine treatment led to a small increase in $\alpha 6(\text{non}\alpha 4)\beta 2^*$ but resulted in a complete loss of $\alpha 6\alpha 4\beta 2^*$ nAChRs. These novel data, coupled with the results of the functional studies, suggest that $\alpha 6\alpha 4\beta 2^*$ nAChRs contribute to the increased dopamine release with burst firing observed after $\alpha 6\beta 2^*$ nAChR blockade under control conditions. Knowledge of these changes in nAChR expression with chronic nicotine administration is critical for the development of targeted therapies with nicotinic receptor ligands for smoking cessation and neurological disorders associated with nAChR deficits such as Parkinson's disease.

The major objective of the present study was to investigate the effect of chronic nicotine treatment on nAChR-mediated dopamine release. Our results in control rats showed that evoked release was similar with single and multiple pulse stimulation, as previously shown, most likely due to the development of short-term depression (Exley et al., 2007; Meyer et al., 2008). Chronic nicotine treatment led to an overall increase in evoked striatal dopamine release that was also similar with nonburst and burst firing. This functional modification with chronic treatment may represent a long-term molecular adaptation to nicotine since previous reports have shown that acute nicotine exposure enhances release with burst but not with nonburst firing (Exley et al., 2007; Rice and Cragg, 2004; Zhang and Sulzer, 2004). Our findings of enhanced release after nicotine treatment correspond with results from some *in vitro* ^3H -dopamine release assays and *in vivo* microdialysis studies (Marshall et al., 1997; Rowell and Wonnacott, 1990; Visanji et al., 2006), although they contrast with other work that reported a decrease (Perry et al., 2007; Westfall and Perry, 1986) or no change (Grilli et al., 2005; Janson et al., 1991). These discrepancies may relate to variations in dosing protocols, the type of preparation used, measurement of exogenous or endogenous dopamine, evaluation of nAChR-induced or stimulus-evoked release, and/or other factors.

An important question is the role of $\alpha 6\beta 2^*$ nAChRs in regulating striatal dopamine release with chronic nicotine dosing as this subtype makes a significant contribution to release under control conditions (Exley et al., 2007; Meyer et al., 2008; Quik and McIntosh, 2006). Our initial studies using striatal slices from control rats showed that the selective $\alpha 6\beta 2^*$ antagonist α -CtxMII blocked ~50% of dopamine release evoked with single pulse stimulation, consistent with recent results (Exley et al., 2007; Meyer et al., 2008). By contrast, block with α -CtxMII significantly increased release with burst firing in slices from control rats. These data at first appear distinct from those of Meyer et al. (2008) who observed no difference with nonburst and burst firing. The most likely explanation for this difference is that the $\alpha 6\beta 2^*$ subtype plays a more prominent role in modulating dopamine release probability at lower stimulus intensity (Exley et al., 2007; Meyer et al., 2008). In the present experiments release was done at 60% of maximum, while maximal stimulation was used in the aforementioned studies.

We subsequently assessed the effects of chronic nicotine treatment on nonburst and burst evoked dopamine release from striatal slices with and without α -CtxMII. α -CtxMII blocked ~50% of dopamine release evoked with single pulse stimulation, similar to our results in control slices. Unexpectedly, however, burst firing elicited a similar pattern of release as a single pulse. Receptor studies were done to elucidate the nAChR subtypes that may mediate these differential functional effects with chronic nicotine dosing. Administration of nicotine in the drinking water resulted in an upregulation of $\alpha 4\beta 2^*$ and downregulation of $\alpha 6\beta 2^*$ nAChRs in striatum of nicotine-treated rats. Thus, this mode of treatment to rats yielded results similar to those previously observed in mice (Khwaja et al., 2007; Lai et al., 2005).

We next investigated whether chronic nicotine treatment altered expression of the two major $\alpha\beta 2^*$ subtypes present in striatum, the $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs. The data from the rat competition studies suggested that there were differential effects of nicotine treatment on these two subtypes with a decline in the $\alpha 6\alpha 4\beta 2^*$ nAChR subtype. Such a result was much more readily evident from our receptor competition studies in $\alpha 4$ nAChR knockout mice which showed that nicotine treatment led to a loss of the $\alpha 6\alpha 4\beta 2^*$ nAChR subtype, but increased the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR subtype. These observations in an *in vivo* model are consistent with previous results using transfected cell culture systems, in which nicotine exposure upregulated either transfected $\alpha 4\beta 2$ or $\alpha 6(\text{non}\alpha 4)\beta 2$ nAChRs (Kuryatov et al., 2005; Sallette et al., 2005; Tumkosit et al., 2006; Walsh et al., 2008). The present findings in wildtype mice also show that nicotine increases $\alpha 4\beta 2^*$ nAChRs, while studies with the $\alpha 4$ knockout mice clearly demonstrate an increase in the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ population. A possible explanation for the decline in $\alpha 6\alpha 4\beta 2^*$ subtype may be that nicotine acts as a chaperone to more sensitively upregulate $\alpha 4^*$ AChRs compared to those containing the $\alpha 6^*$ nAChRs. This would result in a nicotine-induced increase in the $\alpha 4\beta 2^*$ subtype at the expense of $\alpha 6\alpha 4\beta 2^*$ subtype when both these receptors are present simultaneously. On the other hand, nicotine exposure would still result in the increase in $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs.

These findings have important implications for the functional changes observed with nicotine treatment. As mentioned earlier, $\alpha 6\beta 2^*$ nAChR-mediated dopamine release in both control and nicotine-treated animals followed a similar pattern with single pulse stimulation with ~50% decrease in release in the presence of α -CtxMII. Thus the increase in the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR subtype with nicotine treatment most likely compensated for the loss of the $\alpha 6\alpha 4\beta 2^*$ nAChR subtype. By contrast, the pattern of release with burst firing after $\alpha 6\beta 2^*$ nAChR blockade was distinct in control and nicotine-treated rats. One explanation for this difference is that the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ is partially responsible for alleviating the characteristic short-term depression in dopamine release. This could suggest that the $\alpha 6\alpha 4\beta 2^*$ nAChR subtype mediates the differential effects observed under control conditions since these receptors are not present after chronic nicotine treatment. Since not all receptors are necessarily functional, another possibility is that the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR subtype is not involved in mediating dopamine release at higher frequencies, resulting in a similar release with non-burst and burst-stimulated release in the nicotine animals. This might imply that $\alpha 6\alpha 4\beta 2^*$ nAChRs primarily modulate dopamine release with burst firing; however, since this receptor subtype is lost with nicotine treatment, the effect of $\alpha 6\beta 2^*$ nAChR blockade is no longer observed in nicotine-treated animals.

Our studies primarily focused on $\alpha 6\beta 2^*$ nAChR-mediated dopamine release with chronic nicotine treatment. However, the results also show that nicotine treatment increased evoked striatal dopamine release in the presence of the $\alpha 6\beta 2^*$ nAChR blocker α -CtxMII, suggesting that $\alpha 4\beta 2^*$ nAChR function was increased. These data contrast with earlier reports which identified no change in $\alpha 4\beta 2^*$ nAChR-mediated release with chronic nicotine treatment, a discrepancy that most likely relates to the different technique used to evaluate dopamine release, i.e. ^3H -dopamine release from synaptosomal preparations (Khwaja et al., 2007; Lai et al., 2005; Perry et al., 2007). Altogether these data suggest that alterations in both the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs populations contribute to the changes observed in nicotine-treated animals with burst firing.

A question that arises is the relationship between the different nAChR subtypes in the striatum. All $\alpha 6\beta 2^*$ nAChRs appear to be present on dopaminergic nerve terminals since they decline in parallel with dopaminergic measures after nigrostriatal damage (Quik et al., 2003; Quik et al., 2001). However, the $\alpha 6\alpha 4\beta 2^*$ subtype is preferentially lost with moderate nigrostriatal damage whereas the $\alpha 6(\text{non}\alpha 4)\beta 2^*$ subtype is decreased only with more severe dopaminergic damage suggesting that the two $\alpha 6\beta 2^*$ nAChR subtypes are present on different subsets of

dopaminergic neurons (Bordia et al., 2007). Evidence also indicates that the $\alpha 4\beta 2^*$ nAChR is present on yet another population of dopaminergic neurons since lesion studies show that they are decreased only with severe nigrostriatal damage (Kulak et al., 2002). As well, their distinct functional characteristics assessed using cyclic voltammetry suggest they are present on a unique set of dopaminergic neurons compared to the $\alpha 6\beta 2^*$ subtypes (Meyer et al., 2008).

Altogether these data underscore the complexity of nAChR expression and function in the striatum and the potential for a multifaceted regulation of dopaminergic function with chronic nicotine exposure. Moreover, they suggest that long-term nicotine treatment may differentially regulate nAChR subtype expression and function by virtue of their localization in dopaminergic neuron populations that possess distinct regulatory mechanisms.

Acknowledgments

The authors thank Iong Long Ip for excellent technical assistance, and Dr. John Drago for the breeder $\alpha 4(-/-)$ mice.

This work is supported by NIH grants NS42091 and NS47162 to MQ, DA03194 to SRG, DA12242 to SRG and JMM, MH53631 and GM48677 to JMM and grants from the California Tobacco Related Disease Research Program to XAP and MQ.

Abbreviations

nAChRs	nicotinic acetylcholine receptors
α -CtxMII	α -conotoxinMII
RTI-121	3 β -(4-iodophenyl)tropane-2 β -carboxylic acid
BSA	bovine serum albumin
*	the asterisk indicates the possible presence of other nicotinic subunits in the receptor complex

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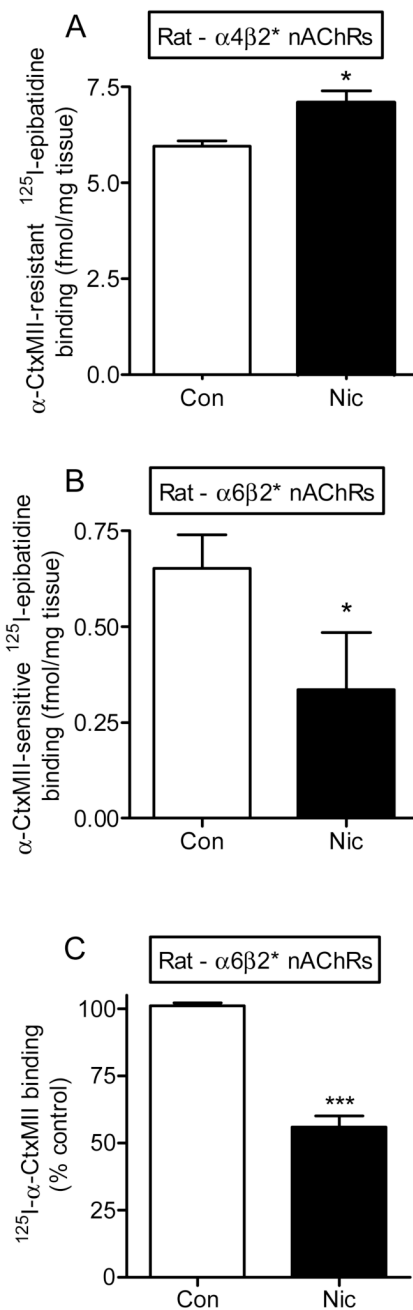


Fig. 1. Chronic nicotine treatment differentially regulates $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs in rat striatum. Rats were given nicotine in the drinking water for 14 days, after which receptor autoradiography was done using ^{125}I -epibatidine and ^{125}I - α -CtxMII. Changes in the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs were determined by measurement of ^{125}I -epibatidine binding in the absence and presence of α -CtxMII (300 nM), with an increase in $\alpha 4\beta 2^*$ nAChRs or α -CtxMII-resistant ^{125}I -epibatidine binding sites (A) and a decrease in $\alpha 6\beta 2^*$ nAChRs or α -CtxMII-sensitive ^{125}I -epibatidine binding sites (B). $\alpha 6\beta 2^*$ nAChRs were also measured using ^{125}I - α -CtxMII, with a significant decline with nicotine treatment (C). Data represent mean \pm SEM of 6-8 rats. Significance of difference from control using a t-test, * $p < 0.05$; *** $p < 0.001$.

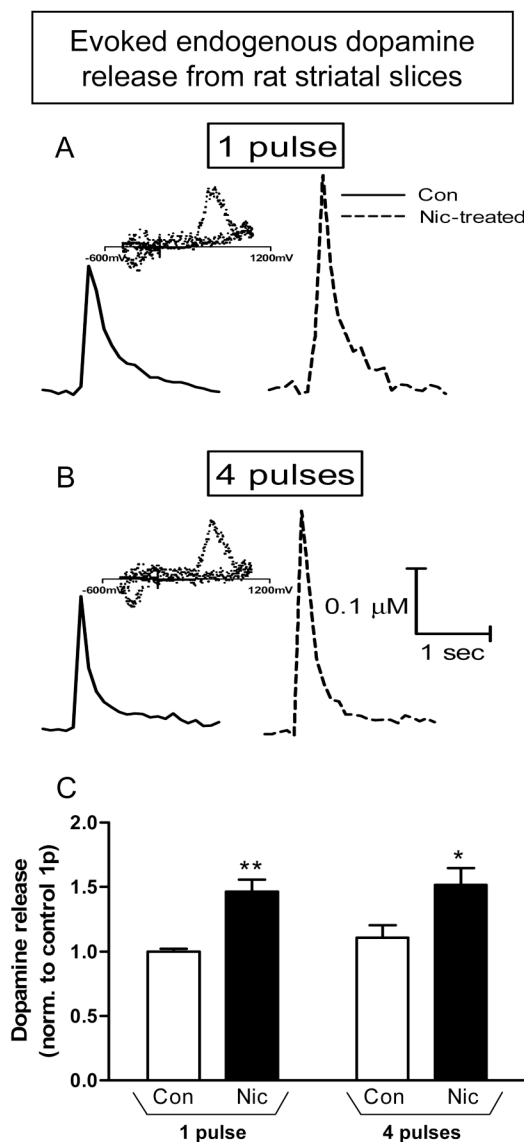


Fig. 2. Chronic nicotine treatment increases electrically-evoked dopamine release in rat striatum. Top and middle panels show representative traces of dopamine release in striatum of control (solid line) and nicotine-treated (dashed line) rats after 1 pulse and 4 pulse stimulation, respectively. Insets: typical voltammograms for dopamine with an oxidation peak at 500-600 mV and a reduction peak around -200 mV. Quantitative analyses of peak dopamine release (bottom panel) for each treatment group normalized to release by 1 pulse in controls. The values represent the mean \pm SEM of 4-6 rats (15-25 observations per animal). Significance of difference from control using a Bonferroni *post hoc* test after a 1 pulse stimulus, ** $p < 0.01$; after a 4 pulse stimulus, * $p < 0.05$.

Evoked endogenous dopamine release from rat striatal slices

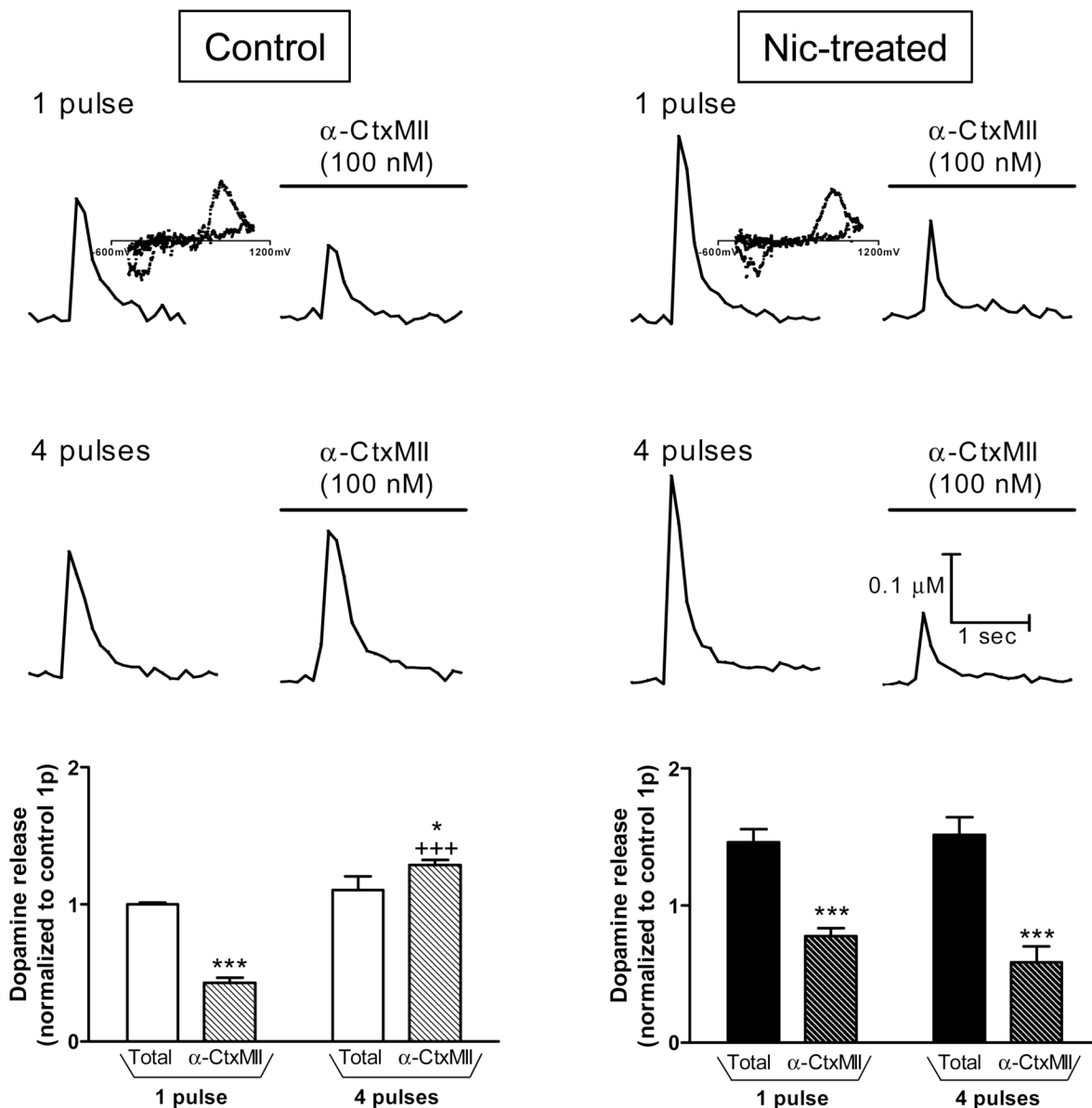


Fig. 3. Chronic nicotine treatment prevents the enhancement of burst-stimulated dopamine release after $\alpha 6\beta 2^*$ nAChR blockade. Representative traces for dopamine release after 1 pulse (top panel) and 4 pulse (middle panel) stimulation in the absence and presence of α -CtxMII for both control (left) and nicotine-treated rats (right). Insets: typical voltammograms for dopamine with oxidation peaks at 500-600 mV and reduction peaks around -200 mV. Quantitative analyses (bottom panel) of peak dopamine release normalized to control total release by 1 pulse in control (left) and nicotine-treated (right) rats induced by non-burst and burst stimulation before and after application of α -CtxMII. The values represent the mean \pm SEM of 6 rats (15-20 observations per animal). Significance of difference from total release using a Bonferroni *post*

hoc test, $*p < 0.05$; $***p < 0.001$. Significance of difference from release with 1 pulse stimulation in the presence of α -CtxMII using a Bonferroni *post hoc* test, $+++p < 0.001$.

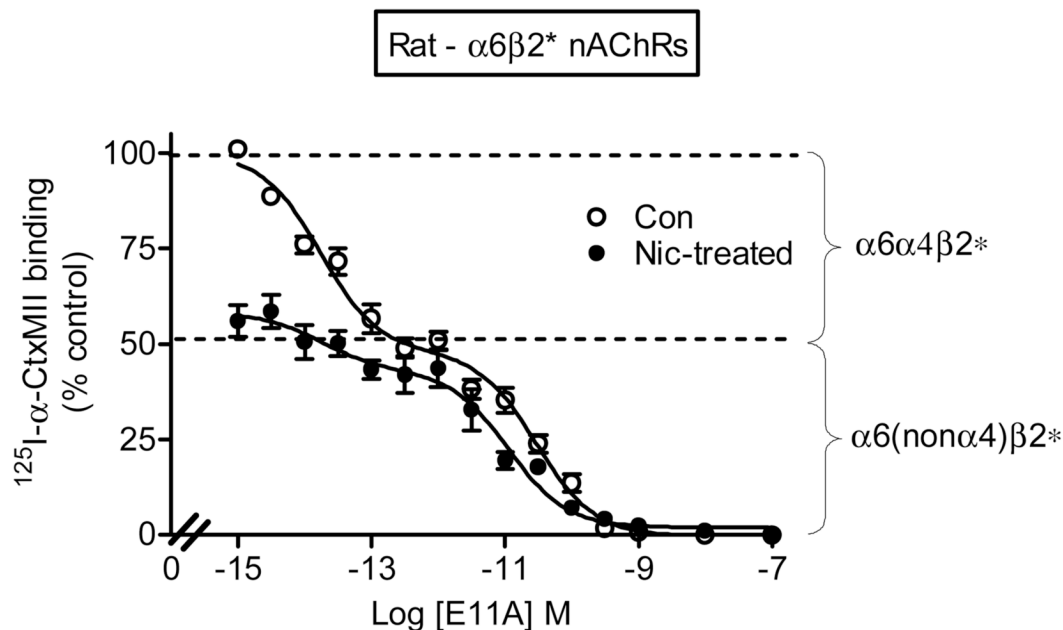


Fig. 4. Chronic nicotine treatment preferentially downregulates a subpopulation of $\alpha 6\beta 2^*$ nAChRs in rat striatum. To further characterize the effect of nicotine treatment on $\alpha 6\beta 2^*$ nAChRs, ^{125}I - α -CtxMII competition assays were done using varying concentrations (10^{-15} to 10^{-7} M) of the α -CtxMII analog E11A. A biphasic inhibition curve (data fit best to two site model) was obtained in control striatum indicating that E11A discriminates between at least two $\alpha 6\beta 2^*$ nAChRs, previously shown to represent the $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ subtypes. Competition analysis of E11A inhibition of ^{125}I - α -conotoxin MII binding in striatum of rats receiving nicotine showed a preferential decline in the very high affinity $\alpha 6\beta 2^*$ nAChR, that is, the $\alpha 6\alpha 4\beta 2^*$ subtype. Symbols represent mean \pm SEM of 6-8 rats. Where the SEM is not depicted, it fell within the symbol.

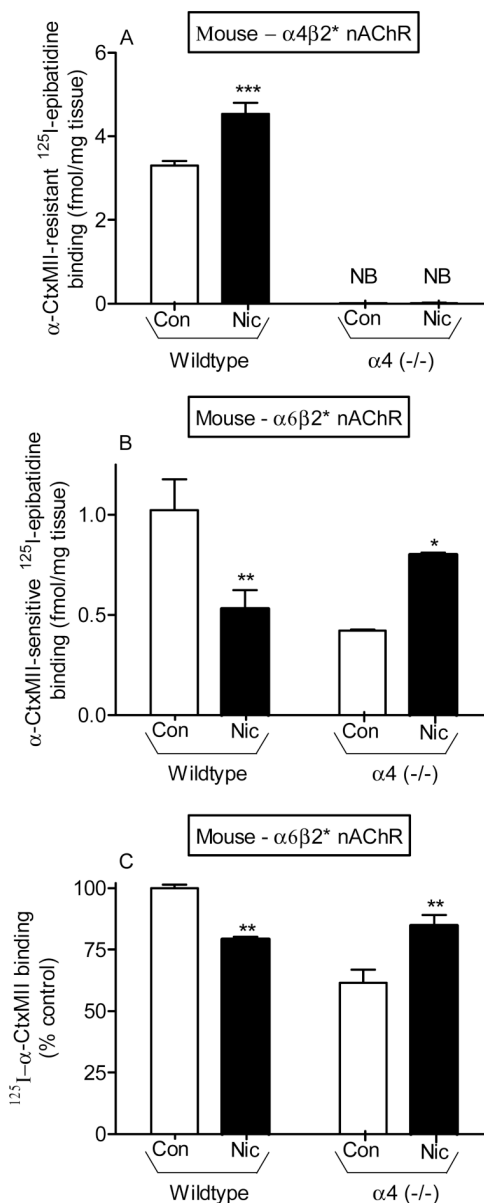


Fig. 5. Nicotine treatment differentially alters $\alpha 6\beta 2^*$ nAChRs in wildtype and $\alpha 4$ (-/-) nAChR mice. Mice were maintained at the final dose of nicotine in the drinking water for 14 days, after which 125 I-epibatidine binding was done in the absence and presence of 300 nM unlabeled α -CtxMII to identify α -CtxMII-resistant ($\alpha 4\beta 2^*$) and α -CtxMII-sensitive ($\alpha 6\beta 2^*$) binding sites. A, Striatal $\alpha 4\beta 2^*$ nAChRs in wildtype mice were significantly increased with nicotine treatment, with no binding in striatum of $\alpha 4$ (-/-) mice, as expected. B, $\alpha 6\beta 2^*$ nAChR binding was significantly decreased in $\alpha 4$ (-/-) mice and nicotine-treated wildtype mice. Unexpectedly, there was a significant increase in $\alpha 6\beta 2^*$ nAChR binding in striatum of $\alpha 4$ (-/-) mice receiving nicotine. Since $\alpha 4$ (-/-) mice do not express $\alpha 6\alpha 4\beta 2^*$ nAChRs, these data suggest there is a selective upregulation of $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs with nicotine treatment. C, For a direct measure of changes in $\alpha 6\beta 2^*$ nAChRs with nicotine treatment, 125 I- α -CtxMII binding was done using striatal sections from wildtype and $\alpha 4$ nAChR-null mutant mice. Again, nicotine

treatment decreased $\alpha 6\beta 2^*$ nAChRs in the wildtype mice. There was also a decrease in the $\alpha 4$ (-/-) mice due to a loss of the $\alpha 6\alpha 4\beta 2^*$ subtype. Again, however, there was a significant increase in $\alpha 6\beta 2^*$ binding in nicotine-treated $\alpha 4$ (-/-) mice, further supporting the idea that there is a selective upregulation of $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs with nicotine treatment. Data represent mean \pm SEM of 3 mice. Significance of difference from control using a Bonferroni *post hoc* test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. NB, indicates no binding.

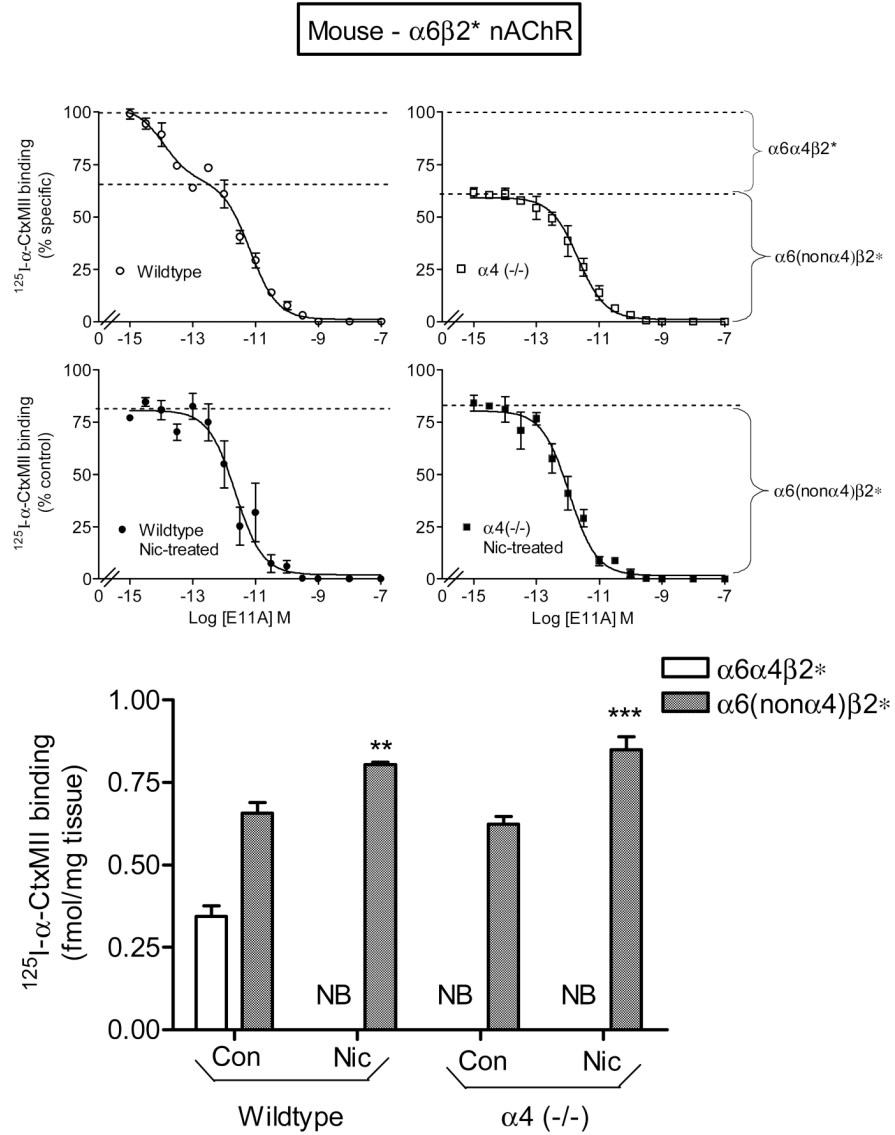


Fig. 6. Decrease in striatal $\alpha 6\alpha 4\beta 2^*$ nAChRs but increase in $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs with nicotine treatment. Wildtype and $\alpha 4$ nAChR-null mutant (-/-) mice were maintained at the final dose of nicotine in the drinking water for 14 days. Striatal $^{125}\text{I}-\alpha\text{-CtxMII}$ binding was subsequently determined in the presence and absence of varying concentrations of E11A (10^{-15} to 10^{-7} M). Two site binding curves were obtained in striatum from wildtype mice suggesting the presence of at least two $\alpha 6\beta 2^*$ nAChR populations. In striatum of $\alpha 4 (-/-)$ mice, E11A inhibited $^{125}\text{I}-\alpha\text{-CtxMII}$ binding in a monophasic manner, as expected since $\alpha 4 (-/-)$ mice do not express $\alpha 6\alpha 4\beta 2^*$ nAChRs. Competition analysis of E11A inhibition of $^{125}\text{I}-\alpha\text{-CtxMII}$ binding in nicotine-treated wildtype and $\alpha 4 (-/-)$ mice yielded similar monophasic curves suggesting the presence of only $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChRs. This site was preferentially increased with nicotine treatment in $\alpha 4 (-/-)$ mice, as depicted in the summary graph (lower panel). Values represent mean \pm SEM of 3 mice. Where the SEM is not depicted, it fell within the symbol. Significance of difference from respective non-treated control using a Bonferroni *post hoc* test, ** $p < 0.01$; *** $p < 0.001$. NB, indicates no binding.

Table 1

Chronic nicotine treatment modulates $\alpha 6\alpha 4\beta 2^*$ and $\alpha 6(\text{non}\alpha 4)\beta 2^*$ nAChR expression

Species	Genotype	Treatment	Preferred model (No. of sites)	E11A		Ratio (IC ₅₀ 2/IC ₅₀ 1)	Fraction of receptors (%)
				IC ₅₀ 1 (CI)	IC ₅₀ 2 (CI)		
Rat	NA	Control	Two	0.017 (0.014 - 0.021)	30 (25 - 35)	1765	51
		Nic-treated	Two	0.017 (0.009 - 0.034)	11 (9.2 - 13.6)	647	26
Mouse	Wildtype	Control	Two	0.011 (0.002 - 0.032)	6.5 (4.3 - 9.7)	590	34
		Nic-treated	One	-----	2.3 (1.5 - 2.9)	-----	-----
	$\alpha 4 (-/-)$	Control	One	-----	2.1 (1.3 - 3.9)	-----	-----
		Nic-treated	One	-----	1.1 (0.7 - 1.5)	-----	-----

125I- α -CtxMII competition studies were done using striatal sections from control and nicotine (nic)-treated rats or wildtype and $\alpha 4$ nAChR-null mutant mice at different concentrations of E11A. Biphasic inhibition by E11A (data best fit to a two-site model) was obtained in the striatum of both control rats and wildtype mice suggesting the presence of at least two $\alpha 6\beta 2^*$ nAChRs. In rats receiving nicotine, competition analysis of the data also yielded a biphasic curve; however there was a preferential decrease in the f1 fraction (f1:f2 = 26:74). In contrast, E11A competition analyses using striatum of non-nicotine-treated $\alpha 4$ nAChR-null mutant mice yielded monophasic curves suggesting a loss in the very high affinity binding site. Similar monophasic curves were obtained in mice (wildtype and $\alpha 4$ nAChR-null mutants) receiving nicotine. Each value represents the mean \pm SEM of 6-8 rats or 3 mice. The numbers in parentheses are the 95% confidence intervals (CI).