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**Chronic Nicotine Exposure Depresses Dopamine Release in Nonhuman
Primate Nucleus Accumbens**

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

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ABSTRACT

Tobacco use is a leading cause of preventable deaths worldwide. However, current smoking cessation therapies have very limited long-term success rates. Considerable research effort is therefore focused on identification of CNS changes with nicotine exposure as this may lead to more successful treatment options. Although recent work suggests that $\alpha6\beta2^*$ nAChRs play a dominant role in dopaminergic function in rodent nucleus accumbens, the effects of chronic nicotine exposure remain to be determined. Here, we used cyclic voltammetry to investigate $\alpha6\beta2^*$ nAChR-mediated release with chronic nicotine treatment in nonhuman primate nucleus accumbens shell. Control studies showed that nAChR-mediated dopamine release occurs predominantly through the $\alpha6\beta2^*$ receptor subtype. Unexpectedly, there was a complete loss of $\alpha6\beta2^*$ nAChR-mediated activity after several months of nicotine treatment. This decline in function was observed with both single and multiple pulse-stimulated dopamine release. Paired pulse studies showed that the facilitation of dopamine release with multiple pulsing observed in controls in the presence of nAChR antagonist was lost with chronic nicotine treatment. Nicotine-evoked ^3H -dopamine release from nucleus accumbens synaptosomes was similar in nicotine and vehicle-treated monkeys, indicating that long-term nicotine administration does not directly modify $\alpha6\beta2^*$ nAChR-mediated dopamine release. Dopamine uptake rates, as well as dopamine transporter and $\alpha6\beta2^*$ nAChRs levels were also not changed with nicotine administration. These data indicate that nicotine exposure, as occurs with smoking, has major effects on cellular mechanisms linked to $\alpha6\beta2^*$ nAChR-mediated dopamine release and that this receptor subtype may represent a novel therapeutic target for smoking cessation.

Introduction

Tobacco use constitutes an urgent worldwide health problem that kills about 5 million people annually, as reported by the World Health organization. Still, an estimated 1.3 billion people worldwide are smokers and this number is likely to increase without proper preventative and therapeutic strategies. It is well established that nicotine represents a component in tobacco smoke responsible for its addictive properties. Although nicotine modifies function in numerous brain regions, its rewarding properties are thought to be primarily linked to increased neurotransmission via nicotinic acetylcholine receptors (nAChRs) in the mesolimbic dopaminergic pathway (Exley and Cragg, 2008; Changeux, 2010; De Biasi and Dani, 2011).

Multiple reports implicate the $\alpha 4\beta 2^*$ nAChR subtype in the reinforcing effects of nicotine (Epping-Jordan et al., 1999; Walters et al., 2006; Picciotto et al., 2008). This includes studies showing that mice lacking the $\beta 2$ nAChR subunit do not self-administer nicotine and that dopaminergic responses to nicotine are decreased in $\alpha 4$ and $\beta 2$ nAChR null mice (Picciotto et al., 1998; Marubio et al., 2003). Additionally, activation of $\alpha 4^*$ nAChRs is sufficient for nicotine reward, tolerance and sensitization (Marubio et al., 2003; Tapper et al., 2004). More recent studies in human smokers also attribute the maintenance of smoking behavior to occupancy of $\alpha 4\beta 2^*$ nAChRs, which possibly prevents withdrawal symptoms (Brody et al., 2006). Importantly, nicotine self administration is reinstated in $\alpha 4$ and $\beta 2$ knockout mice after re-expression of these subunits in the ventral tegmental area (Pons et al., 2008).

$\alpha 6\beta 2^*$ nAChRs are also widely expressed in the mesolimbic dopaminergic pathway (Gotti et al., 2010). Their potential functional relevance is evident from a body of work showing that $\alpha 6\beta 2^*$ nAChRs modulate the majority (75-100%) of nAChR-mediated release in the nucleus accumbens and striatum of rodents and monkeys (McCallum et al., 2006; Exley et al., 2008;

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Perez et al., 2008; Perez et al., 2009; Exley et al., 2011). In addition, recent behavioral studies show that $\alpha6\beta2^*$ nAChRs play a critical role in nicotine conditioned place preference, self-administration and withdrawal (Pons et al., 2008; Jackson et al., 2009; Brunzell et al., 2010). Thus, the $\alpha6\beta2^*$ nAChR subtype may also play an important role in nicotine addiction.

The goal of this study was to determine the effect of chronic nicotine treatment on the $\alpha6\beta2^*$ nAChR-mediated regulation of dopamine release in nonhuman primates. We focused on the nucleus accumbens shell as this region is critical in nicotine-mediated reinforcement and reward, although other regions such as the caudate-putamen and accumbens core also contribute (Wise, 2009; Changeux, 2010). Dopamine release was determined using cyclic voltammetry in response to both a single pulse and a stimulus train, with the latter typically associated with reward (Schultz, 2002). The results show that $\alpha6\beta2^*$ nAChRs play a key role in the control of dopamine release in the accumbens shell in primates. In addition, they are the first to demonstrate that chronic nicotine exposure significantly decreases $\alpha6\beta2^*$ nAChR-mediated dopamine release in the accumbens shell.

Materials and Methods

Animal Treatment. Adult female and male squirrel monkeys (*Saimiri sciureus*) weighing 0.7-1.0 kg were purchased from Worldwide Primates (Miami, FL) and quarantined for one month according to California state regulations. Animals were housed separately in a room maintained at $27 \pm 3^\circ\text{C}$, with a 12:12-h light/dark cycle. Food (consisting of monkey chow fruits and vegetables) was given once daily, and water *ad libitum*. Animals were randomly divided into a vehicle-treated or a nicotine-treated group. The vehicle used was diluted Gatorade (50% in water), which was necessary to mask the bitter taste of nicotine. All animals were first given

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Gatorade in the drinking water for 3-4 d. Nicotine (free base) was then added to the Gatorade solution of the nicotine-treated group starting at a concentration of 50 $\mu\text{g/ml}$ and increased to a final concentration of 650 $\mu\text{g/ml}$ over 3 wk. Gatorade or Gatorade containing nicotine was also added to the food. One month after reaching the final nicotine concentration, blood was drawn from the femoral vein under ketamine anesthesia (15-20 mg/kg i.m.) and assayed for plasma cotinine using an ELISA kit (Orasure Technologies, Bethlehem, PA). All animals were maintained on Gatorade or nicotine (650 $\mu\text{g/ml}$) for 3-6 months until they were euthanized. There was no difference in dopamine release at the different treatment time points, and therefore the data was pooled.

Monkeys were euthanized according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. They were injected with 1.5 ml euthanasia solution intraperitoneally (390 mg sodium pentobarbital and 50 mg phenytoin sodium/ml), followed by 1.5 ml/kg of the same solution administered intravenously. All studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at SRI International.

Tissue Preparation. The brains were rapidly removed and rinsed in cold phosphate-buffered saline. They were placed in a squirrel monkey brain mold and cut into 2 mm-thick blocks using stainless steel blades. The slice at level A14.0-15.0 was bisected along the midline. The nucleus accumbens from one half of the brain slice was dissected for the measurement of synaptosomal ^3H -dopamine release or immediately frozen in isopentane on dry ice and stored at -80°C . These blocks were later used for preparation of 10 μm -thick sections 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 autoradiography.

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The slice from the other half of the brain was immediately placed in ice-cold, pre-oxygenated (95% O₂/5% CO₂) physiological buffer consisting of (in mM) 125 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 2.4 CaCl₂, 1.2 MgCl₂, 20 HEPES, 11 glucose, and 25 NaHCO₃, pH 7.4, for the voltammetry experiments. Striatal slices (400 μm thick) were prepared using a vibratome (Leica VT1000S) and incubated at room temperature for 2 h in oxygenated buffer. 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.

Cyclic Voltammetry. Carbon fiber microelectrodes (7 μm in diameter; tip length ~100 μm) were constructed as previously described (Perez et al., 2008). 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 μM dopamine. Dopamine release was measured as the maximal peak response obtained after electrical stimulation.

Electrically-evoked dopamine release was measured in the nucleus accumbens shell (Emmers and Akert, 1963). Electrical stimulation was applied using a bipolar stimulating electrode (Plastics One, Roanoke, VA) connected to a linear stimulus isolator (WPI, Sarasota, FL) and

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triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). The stimulating electrode was consistently placed on the dorsal half of the nucleus accumbens shell so that it just touched the surface of the slice and the carbon fiber electrode was positioned ~100 μm away. Evoked release was elicited by either a single electrical pulse applied every 2.5 min or a train of 2-4 pulses (2-4 ms in duration) at 30 or 100 Hz applied every 5 min as previously described (Perez et al., 2009). It was not feasible to assess a wider range of frequencies due to the limited amount of primate tissue available. Control evoked release was first 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 μM mecamylamine to block all nAChR subtypes. The effect of mecamylamine alone is similar to that which occurs with mecamylamine plus α -CtxMII (data not shown). Although mecamylamine interacts with NMDA receptors at high concentrations, this is most likely not a factor in our system since similar results were obtained with α -CtxMII, which is selective for $\alpha 6\beta 2^*$ nAChRs. Perfusion 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 mecamylamine were recorded over at least a 2 h period. The reported effects with each drug represent the average of those signals obtained once a stable maximal response was established.

For the paired-pulse analyses, release by one pulse was subtracted from that in response to two pulses to determine the amount of release by the second pulse at each specified frequency. Paired-pulse ratios were then calculated by dividing the amount of release stimulated with the second pulse by the amount of release stimulated with a single pulse.

³H-Dopamine release from striatal synaptosomes. A crude synaptosomal fraction was prepared as previously described (McCallum et al., 2006). Tissue was homogenized in 2 ml ice-

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cold 0.32 M sucrose buffered with HEPES (pH 7.5). A P1 pellet was obtained and the resuspended synaptosomes were incubated in 37°C uptake buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH₂PO₄, 3.2 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES, pH 7.5, 10 mM glucose, 25 mM NaHCO₃, 1 mM ascorbic acid and 0.01 mM pargyline) for 10 min before adding 100 nM ³H-dopamine (30-60 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA). Incubation with ³H-dopamine was done for 5 min, 80 µl aliquots of synaptosomes were then pipetted onto 5 mm diameter A/E glass-fiber filters mounted on polypropylene platforms. Synaptosomes were perfused for 10 min with perfusion buffer (uptake buffer plus 0.1% bovine serum albumin and 10 µM nomifensine) at a rate of 1 ml/min before fraction collection began. Release of ³H-dopamine from synaptosomes on each platform was evoked by an 18 sec exposure to one of a range of nicotine concentrations (0.03 µM-100 µM) or K⁺ (20 mM). α-CtxMII (50 nM) was perfused for 3 min before nicotine exposure to distinguish between α4β2* and α6β2* nAChR-mediated release. Twelve 18 sec fractions (including basal release before and after the stimulated release) were collected, and radioactivity determined by liquid scintillation counting.

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 ³H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) for several days. Nonspecific binding was assessed in the presence of 100 µM nicotine and was similar to the film blank.

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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 % BSA (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 ³H-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 hr 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 ³H-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nomifensine (100 μ M) was used to define nonspecific binding.

Data Analyses. ³H-dopamine release was plotted as counts per minute (cpm) versus fraction number using a curve-fitting algorithm of SigmaPlot 5.0 for DOS. Basal release was calculated

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by selecting fractions before and after the peak and plotting them as a single exponential decay function. Baseline release was subtracted from each fraction and fractions of release that were at least 10% higher than baseline were added to achieve total release units. Release units were normalized to the wet weight of the tissue sample on each filter.

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 ^3H standards. These were calibrated for ^{125}I autoradiography using previously described correction factors, including exposure time, section thickness and concentration of radioactivity (Artymyshyn et al., 1990). Care was taken to ensure that sample optical density readings were within the linear range.

All statistics were conducted using GraphPad Prism version 4.03 (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 $p \leq 0.05$ was considered significant. All values are expressed as the mean \pm SEM of the indicated number of animals. Values for each animal represent the average of 6-15 signals from 1-2 slices.

Results

$\alpha 6\beta 2^*$ nAChRs are primarily responsible for nAChR-mediated dopamine release in vehicle-treated monkey nucleus accumbens. Dopamine neurons normally fire in a tonic single-spike mode interspersed with transient burst activity associated with the presentation of reward-

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related stimuli (Schultz, 2002; Exley and Cragg, 2008). Voltammetry studies using rodent nucleus accumbens show that $\alpha 4(\text{non}\alpha 6)\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR stimulation affects dopamine release resulting from both types of firing (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Perez et al., 2009). Here we investigate the role of $\alpha 4(\text{non}\alpha 6)\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs in the regulation of dopamine release with a single pulse or a train of pulses in control nucleus accumbens shell slices of nonhuman primates. Fig. 1 (top) depicts representative traces of stimulated dopamine release done in the absence (control) and presence of the $\alpha 6\beta 2^*$ antagonist α -CtxMII (100 nM) or the general nAChR blocker mecamylamine (Mec, 100 μ M). Quantitative analyses of dopamine release shows that single pulse-stimulated dopamine release was decreased by ~30% in the presence of α -CtxMII ($p < 0.01$) with no further decline in the presence of mecamylamine. Although α -CtxMII binds both $\alpha 3\beta 2^*$ and $\alpha 6\beta 2^*$ nAChRs in monkey striatum, $\alpha 6\beta 2^*$ nAChRs form the greater majority of α -CtxMII binding sites with $\alpha 3\beta 2^*$ nAChRs representing a much smaller fraction (Quik et al., 2005). In addition, previous studies in rodents (Exley et al., 2008), which do not express striatal $\alpha 3^*$ nAChRs, yield similar results as those in monkeys. Thus, single-pulse stimulated dopamine release from control monkey nucleus accumbens shell appears primarily mediated by $\alpha 6\beta 2^*$ nAChRs, and possibly $\alpha 3\beta 2^*$ nAChRs, with very little if any contribution from $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs.

We also assessed dopamine release evoked by a train of pulses. Release was similar in the absence or presence of α -CtxMII or mecamylamine (Fig. 1 bottom), in agreement with previous studies in rodent nucleus accumbens (Zhang and Sulzer, 2004; Meyer et al., 2008; Perez et al., 2009; Zhang et al., 2009). This apparent lack of effect of nAChR blockade with a stimulus train is thought to be due to the ability of blockers to enhance the response of dopamine terminals to

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burst stimuli. In the absence of antagonists, endogenous acetylcholine promotes initial dopamine release by stimulating nAChRs (Zhou et al., 2001; Rice and Cragg, 2004; Zhang and Sulzer, 2004). However, with burst stimulation, the initial high probability of dopamine release limits release with subsequent pulses, a phenomenon known as short-term depression (Rice and Cragg, 2004; Zhang and Sulzer, 2004). In the presence of nAChR antagonists, dopamine release is partially inhibited so that the first pulse induces a smaller amount of dopamine release due to receptor blockade, thus relieving short-term depression. Subsequent pulses within a train of pulses are then capable of eliciting more dopamine release such that overall release with high stimulation frequencies is similar in the absence and presence of antagonists.

In summary, our data show the $\alpha 6\beta 2^*$ nAChR subtype dominates in the control of nAChR-mediated dopamine release in the nucleus accumbens of nonhuman primates.

Chronic nicotine treatment depresses dopamine release in nucleus accumbens shell.

To date, voltammetry studies have focused on the effect of acute nicotine superfusion on dopamine release in tissue slices (Rice and Cragg, 2004; Zhang and Sulzer, 2004; Exley et al., 2008; Zhang et al., 2009). However, nicotine dependence is a chronic condition that leads to additional long-term modifications in the cholinergic, dopaminergic and other systems. Therefore, we next did a series of experiments to investigate the effect of chronic nicotine exposure on dopamine release in nucleus accumbens shell. For these studies, monkeys were given drinking water containing 50% Gatorade alone or 50% Gatorade plus nicotine for 3-6 months. The final nicotine dose resulted in plasma cotinine levels similar to those in smokers (Table 1) (Matta et al., 2007). The monkeys were then euthanized and dopamine release measured in response to a single-pulse or a train of pulses delivered at 30 or 100 Hz.

Chronic nicotine treatment greatly decreased ($p < 0.001$) evoked dopamine release in nucleus

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accumbens shell in response to both single and multiple pulse stimulation by ~70% (Fig. 2). Dopamine release significantly increased in a frequency-dependent manner for both vehicle-treated ($p < 0.001$) and nicotine-treated ($p < 0.05$) animals.

We then evaluated the effect of chronic nicotine treatment on $\alpha 6\beta 2^*$ and $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR-modulated dopamine release. To approach this, we again tested release in the absence (control) and presence of the $\alpha 6\beta 2^*$ antagonist α -CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Chronic nicotine treatment resulted in a loss of the α -CtxMII and mecamylamine-induced decrease in single-pulse stimulated dopamine release and also attenuated non-nAChR-regulated release (Fig. 3 top panel). Experiments were next done at higher stimulation frequencies, that is, 4 pulses at 30 Hz or 100 Hz (Fig. 3 middle and bottom panels). Chronic nicotine treatment led to dramatic declines in dopamine release evoked by a train of pulses in both the absence and presence of the nAChR blockers.

The present findings thus show that chronic nicotine treatment depressed $\alpha 6\beta 2^*$ nAChR-mediated dopamine release in nucleus accumbens shell, as well as release regulated by non-nAChR-mediated mechanisms.

Chronic nicotine treatment prevents the enhanced paired-pulse ratios observed with nAChR blockade in nucleus accumbens. As an approach to further understand how chronic nicotine treatment modifies nAChR-mediated dopamine release, paired-pulse stimulation studies were done. Paired-pulse ratios, defined as the response of a second stimulus within a train divided by that of the first, provide a measure of dopamine release probability. For instance, interventions that decrease the probability of release with the first pulse but facilitate release by a second pulse within a train of pulses will result in enhanced paired-pulse ratios. Such studies have proved useful in understanding the mechanisms underlying dopamine release with burst firing in

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the absence and presence of nAChR antagonists (Rice and Cragg, 2004; Zhang and Sulzer, 2004).

Representative traces of dopamine release elicited by 1 or 2 pulses at 100 Hz for vehicle-treated and nicotine-treated animals are shown in Fig. 4. Quantitative analyses showed that paired-pulse ratios under control conditions were similar in the vehicle-treated and chronic nicotine-treated groups (Fig. 4). NACHR blockade with α -CtxMII or mecamylamine enhanced paired-pulse ratios in vehicle-treated nucleus accumbens with 100 Hz stimulations (Fig. 4). By contrast, there was no increase in paired-pulse ratios in the nicotine-treated animals (Fig. 4). Similar results were observed with the lower stimulation frequency of 30 Hz (data not shown). Thus, chronic nicotine treatment blocks the facilitation of high frequency stimulated dopamine release that is observed with nAChR blockade in vehicle-treated nucleus accumbens.

Synaptosomal nAChR-mediated ^3H -dopamine release is not altered after chronic nicotine treatment. Another approach commonly used to investigate dopaminergic function is nicotine-evoked ^3H -dopamine release from synaptosomes (Grady et al., 2007). This technique provides a direct measure of nAChR-mediated release, since it involves an isolated nerve terminal preparation. Release was measured in the absence and presence of α -CtxMII, to distinguish between the $\alpha 4(\text{non}\alpha 6)\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR-mediated components. In vehicle treated animals (Fig. 5), the proportion of release regulated by $\alpha 6\beta 2^*$ nAChRs (~80%) was greater than that regulated by $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs (~20%), similar to previous findings using the entire accumbens (McCallum et al., 2006). Somewhat unexpectedly, there was no significant difference in $\alpha 6\beta 2^*$ or $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR-evoked ^3H -dopamine release from nucleus accumbens shell synaptosomes of vehicle-treated and nicotine-treated monkeys (Fig. 5). Release was done at varying nicotine concentrations to test the possibility that there may be differential

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effects of chronic nicotine dosing at high or low affinity nAChRs. There were no significant differences in the EC₅₀ values for either $\alpha 4(\text{non}\alpha 6)\beta 2^*$ or $\alpha 6\beta 2^*$ -nAChR stimulated dopamine release in the nicotine- compared to vehicle-treated animals. The EC₅₀ values were 2.56 ± 1.40 μM (nicotine-treated) and 1.86 ± 0.29 μM (vehicle-treated) for $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR-mediated release, and 1.15 ± 0.45 μM (nicotine-treated) and 0.93 ± 0.13 μM (vehicle-treated) for $\alpha 6\beta 2^*$ nAChR-mediated release. These data indicate that chronic nicotine treatment does not directly affect mechanisms involved in the regulation of dopamine release by nAChRs.

We also examined the effect of chronic nicotine treatment on K^+ -evoked ^3H -dopamine release from synaptosomes to allow for a clearer comparison between the results from the voltammetry and synaptosomal preparations. K^+ -evoked release from synaptosomes may more closely resemble the electrically-evoked dopamine release used in voltammetry. As shown in Fig. 5, no significant difference in K^+ -stimulated (20 mM) release was observed between the vehicle-treated (49.7 ± 4.16 release units) and nicotine-treated (58.0 ± 5.72 release units) group, in agreement with previous studies (McCallum et al., 2006). These results further support the idea that extended nicotine exposure does not affect the overall exocytotic mechanism.

Chronic nicotine treatment does not affect dopamine transport. 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 dopamine transport. No differences in dopamine transporter densities were found between vehicle- and nicotine-treated animals (Table 2).

Experiments were also done to determine if chronic nicotine treatment modified dopamine release uptake constants in the voltammetric studies. The decaying portion of each dopamine peak was fitted to a one-phase exponential decay at points where the amount of release matched

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in vehicle- and nicotine-treated animals. Chronic nicotine treatment did not modify dopamine uptake rates (Table 2). Similarly, neither α -CtxMII, mecamylamine or frequency of stimulation had a significant effect on uptake rates in either treatment group (data not shown). These data indicate that the changes in dopamine release observed with chronic nicotine treatment arise from changes in the amount of release.

Chronic nicotine treatment increases $\alpha 4\beta 2^*$ nAChRs but does not affect $\alpha 6\beta 2^*$ nAChR expression. Experiments were performed to determine the effect of chronic nicotine treatment on nAChR binding sites. To identify $\alpha 4\beta 2^*$ nAChRs, binding of ^{125}I -epibatidine was done in the presence of α -CtxMII using autoradiography (Fig. 6). Significant increases in $\alpha 4\beta 2^*$ nAChRs ($p < 0.05$) in the nucleus accumbens shell were observed in the nicotine-treated animals (Table 3). $\alpha 6\beta 2^*$ nAChRs, identified using ^{125}I - α -CtxMII were not affected with nicotine treatment (Table 3). Thus, the decline in nAChR-mediated DA release with chronic nicotine dosing is not due to declines in nAChRs.

Discussion

The major nAChRs present in the rodent and nonhuman primate nucleus accumbens are the $\alpha 6\beta 2^*$ and $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs, with little expression of $\alpha 7$ nAChRs (Gotti et al., 2010). Previous voltammetric studies in rodents showed that $\alpha 6\beta 2^*$ nAChRs primarily regulate the effects of nicotine on dopamine release in the nucleus accumbens, with little contribution from $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs (Exley et al., 2008). In vehicle-treated monkeys, the selective $\alpha 6\beta 2^*$ nAChR antagonist α -CtxMII was as effective as the general nAChR antagonist mecamylamine in reducing single-pulse-evoked dopamine release from nucleus accumbens shell. In addition, release evoked by train stimuli was also similar in the presence of α -CtxMII or mecamylamine.

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These observations indicate that $\alpha 6\beta 2^*$ receptors are also the major contributors to nAChR-evoked dopamine release in nucleus accumbens shell of nonhuman primates, a model that offers the advantage that it more closely resembles humans at a genetic, neuroanatomical and neurochemical level.

We next investigated the effect of chronic nicotine exposure on $\alpha 6\beta 2^*$ nAChR-modulated dopamine release. Monkeys were chronically exposed to nicotine for several months via the drinking water. This mode of administration was selected because it is not invasive or stressful, and visual observation indicates that nicotine intake was intermittent. The dose was titrated to yield plasma cotinine levels similar to those with smoking (Matta et al., 2007). The results show that chronic nicotine dosing dramatically reduced nAChR-mediated dopamine release stimulated by both a single-pulse and a train of pulses.

To investigate the mechanism(s) whereby chronic nicotine dosing reduced burst-induced dopamine release, paired-pulse experiments were done. Such studies help identify whether chronic nicotine dosing affects the enhanced dopamine release that normally occurs with a second pulse in the presence of nAChR blockade or desensitization (Rice and Cragg, 2004; Zhang and Sulzer, 2004). The results show that facilitation induced by nAChR antagonists is disrupted with chronic nicotine dosing. One explanation for these findings is that endogenous acetylcholine levels are decreased with long-term nicotine treatment, which might be expected to decrease electrically-evoked dopamine release as observed in the present study. Indeed, variations in acetylcholine are known to influence single and burst stimulated dopamine release (Zhou et al., 2001; Exley et al., 2008). Alternately, prolonged nAChR desensitization with chronic nicotine exposure may modify other inputs and/or signaling mechanisms. For instance, changes in the electrical threshold for stimulation may contribute to an altered response of

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dopamine terminals to distinct firing patterns. Further experimentation is required to better understand the mechanisms by which long term nicotine depresses dopamine release.

As an approach to determine if the reduced neuronal responsiveness with chronic nicotine treatment was due to a direct effect on nAChR-mediated dopamine release, we measured nicotine-evoked ^3H -dopamine release from nucleus accumbens shell synaptosomes. NACHR-mediated release was similar in vehicle- and nicotine-treated monkeys, indicating that changes in endogenously evoked dopamine release measured with voltammetry are not due to effects on the nAChR-linked dopamine release mechanism. These data suggest that the depression of dopamine release in accumbens slices assessed using cyclic voltammetry is not due to functional alterations in the nAChR-mediated release machinery but rather involves nAChR-mediated changes in neuronal circuitry that are only evident in a slice preparation. As a further approach to investigate this idea, we measured K^+ -evoked ^3H -dopamine release from striatal synaptosomes, which may more closely mimic electrical stimulation in slices. In this case, one would predict similar changes in the two preparations, that is, a decrease in dopamine release since the mechanism by which release is ultimately controlled under both conditions is depolarization of presynaptic terminals. However, K^+ -evoked ^3H -dopamine release from striatal synaptosomes was similar in vehicle-treated animals as compared to monkeys on long term nicotine treatment. These results may imply that synaptosomes are not fully representative of the mechanism that control release in intact presynaptic terminals.

In the present study, nicotine treatment was maintained until the time of death. However, it appears unlikely that residual nicotine in the tissue is responsible for the decreased dopamine release in the nicotine-treated animals for several reasons. First, the accumbens slices are superfused for ≥ 4 hours before initiation of the release studies, which should allow adequate time

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to wash out residual nicotine. Second, studies in nicotine-treated rats show that residual nicotine in the brain does not interfere with nAChR binding or function (Nguyen et al., 2004; Matta et al., 2007). Residual nicotine might decrease nAChR binding in accumbens of nicotine-treated animals. However, receptor autoradiography showed that $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs are upregulated, with no change in $\alpha 6\beta 2^*$ nAChRs. These latter data are similar to previous findings in chronically treated monkeys in which nicotine had been withdrawn for 18-24 hours (McCallum et al., 2006). Finally, if residual nicotine were present in the slices, we would observe decreases in low-frequency-stimulated release accompanied by increases in the relative dopamine release in response to burst-like stimulation (Rice and Cragg, 2004; Zhang and Sulzer, 2004), which contrasts with the results in the nicotine-treated animals.

The effects of chronic nicotine treatment on dopamine release in the primate nucleus accumbens are distinct from our previous observations in monkey putamen (Perez et al., 2008; Perez et al., 2009). In vehicle-treated monkey putamen, $\alpha 6\beta 2^*$ nAChRs regulate 80-100% of single pulse nAChR-modulated dopamine release and $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs the remainder. However, nicotine treatment did not reduce overall or nAChR-mediated dopamine release in monkey putamen as it did in nucleus accumbens. Instead, it modified the pattern of high frequency stimulated dopamine release in the presence of nAChR antagonists (Perez et al., 2009). These combined findings highlight the distinct roles that the striatum and nucleus accumbens may play in nicotine dependence.

One explanation for these varying effects of nicotine in the nucleus accumbens versus striatum may relate to differential expression of $\alpha 6\beta 2^*$ and $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR subtypes in these brain regions. Immunoprecipitation studies show that there are different proportions of $\alpha 6\alpha 4\beta 2\beta 3$ and $\alpha 6\beta 2\beta 3$ in rat dorsal versus ventral striatum (Gotti et al., 2010). The presence of

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distinct nAChR subtypes may contribute to the unique regulatory mechanisms and functional roles of the accumbens compared to the striatum (McCallum et al., 2006; Gotti et al., 2010). For instance, previous work has shown that $\alpha 6\alpha 4\beta 2\beta 3$ and $\alpha 6\beta 2\beta 3$ receptors are uniquely regulated by nicotine treatment, with a decline in the former and increase in the latter subtype (Perez et al., 2008). Functional modifications may be linked to changes in expression of only one or other $\alpha 6\beta 2^*$ nAChR subtype. In the nucleus accumbens this may relate to changes in $\alpha 6\alpha 4\beta 2\beta 3$ nAChRs as these seem to be the primary receptor population regulating dopamine release in this brain region (Exley et al., 2012).

Somewhat unexpectedly, chronic nicotine treatment also decreased non-nAChR-mediated dopamine release. One explanation for this finding is that chronic nicotine induces long-term changes in the mesocorticolimbic pathway that alter the response of dopaminergic terminals in the nucleus accumbens to an electrical stimulus. In fact, nicotine treatment upregulates mesolimbic $\alpha 4\beta 2^*$ nAChRs on GABAergic neurons in the ventral tegmental area, which leads to an increase in their firing and a decline in dopaminergic activity (Nashmi et al., 2007). Additionally, chronic nicotine may desensitize $\alpha 6\beta 2^*$ and/or $\alpha 4\beta 2^*$ nAChRs on GABAergic neurons in the ventral tegmental area to enhance mesolimbic glutamatergic transmission (Mansvelder et al., 2002; Nashmi et al., 2007; Yang et al., 2011). This altered GABAergic and glutamatergic activity in the ventral tegmental area may lead to downstream changes that depress dopaminergic activity in the nucleus (Thomas et al., 2000). Further evidence for a depressive effect of prolonged nicotine exposure stems from studies showing that chronic nicotine treatment attenuates the threshold for long term depression in monkey striatal slices (Quik et al., 2006). Chronic nicotine dosing may also enhance D2 inhibition of dopaminergic function either directly or by decreasing activity of cholinergic interneurons. Evidence for this possibility stems from

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recent studies showing increases in D2 dopamine receptors with nicotine treatment (Novak et al., 2010). Finally, the observed decline in release observed with chronic nicotine exposure is unlikely due to a decrease in dopamine content, as our previous studies showed no change in dopamine and metabolite levels in nucleus accumbens of nicotine-treated animals as compared to controls (McCallum et al., 2006).

Voltammetry studies have previously assessed the effects of nicotine withdrawal on dopamine release. These showed that there was a decline in both low and high frequency-stimulated dopamine release one day after nicotine removal, as well as a decrease in nAChR-modulated release (Zhang et al., 2011). Our data suggest that the decrease in release observed with withdrawal may also be related to prolonged nicotine use.

A final question that arises concerns the implications of depressed dopamine release for nicotine addiction. One hypothesis is that nAChR upregulation and inactivation underlie nicotine dependence (Brody et al., 2006). Continued smoking is driven by the urge to avoid withdrawal symptoms by maintaining receptor occupancy. Numerous studies have demonstrated a role for $\alpha 4\beta 2^*$ nAChRs, with more recent work also indicating an involvement of $\alpha 3$, $\beta 4$ and/or $\alpha 5$ containing nAChRs (De Biasi and Dani, 2011). Our results show that $\alpha 6\beta 2^*$ nAChRs in the primate nucleus accumbens play a key role in the regulation of the dopamine system, consistent with previous behavioral studies in rodents (Pons et al., 2008; Jackson et al., 2009; Brunzell et al., 2010; Gotti et al., 2010). These combined observations suggest that $\alpha 6\beta 2^*$ nAChRs are also involved in nicotine dependence and withdrawal. Thus, drugs targeting $\alpha 6\beta 2^*$ nAChRs may be useful for smoking cessation.

In summary, the present results are the first to show that the regulation of dopamine release by nAChRs in the nucleus accumbens shell of nonhuman primates occurs primarily through

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$\alpha\beta 2^*$ nAChRs. Moreover, they demonstrate that chronic nicotine treatment significantly decreased dopamine release stimulated by both a single-pulse and a train of pulses. These findings indicate that chronic nicotine exposure has major depressant effects on dopamine release in nonhuman primate nucleus accumbens, and that $\alpha\beta 2^*$ nAChRs play a critical role.

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Authorship Contributions

Participated in research design: Perez, Quik.

Conducted experiments: Ly, Perez

Contributed new agents or analytical tools: McIntosh

Performed data analysis: Ly, Perez

Wrote or contributed to the writing of the manuscript: Perez, Quik

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Footnotes

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Figure legends

Fig. 1. Single pulse- and burst-stimulated endogenous dopamine release from vehicle-treated monkey nucleus accumbens is mediated by $\alpha 6\beta 2^*$, and not $\alpha 4\beta 2^*$, nAChRs. *Left panels:* Representative traces of dopamine release determined in the absence (control) and presence of the $\alpha 6\beta 2^*$ antagonist α -CtxMII (100 nM), or the general nAChR blocker mecamylamine (100 μ M). Scale bar represents 10 nM and 1 s. *Right panels:* Quantitative analyses of dopamine release show that both α -CtxMII and mecamylamine decrease single-pulse stimulated dopamine release to the same extent. Dopamine release stimulated via a train of four pulses at 100 Hz was significantly greater than that stimulated by a single pulse and was unaffected by either antagonist. These data indicate that 100% of nAChR-modulated dopamine release in vehicle-treated nucleus accumbens occurs through $\alpha 6\beta 2^*$ nAChRs. The values represent the mean \pm SEM of 6-7 monkeys. Significance of difference from control release using a Newman Keuls *post hoc* test, * $p < 0.05$; ** $p < 0.01$.

Fig. 2. Chronic nicotine treatment decreases low and high frequency stimulated dopamine release in monkey nucleus accumbens. Nicotine (free base) was added to Gatorade containing drinking water starting at 50 μ g/ml and increased to a concentration of 650 μ g/ml over a 4 wk period. Animals were maintained at this final nicotine dose for 3-6 months until they were euthanized. *Top panels:* Representative traces of dopamine release evoked by either a single pulse or a train of four pulses applied at 30 or 100 Hz. Scale bar represents 10 nM and 1 s. *Bottom panels:* dopamine release was significantly decreased with chronic nicotine treatment regardless of the stimulation frequency. The values represent the mean \pm SEM of 6-8 monkeys.

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Significance of difference from vehicle-treated animals using a Student's t-test, *** $p < 0.001$.

Fig. 3. Chronic nicotine treatment abolishes the effect of $\alpha 6\beta 2^*$ nAChR antagonism and decreases non-nAChR-mediated dopamine release in nucleus accumbens. *Top panel:* Dopamine release in accumbal slices was determined in the absence (control) and presence of the $\alpha 6\beta 2^*$ antagonist α -CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μ M). Quantitative analyses of dopamine release show that nAChR-modulated dopamine release occurs through $\alpha 6\beta 2^*$ nAChRs in vehicle-treated nucleus accumbens. Chronic nicotine treatment significantly decreased control single-pulse stimulated dopamine release in monkey nucleus accumbens. In addition, single pulse-stimulated dopamine release is not decreased in the presence of either nAChR antagonists with nicotine treatment, suggesting that nAChRs no longer influence dopamine neurotransmission. *Bottom two panels:* Release was determined after a train of four pulses at 30Hz (second panel) or 100Hz (third panel). Quantitative analyses show that nAChR blockade does not significantly affect high frequency stimulated release in either treatment group at the higher stimulation frequencies. The values represent the mean \pm SEM of 6-7 monkeys. Significance of difference from control release using a Bonferroni comparison *post hoc* test, *** $p < 0.001$. Significance of difference from vehicle-treated under each given condition using a Bonferroni comparison *post hoc* test, +++ $p < 0.001$.

Fig. 4. Exposure to nAChR antagonists does not enhance paired-pulse ratios in the nucleus accumbens of nic-treated monkeys. Dopamine release was stimulated by one (1p) or two (2p) pulses at 100Hz in the absence (control) and presence of the $\alpha 6\beta 2^*$ antagonist α -CtxMII (100 nM), the general nAChR blocker mecamylamine (100 μ M). *Top two panels:* Representative

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traces of dopamine release. Release stimulated by one pulse was subtracted from that evoked by two pulses to determine release by the second pulse (p2). Paired-pulse ratios were calculated by dividing release stimulated with the second pulse by that stimulated with a single pulse. Scale bar represents 10 nM and 1 s. *Bottom panel:* Quantitative analyses show that there is a significant increase in paired-pulse ratios in vehicle-treated but not nic-treated monkeys in the presence of nAChR antagonists. The values represent the mean \pm SEM of 3-5 monkeys. Significance of difference using a Bonferroni comparison *post hoc* test from control release, ** $p < 0.01$; *** $p < 0.001$; from vehicle-treated, ⁺ $p < 0.05$.

Fig. 5. ³H-Dopamine release from monkey nucleus accumbens synaptosomes is not altered with chronic nicotine treatment. Dose-response curves for nicotine-evoked ³H-dopamine (DA) release were determined in the presence and absence of α -CtxMII (50 nM) to differentiate the components of release mediated by the $\alpha 4\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR populations. Release remaining in the presence of α -CtxMII was defined as release mediated by $\alpha 4\beta 2^*$ nAChRs, with the $\alpha 6\beta 2^*$ nAChR-mediated component determined by subtraction of the $\alpha 4\beta 2^*$ component from total release. K⁺-stimulated ³H-dopamine release was done in the presence of 20 mM K⁺. The values represent the mean \pm SEM of 3-5 monkeys.

Fig. 6. Autoradiograms depicting the effect of chronic nicotine treatment on $\alpha 4(\text{non}\alpha 6)\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR expression. ¹²⁵I-Epipatidine binding in the presence of α -CtxMII was done to determine $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs, while ¹²⁵I- α -CtxMII binding was used as a measure of $\alpha 6\beta 2^*$ nAChR levels. Chronic nicotine treatment increased $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChRs, with no changes in $\alpha 6\beta 2^*$ nAChRs. Non-specific binding (Blank) was done in the presence of 100 μ M nicotine.

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AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cd, caudate; Cx. Cortex; Put, putamen.

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TABLE 1

Plasma cotinine levels in monkeys receiving chronic nicotine treatment in their drinking water
Animals were given nicotine in their drinking water containing Gatorade to mask the bitter taste of nicotine. Nicotine was administered starting at a low dose of 50 µg/ml and gradually increased to a final concentration of 650 µg/ml. Blood was collected to measure plasma cotinine levels. The plasma cotinine levels were similar to that in smokers. Values are mean ± SEM of the indicated number of animals.

No. of animals	Nicotine dose (µg/ml)	Cotinine level (ng/ml)
10	0	0
10	650	455 ± 57.5

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TABLE 2

Chronic nicotine treatment does not affect dopamine transport in nucleus accumbens shell

Dopamine transporter densities were determined using ^{125}I -RTI-121 autoradiography as described in Materials and Methods. To calculate dopamine uptake rate, constants signals from the voltammetry experiments were fit to a one-phase exponential decay. Care was taken to fit portions of the decaying phase of each dopamine signal where the amount of release matched in both treatment groups. Each value represents the mean \pm SEM of 6-10 animals per group.

Treatment group	Dopamine transporter (fmol/mg tissue)	Dopamine uptake rate constant (s^{-1})
Vehicle-treated	12.8 ± 1.52	4.2 ± 0.22
Nic-treated	11.9 ± 1.53	4.7 ± 0.53

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TABLE 3

Chronic nicotine treatment increases $\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR expression but does not affect $\alpha 6\beta 2^*$ nAChR density in nucleus accumbens shell

^{125}I -Epibatidine in the presence of α -CtxMII and ^{125}I - α -CtxMII binding assays were done to determine $\alpha 4(\text{non}\alpha 6)\beta 2^*$ and $\alpha 6\beta 2^*$ nAChR expression, respectively, as described in Materials and Methods. Each value represents the mean \pm SEM of 10 animals per group.

nAChR subtype	Binding (fmol/mg)		% Vehicle-treated
	Vehicle-treated	Nicotine-treated	
$\alpha 4(\text{non}\alpha 6)\beta 2^*$ nAChR	2.82 \pm 0.29	3.75 \pm 0.32*	132 \pm 9.09*
$\alpha 6\beta 2^*$ nAChR	0.81 \pm 0.10	0.90 \pm 0.13	111 \pm 15.5

Significance of difference from vehicle-treated using a t-test: * $p < 0.05$.

Figure 1

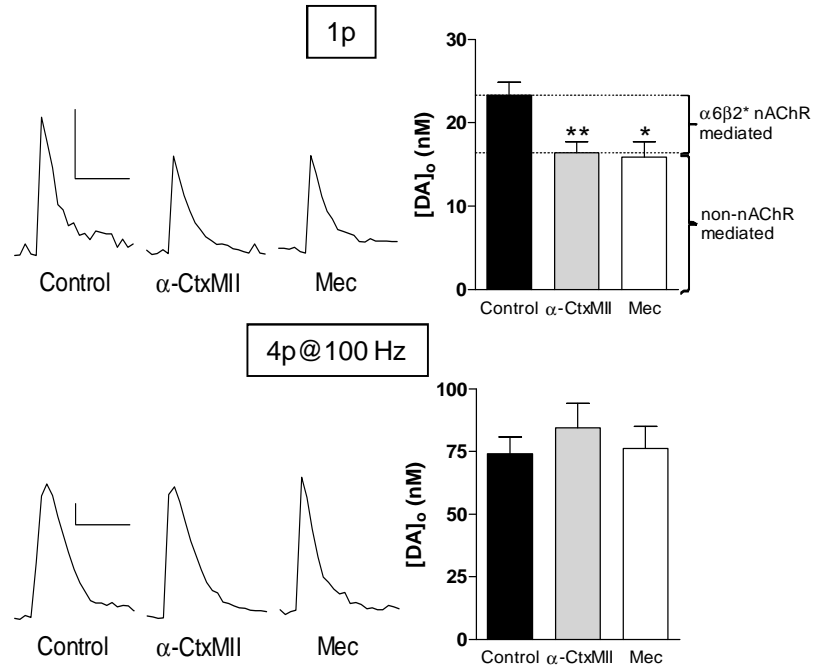


Figure 2

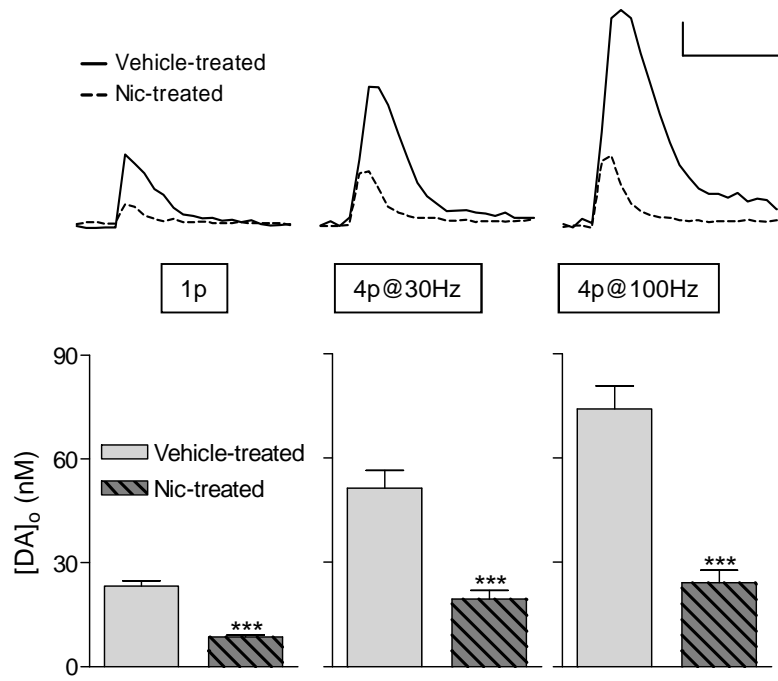


Figure 3

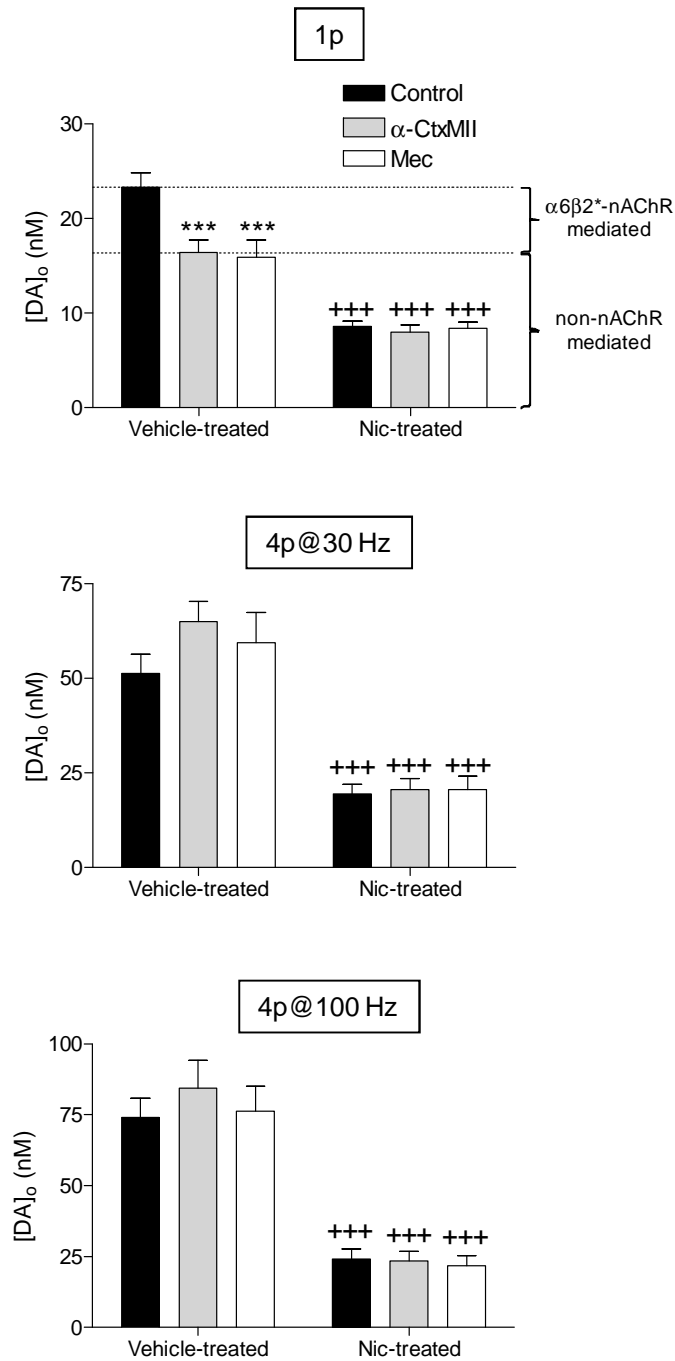


Figure 4

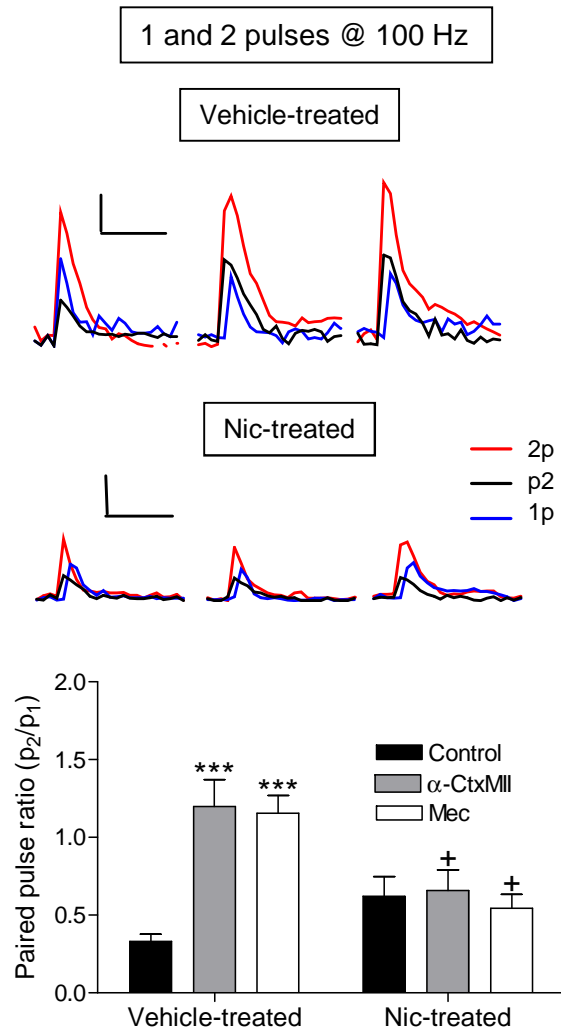


Figure 5

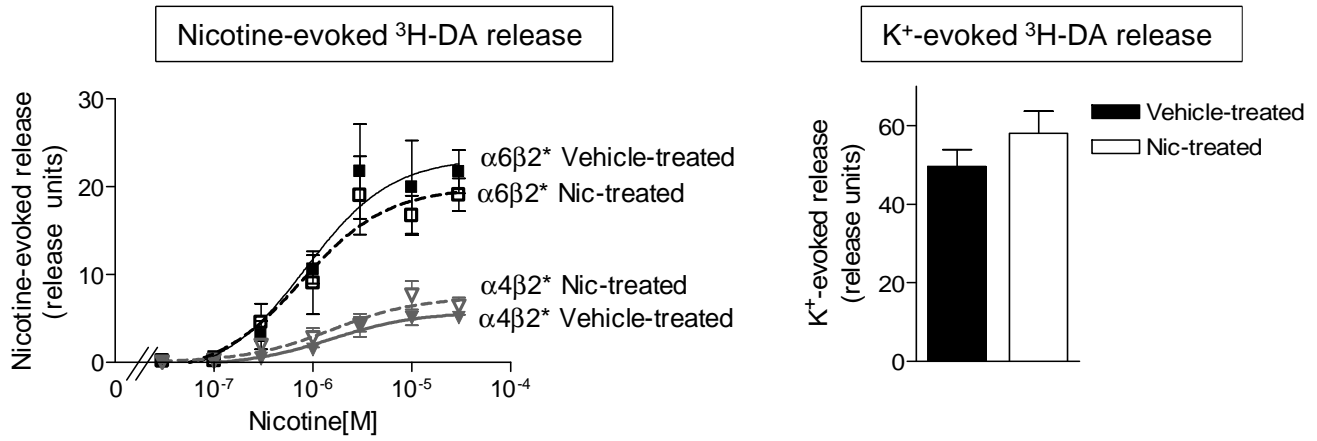


Figure 6

