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Prominent Role of $\alpha3/\alpha6\beta2^*$ nAChRs in Regulating Evoked Dopamine Release in Primate Putamen: Effect of Long-Term Nicotine Treatment

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ABSTRACT

Brain dopaminergic systems are critical in motor control as evidenced by findings that their disruption results in movement disorders such as Parkinson's disease. Nicotinic acetylcholine receptor (nAChR) activation plays an important role in regulating striatal dopaminergic function. Rodent studies show that short-term nicotine exposure influences stimulated striatal dopamine release with responsiveness dependent on neuronal activity. However, studies have not yet been done in nonhuman primates, nor has work been done to evaluate the effect of long-term nicotine exposure, which is relevant for therapies for chronic neurological disorders. Here, we used voltammetry to assess the role of nAChRs on evoked dopamine release from monkey putamen slices. In both ventral and dorsal putamen, $\alpha3/\alpha6\beta2^*$ nAChRs regulated $\geq$80% of non–burst- (single pulse) nAChR-modulated dopamine release, and $\alpha4\beta2^*$ nAChRs regulated the remainder. Similar results were observed with burst-firing in ventral but not dorsal putamen, indicating that nAChR-modulated effects on release depend on the subregion and firing frequency. Next, we investigated the consequence of long-term nicotine exposure via the drinking water on nAChR-modulated responsiveness. Nicotine treatment altered both non–burst- and burst-stimulated dopamine release in ventral but not dorsal putamen. Altogether, these data support a predominant role for $\alpha3/\alpha6\beta2^*$ nAChRs in the regulation of evoked dopamine release in nonhuman primate putamen. They also show that long-term nicotine treatment selectively modifies nAChR-modulated release in distinct striatal subregions. These findings have implications for the development of treatments for addiction and neurological disorders with nAChR dysfunction.

The nigrostriatal dopaminergic system plays a critical role in motor function under physiological conditions and in neurodegenerative disorders such as Parkinson's disease. Striatal dopaminergic afferents are in intimate contact with numerous neuronal elements from other neurotransmitter systems, including those of the cholinergic system (Zhou et al., 2002; Exley and Cragg, 2008). Indeed, there exists an extensive overlap of dopaminergic and cholinergic markers in the striatum, providing the anatomical basis for the close functional interrelationship between these two neurotransmitter systems (Zhou et al., 2002).

Acetylcholine, secreted from cholinergic interneurons, modulates striatal dopamine release primarily via activation of nicotinic acetylcholine receptors (nAChRs), which are pentameric ligand-gated ion channels. The principal nAChRs in the striatum are the $\alpha4\beta2^*$ and $\alpha6\beta2^*$ subtypes (the asterisk denotes the possible presence of other subunits in the receptor complex) (Grady et al., 2007; Quik et al., 2007; Exley and Cragg, 2008). Receptor expression studies in rodents indicate that $\alpha4\beta2^*$ nAChRs are in the majority (~85%) compared with the $\alpha6\beta2^*$ nAChR subtype (~15%). However, functional studies (synaptosomal nAChR-evoked [^{3}H]dopamine release) show that $\alpha4\beta2^*$ nAChRs control only ~70% and $\alpha6\beta2^*$ nAChRs 30% of striatal dopamine release. This discrepancy between receptor expression and function may relate to the fact that $\alpha4\beta2^*$ nAChRs are located on both dopaminergic

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ABBREVIATIONS: nAChRs, nicotinic acetylcholine receptors; α-CtxMII, α-conotoxinMII; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid; *, the possible presence of other nicotinic subunits in the receptor complex; aCSF, artificial cerebrospinal fluid; FSCV, fast-scan cyclic voltammetry.

and nondopaminergic neurons, whereas $\alpha6\beta2^*$ nAChRs are localized primarily on striatal dopaminergic terminals (Quik and McIntosh, 2006). Fast-scan cyclic voltammetry (FSCV), which measures the release of evoked dopamine in striatal slices, recently showed an even more prominent role for $\alpha6\beta2^*$ nAChRs, with ≥50% nAChR-modulated dopamine release regulated by $\alpha6\beta2^*$ nAChRs in rodents (Exley et al., 2008; Meyer et al., 2008).

The $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ subtypes are also the main nAChRs that modulate stimulated dopamine release in nonhuman primate striatum (Quik and McIntosh, 2006). Because both the $\alpha3$ and $\alpha6$ subunits are present in monkey striatum, ^{125}I-α-conotoxinMII binding sites are designated as $\alpha3/\alpha6\beta2^*$ nAChRs. The $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ populations are expressed in roughly similar proportions in monkey striatum. However, $\alpha3/\alpha6\beta2^*$ nAChR stimulation evokes significantly greater synaptosomal [^{3}H]dopamine release, with $\alpha3/\alpha6\beta2^*$ nAChRs responsible for ~70% release and the $\alpha4\beta2^*$ nAChR responsible for the remainder (McCallum et al., 2006a). Thus, in monkeys, $\alpha3/\alpha6\beta2^*$ nAChRs are present at a greater density and play a greater role in regulating striatal dopamine function, at least as evaluated using synaptosomal [^{3}H]dopamine release.

The question that next arises is the contribution of the $\alpha3/\alpha6\beta2^*$ nAChR subtype to nonhuman primate dopaminergic function using FSCV, which offers a dynamic measure of evoked striatal dopamine release. We therefore initiated experiments to evaluate the role of $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChRs in modulating dopamine release probability at high- and low-frequency firing. Release was investigated in both ventral and dorsal putamen because of the demonstrated heterogeneity between these subregions (Cragg et al., 2002). Last, we investigated the effects of long-term nicotine administration because of its relevance for treating chronic neurological disorders. The results are the first to demonstrate a predominant role for $\alpha3/\alpha6\beta2^*$ nAChRs in regulating the evoked striatal dopamine release in nonhuman primates.

Materials and Methods

Animal Treatment. Adult female squirrel monkeys (*Saimiri sciureus*) weighing between 0.6 and 0.8 kg were purchased from Worldwide Primates (Miami, FL) and were quarantined for 1 month according to California state regulations. All animals were housed separately in a room maintained at 27 ± 3°C, with a 13:11-h light/dark cycle. They were given food (consisting of monkey chow, fruits, and vegetables) once daily, and water ad libitum. Animals were randomly divided into the two treatment groups: control and nicotine-treated. After acclimation to the home environment, all animals were given commercially available Gatorade in the drinking water for ~2 weeks. Gatorade was used as a vehicle to mask the bitter taste of nicotine. Nicotine (free-base) was added to the Gatorade solution of the nicotine-treated group starting at a concentration of 50 μg/ml. It was gradually increased to a final concentration of 650 μg/ml over 3 weeks. 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 enzyme-linked immunosorbent assay kit (Orasure Technologies, Bethlehem, PA). Cotinine levels in nicotine-treated animals were 489 ± 51.1 ng/ml, which are similar to levels found in smokers (Matta et al., 2007). All animals were maintained on vehicle or nicotine (650 μg/ml) for ~8 months, after which they were euthanized. Nicotine was removed from the drinking solution 24 h before death. This time point was selected because there is little change in nicotine-induced receptor up- or down-regulation or in behavior, but effects of residual tissue nicotine would be minimal in the binding and functional studies.

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 of euthanasia solution intraperitoneally (390 mg of sodium pentobarbital and 50 mg/ml phenytoin sodium), followed by 1.5 ml/kg of the same solution administered intravenously. Ketamine was not used because a previous work had shown that it interferes with nicotinic receptor function (Coates and Flood, 2001). All studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Parkinson's Institute.

Tissue Preparation. The brains were rapidly removed, rinsed in cold saline, and sectioned along the midline. One half was placed in a squirrel monkey brain mold and cut into 6-mm thick blocks using stainless steel blades. The blocks were immediately frozen in isopentane on dry ice and stored at −80°C. These blocks were later used for preparation of 20-μm thick sections using a cryostat (Leica Microsystems, Inc., Deerfield, IL) cooled to −15°C. Frozen sections were thaw-mounted onto Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA), air-dried, and stored at −80°C for autoradiography.

The other half of the brain was cut into 2-mm sections and used as follows. Ten-milligram tissue aliquots of ventral and dorsal putamen were dissected for measurement of [^{3}H]dopamine release. Five-milligram aliquots of ventral and dorsal putamen were used for assay of striatal dopamine. The slice at level A11.0 to 12.0 was used for the determination of evoked dopamine release using cyclic voltammetry, as described previously (Perez et al., 2008b). It was bisected at the level of the internal capsule, and the portion containing the putamen was placed in ice-cold, preoxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) consisting of 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, and 25 mM $NaHCO_3$, pH 7.4. Striatal slices (400 μm thick) were prepared using a vibratome (Leica VT1000S) and were incubated at room temperature for 2 h in oxygenated aCSF. Thirty minutes before recording, the slice was transferred to a submersion-recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at 1 ml/min with 30°C oxygenated aCSF, and allowed to equilibrate.

Electrochemical Measurement of Dopamine Release. For the FSCV experiments, carbon fiber microelectrodes were constructed as described previously (Perez et al., 2008b). The electrode was positioned 75 μm below the surface of the slice, and its 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 mV/ms. This triangular wave was repeated every 100 ms at a sampling frequency of 50 Hz. The observed signals primarily represent striatal dopamine, because there is only minimal noradrenergic innervation to the squirrel monkey putamen with levels of striatal noradrenaline ~2% those of dopamine (Hsu et al., 1971). Current was recorded with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Triangular wave generation and data acquisition were controlled by pClamp 9.0 software (Molecular Devices). Electrical stimulation was applied using a bipolar tungsten stimulating electrode (Plastics One, Roanoke, VA) connected to a linear stimulus isolator (WPI, Saratoga, FL) and triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). The stimulating electrode was placed on the surface of the slice, 100 to 200 μm away from the carbon fiber electrode. Background current was digitally subtracted, and the peak oxidation currents were converted into concentration after postexperimental calibration of the carbon fiber electrode with a fresh solution of 1 μM dopamine in experimental buffer.

After a 2-h incubation period, the slice was transferred to a submersion recording chamber (Campden Instruments Ltd., Lafayette, IN), perfused at 1 ml/min with oxygenated aCSF at 30°C and allowed to equilibrate for 30 min. Because of the limited availability of

monkey tissue, evoked release was only determined by either a single rectangular electrical pulse applied every 2.5 min or by a burst of four pulses at 100 Hz applied every 5 min with a stimulus intensity of 8 V, as described previously (Perez et al., 2008b). The burst stimulation paradigm was chosen based on previous rodent studies, which showed that maximal effects of the drugs on nAChR-modulated responses occur at this frequency (Rice and Cragg, 2004; Zhang and Sulzer, 2004). Moreover, phasic firing in primates is known to reach up to 100 Hz in frequency (Schultz, 1986). Dopamine release was measured in the same area of the ventral or dorsal putamen from the different animals to minimize variability (Perez et al., 2008b). Total evoked release by both a single and a burst of pulses was first assessed in physiological buffer. nAChR-modulated release was assessed in the presence of 100 nM α-conotoxinMII (α-CtxMII) and/or 100 μM mecamylamine. These concentrations were chosen based on previous studies showing they yielded maximal blockade of $\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChRs (Exley et al., 2008; Perez et al., 2008a). Perfusion of the slice with α-CtxMII resulted in a maximal decrease in release within ~15 min and with mecamylamine by 10 min. Signals remained stable throughout data collection for each experimental condition. The reported effects on release with each antagonist represent the average of those signals obtained once a stable maximal response was established.

[^{3}H]Dopamine Release from Striatal Synaptosomes. A crude synaptosomal fraction was prepared as described previously (McCallum et al., 2006a). In brief, tissue was homogenized in 2 ml of ice-cold 0.32 M sucrose buffered with HEPES, pH 7.5. A P1 pellet was obtained by centrifuging the homogenate at 12,000*g* for 20 min. Resuspended synaptosomes were incubated in 37°C uptake buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH_2PO_4, 3.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 25 mM $NaHCO_3$, 1 mM ascorbic acid, and 0.01 mM pargyline) for 10 min before adding 100 nM [^{3}H]dopamine (3,4-[ring-2,5,6-^{3}H], 30–60 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA). Incubation with [^{3}H]dopamine (4 μCi for 0.8 ml of synaptosomes) was done for 5 min, then 80-μl aliquots of synaptosomes (0.5 to 2 mg of tissue) were pipetted onto 5-mm diameter A/E glass-fiber filters (Gelman, Inc., Ann Arbor, MI) 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 [^{3}H]dopamine from synaptosomes on each platform was evoked by an 18-s exposure to one of a range of nicotine concentrations (0.03–30 μM). When used, α-CtxMII (50 nM) was perfused for 3 min before nicotine exposure to distinguish between $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChR-mediated release. Fifteen 18-s fractions (including basal release before and after the stimulated release) were collected, and radioactivity was determined by liquid scintillation counting.

Release was plotted as cpm versus fraction number using a curve-fitting algorithm of SigmaPlot 5.0 for DOS (Systat Software, Inc., San Jose, CA). Basal release was calculated 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 cpm released. The corrected cpm was then normalized to the wet weight of the tissue sample on each filter to obtain units of cpm per milligram of tissue. R_{max} values were obtained from dose-response curves by fitting data to a nonlinear regression equation using Prism (GraphPad Software, San Diego, CA).

^{125}I-Epibatidine Autoradiography. Binding of ^{125}I-epibatidine (2200 Ci/mmol) was done as reported previously (Bordia et al., 2007). Slides were preincubated at 22°C for 15 min in buffer containing 50 mM Tris, pH 7.5, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$. They were incubated for 40 min with 0.015 nM ^{125}I-epibatidine in the presence or absence of α-CtxMII (300 nM). They were then washed, dried, and exposed to Kodak MR film (PerkinElmer Life and Analytical Sciences) with ^{125}I-microscale standards for several days. Nonspecific binding was assessed in the presence of 100 μM nicotine and was similar to the film blank.

^{125}I-α-CtxMII Autoradiography. Binding of ^{125}I-α-CtxMII (specific activity, 2200 Ci/mmol) was done as reported previously (Bordia et al., 2007). Striatal sections were preincubated at room temperature for 15 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$ 1 mM $MgSO_4$, 20 mM HEPES, and 0.1% bovine serum albumin, pH 7.5) plus 1 mM phenylmethylsulfonyl fluoride. 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 μg/ml each of aprotinin, leupeptin and pepstatin A plus 0.5 nM ^{125}I-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 0.1× 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 ^{125}I-microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nicotine (100 μM) was used to determine nonspecific binding.

Dopamine Transporter Autoradiography. Binding to the dopamine transporter was measured using [^{125}I]RTI-121 (2200 Ci/mmol; PerkinElmer) as described previously (Bordia et al., 2007). Thawed sections were preincubated twice for 15 min each at room temperature in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl and then incubated for 2 h in buffer with 0.025% bovine serum albumin, 1 μM fluoxetine, and 50 pM [^{125}I]RTI-121. Sections were washed at 0°C for 4 × 15 min each in buffer and once in ice-cold water, air-dried, and exposed for 2 days to Kodak MR film (PerkinElmer) with ^{125}I-microscale standards (GE Healthcare). Nomifensine (100 μM) was used to define nonspecific binding.

Dopamine Measurements. Striatal tissue samples (5 mg) were placed in a 0.25-ml volume of ice-cold 0.4 M perchloric acid and then sonicated and centrifuged at 14,000*g* for 12 min. The supernatants were used for the measurement of dopamine using high-pressure liquid chromatography with electrochemical detection (Coulochem II detector; ESA Inc., Chelmsford, MA). The mobile phase was delivered at a flow rate of 1 ml/min and consisted of a mixture of 90 mM sodium acetate, 35 mM citric acid, 130 μM EDTA, and 230 μM 1-octanesulfonic acid. Protein concentrations were determined using the method of Lowry et al. (1951).

Statistical Analyses. All statistics were conducted using Prism (GraphPad Software). Statistical comparisons were performed using one-way analysis of variance followed by a Newman-Keuls multiple comparisons test or two-way analysis of variance followed by Bonferroni post hoc test. A value of $p < 0.05$ was considered significant. All values are expressed as the mean ± S.E.M. of the indicated number of animals.

Results

$\alpha3/\alpha6\beta2^*$ nAChR Blockade Results in a Major Decrease in Evoked Dopamine Release with Single-Pulse Stimulation. Although previous work had shown that dopamine release and uptake share many characteristics in the putamen and caudate, there seems to be a continuum of distinct functional domains from ventral to dorsal areas within these two brain regions, with dopamine release and uptake being significantly greater in the dorsal compared with the ventral regions (Cragg et al., 2000, 2002). We therefore investigated $\alpha3/\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChR-modulated functional properties in the ventral and dorsal striatal areas, with a focus on the putamen because it is more vulnerable in Parkinson's disease (Hornykiewicz, 2001). To approach this, we used FSCV to measure evoked dopamine release from striatal slices in the absence and presence of the $\alpha3/\alpha6\beta2^*$-selective nAChR antagonist α-CtxMII and mecamylamine, a

neuronal nAChR receptor antagonist that blocks both $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChRs.

Representative traces (Fig. 1, top) show that α-CtxMII (100 nM) pretreatment decreased release by ~50% with single-pulse stimulation in ventral putamen. Control values declined significantly ($p < 0.001$) from 0.100 ± 0.004 to 0.055 ± 0.004 μM (n = 10–15 observations from one monkey), with results representative of seven animals. Subsequent addition of 100 μM mecamylamine to the perfusion buffer further decreased ($p < 0.001$) evoked dopamine release to 0.038 ± 0.001 μM. Release in the presence of mecamylamine was significantly ($p < 0.01$) lower compared with that in the presence of α-CtxMII in five of seven monkeys.

In the dorsal putamen, representative traces (Fig. 1, bottom) also demonstrate a decrease (~70%) in single pulse-stimulated dopamine release with α-CtxMII. Values (mean ± S.E.M. of 10–15 observations from one monkey) were significantly ($p < 0.001$) reduced from 0.124 ± 0.005 μM 0.047 ± 0.002 μM. These results are representative of four animals. In this region, however, perfusion with mecamylamine did not further decrease release (0.055 ± 0.005 μM) as it had in ventral putamen.

These data show that nAChRs modulate 50 to 70% of electrically evoked dopamine release in putamen. In the ventral putamen, the $\alpha3/\alpha6\beta2^*$ nAChR subtype is responsible for 88% of this release, and the $\alpha4\beta2^*$ nAChR is responsible for the remainder, whereas in the dorsal putamen, all nAChR-modulated release was modulated by $\alpha3/\alpha6\beta2^*$ nAChRs under the current stimulus conditions.

Differential Dopaminergic Responsiveness to nAChR Blockade with Burst Stimulation in Ventral and Dorsal Putamen. The results in the preceding section show $\alpha3/\alpha6\beta2^*$ nAChRs control the majority of nAChR-modulated dopamine release with nonburst (single-pulse) stimulation. Previous studies in rodents have shown that nAChR subtypes differentially regulate dopamine release with nonburst and burst stimulation (Exley et al., 2008; Meyer et al., 2008). We therefore next conducted experiments to determine the effect of α-CtxMII and mecamylamine on dopamine release in ventral and dorsal putamen in response to burst stimulation (4 pulses/100 Hz). Evoked release was performed under only two stimulus conditions (a burst of four pulses at 100 Hz applied every 5 min or a single rectangular electrical pulse applied every 2.5 min as described above), because available monkey tissue was limited.

In ventral putamen, representative traces show that pretreatment of the slice with 100 nM α-CtxMII reduced release ~30% with burst stimulation (Fig. 2, top). Toxin exposure significantly ($p < 0.01$) decreased release from 0.141 ± 0.009 to 0.100 ± 0.007 μM (n = 4–6 observations from one monkey and are representative of five of six animals). Perfusion of the slice with mecamylamine reduced dopamine release by an additional 17% to 0.075 ± 0.005 μM in four of the six animals.

In contrast, in dorsal putamen, representative traces indicate that dopamine release induced by burst stimulation was similar in the absence and presence of either α-CtxMII or mecamylamine (Fig. 2, bottom). Evoked dopamine release in the absence of antagonist was 0.121 ± 0.006 μM, in the

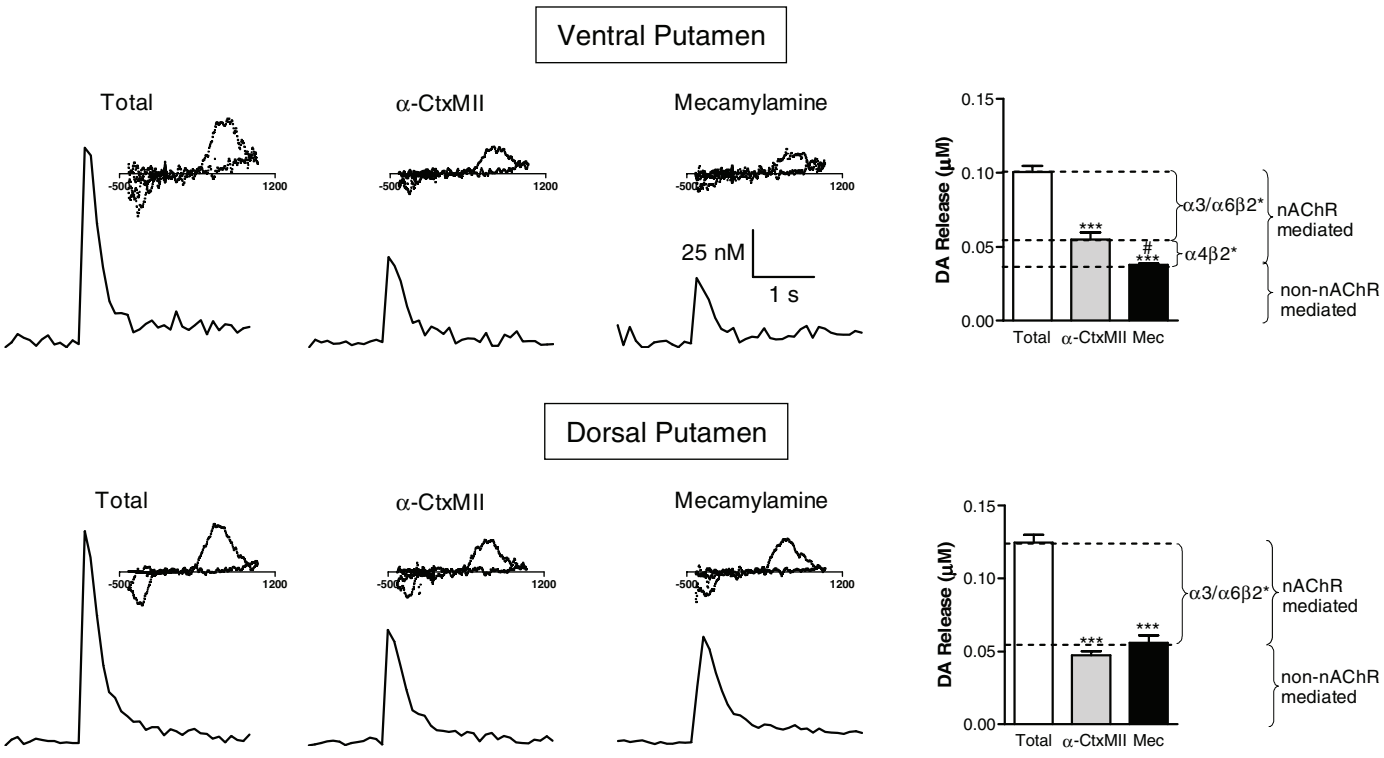

Fig. 1. Single (one pulse) pulse-stimulated endogenous dopamine release in primate putamen is predominantly influenced by $\alpha3/\alpha6\beta2^*$ nAChRs. Traces of dopamine release in ventral (top) and dorsal putamen (bottom) in the absence (Total) and presence of the $\alpha3/\alpha6\beta2^*$ antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Insets, typical voltammograms for dopamine with an oxidation peak at 500 to 600 mV and a reduction peak of approximately −200 mV. Quantitative analyses of peak dopamine release show that in the ventral putamen, ~80% of nAChR-modulated dopamine release occurs through $\alpha3/\alpha6\beta2^*$ nAChRs and ~20% by $\alpha4\beta2^*$ nAChRs, whereas in dorsal putamen, 100% of nAChR-modulated dopamine release occurs through $\alpha3/\alpha6\beta2^*$ nAChRs. The values represent the mean ± S.E.M. of 10 to 15 observations from 1 monkey. Results are representative of five animals. Significance of difference from total release using a Newman-Keuls multiple comparison post hoc test, ***, $p < 0.001$; significance of difference from release in the presence of α-CtxMII, #, $p < 0.01$.

presence of α-CtxMII was 0.117 ± 0.005 μM, and with mecamylamine was 0.125 ± 0.006 μM. Results represent four to six observations from one monkey and are representative of four animals. Altogether, these data show that the nAChR subtypes in dorsal putamen differentially influence dopamine release with burst stimulation compared with ventral putamen.

Synaptosomal [^{3}H]Dopamine Release Assay Also Shows a Prominent Role for α3/α6β2* nAChRs. Another approach to assess striatal nAChR-modulated dopaminergic function is the measurement of nicotine-evoked synaptosomal [^{3}H]dopamine release (McCallum et al., 2006a; Grady et al., 2007). Comparison of the FSCV results using single-pulse stimulation with those using the synaptosomal [^{3}H]dopamine release assay in the same monkeys (Fig. 3) showed that a large proportion of release was also modulated through α3/α6β2* nAChRs in ventral and dorsal putamen using this latter technique. For instance, 71% of total nicotine-stimulated [^{3}H]dopamine release in ventral putamen was due to α3/α6β2* and 29% to α4β2* nAChR activation (Fig. 3). Values were similar in dorsal putamen, with α3/α6β2* and α4β2* nAChRs mediating 62 and 38% of total nicotine-evoked [^{3}H]dopamine release, respectively. Altogether, these combined results demonstrate a predominant role for α3/α6β2* nAChRs in mediating dopamine release in the primate dorsal and ventral putamen compared with α4β2* nAChRs.

Long-Term Nicotine Treatment of Nonhuman Primates. Because numerous studies show that long-term nicotine treatment modulates nicotinic receptor responsiveness (Flores et al., 1992; Marks et al., 1992; Pauly et al., 1996), we next investigated the effect of long-term nicotine exposure on α3/α6β2* and α4β2* nAChR-modulated dopamine release in primate putamen using FSCV. Nicotine was provided in the drinking water according to the dosing regimen depicted in Fig. 4A. This approach offers the advantage that it readily allows for long-term administration (Quik et al., 2006). In brief, the nicotine dose was increased over a 3-week period from 50 μg/ml to the final dose of 650 μg/ml, at which the animals were maintained for an additional ~8 months. To evaluate effectiveness of the treatment, we measured α4β2* and α3/α6β2* nAChR expression (Fig. 4, B and C) using quantitative receptor autoradiography. There was no change in ^{125}I-α-CtxMII binding, which labels α3/α6β2* nAChRs (Fig. 4B). Previous studies demonstrated a small (10–15%) decrease in binding with long-term nicotine dosing (McCallum et al., 2006a); the difference between these latter and the current studies may be due to differences in the ages of the monkeys. For instance, the average age of this set of monkeys was between 5 and 10 years, whereas the monkeys in the earlier studies ranged between 7 and 20 years. Aged monkeys may be more susceptible to the biological effects of nicotine. Our previous work in mice demonstrated more robust changes in α4β2*and α6β2* nAChRs with age (Lai et al., 2005). We therefore also determined the binding of ^{125}I-epibatidine in the presence of α-CtxMII to identify α4β2* nAChRs (α-CtxMII-resistant ^{125}I-epibatidine sites). Oral nic-

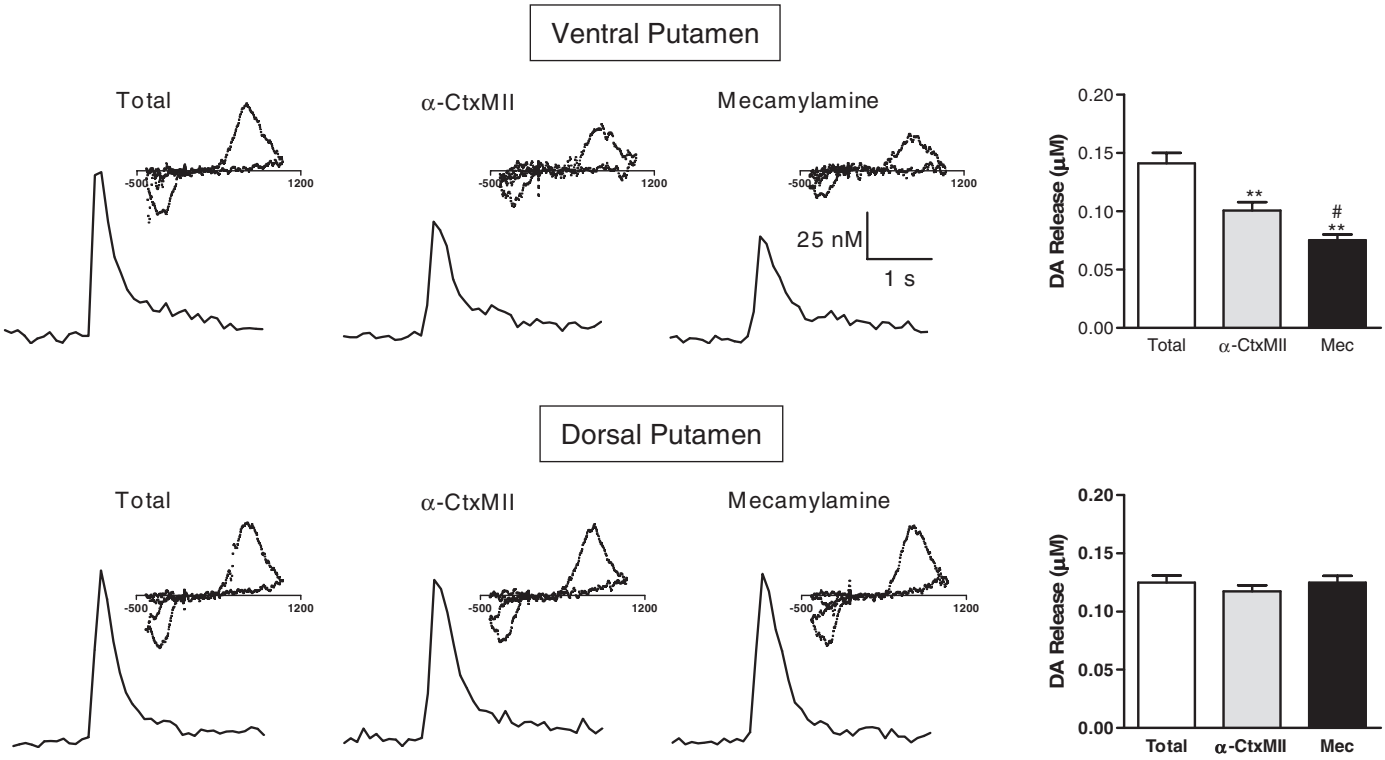

Fig. 2. Burst (four pulses) stimulated endogenous dopamine release in primate putamen is differentially affected by nAChR blockade in ventral compared with dorsal putamen. Traces of dopamine release in ventral (top) and dorsal putamen (bottom) in the absence (Total) and presence of the α3/α6β2* antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Insets, typical voltammograms for dopamine with an oxidation peak at 500 to 600 mV and a reduction peak of approximately −200 mV. Quantitative analyses of peak dopamine release for each brain region show that in the ventral putamen α3/α6β2* nAChR-blockade with α-CtxMII decreased dopamine release by ~30%, whereas application of mecamylamine further decreased it to ~46% of total release. In contrast, the antagonists did not affect peak dopamine release in the dorsal putamen. The values represent the mean ± S.E.M. of four to six observations from one monkey. Results are representative of four animals. Significance of difference from total release using a Newman-Keuls multiple comparison post hoc test, **, $p < 0.01$; significance of difference from release in the presence of α-CtxMII, #, $p < 0.05$.

otine treatment significantly increased $\alpha4\beta2^*$ nAChRs in both ventral ($p < 0.05$) and dorsal ($p < 0.01$) putamen (Fig. 4C).

Long-Term Nicotine Treatment Alters Non–Burst- and Burst-Stimulated nAChR-Modulated Dopamine Release in Ventral but Not Dorsal Putamen. The effect of long-term nicotine treatment on evoked dopamine release in ventral putamen in the absence and presence of nicotinic antagonists is depicted in Figs. 5 and 6. In ventral putamen of control animals, α-CtxMII significantly ($p < 0.001$) decreased single pulse-stimulated dopamine release from 0.119 ± 0.008 to 0.063 ± 0.006 μM ($n = 7$ monkeys), as evident from the traces in Fig. 1. In nicotine-treated animals, there was a trend for a decline in total release in the presence of α-CtxMII with single-pulse stimulation (from 0.116 ± 0.016 to 0.074 ± 0.019 μM with $n = 4$ monkeys). However, this was not significant most likely because the toxin reduced the release in putamen of only two of the four monkeys. With burst stimulation (Figs. 5 and 6), α-CtxMII significantly decreased release from 0.134 ± 0.013 to 0.089 ± 0.013 μM in ventral putamen of control animals ($n = 6$ monkeys), as evident from the traces in Fig. 2. In ventral putamen of nicotine-treated monkeys (Fig. 6), the amount of dopamine released was comparable in the absence and presence of α-CtxMII (0.115 ± 0.015 and 0.138 ± 0.022 μM, $n = 3$ monkeys, respectively). Similar results were obtained with the general nAChR antagonist mecamylamine (Figs. 5 and 6). Thus, both non–burst- and burst-evoked dopamine release were modified by nicotine treatment in ventral putamen.

By contrast, nAChR-modulated dopamine release in dorsal putamen was unaffected by long-term nicotine treatment with both nonburst and burst stimulation (Fig. 7). In dorsal putamen (Fig. 7) of control monkeys ($n = 4$), α-CtxMII significantly ($p < 0.01$) decreased nonburst release from 0.097 ± 0.010 to 0.0488 ± 0.072 μM, as evident from the traces in Fig. 1. Comparable results were obtained with single-pulse stimulation in dorsal putamen of nicotine-treated monkeys ($n = 4$ monkeys). With burst stimulation, dopamine release in dorsal putamen in the absence and presence of α-CtxMII was also alike in control ($n = 4$) and nicotine-treated monkeys ($n = 4$). Similar results were obtained with mecamylamine (Fig. 7). Overall, these results in ventral and dorsal putamen show that long-term nicotine treatment results in regionally selective alterations in evoked dopamine release.

Long-Term Nicotine Exposure Does Not Affect Dopamine Levels or Dopamine Transporter Expression. To further examine striatal alterations that may account for the observed functional changes, we assessed the effects of long-term nicotine treatment on various markers of striatal dopamine terminal integrity known to influence dopamine release. We examined the levels of dopamine and dopamine transporter expression in ventral and dorsal putamen. As shown in Table 1, nicotine treatment did not have a significant effect on these measures in ventral or dorsal putamen.

Discussion

The present results show that $\alpha3/\alpha6\beta2^*$ nAChRs play an important role in regulating dopamine release in primate putamen. Blockade of $\alpha3/\alpha6\beta2^*$ nAChRs resulted in an $\geq$80% decrease in nonburst nAChR-modulated dopamine release in both ventral and dorsal putamen. These data suggest that acetylcholine released spontaneously from striatal cholin-

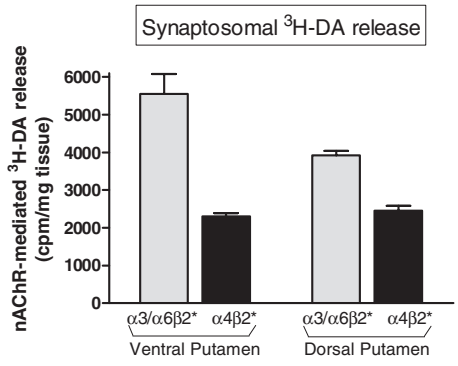

Fig. 3. Evaluation of striatal $\alpha3/\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChR-stimulated dopamine release using synaptosomal [^{3}H]dopamine (DA) release assay. [^{3}H]DA release yields a measure of nicotine-evoked release in an isolated synaptosomal preparation in the absence of influences from other regulatory neuronal inputs. Voltammetry, on the other hand, provides a measure of electrically evoked dopamine release, which is influenced by other striatal inputs and endogenous acetylcholine tone. [^{3}H]DA release was determined in the presence and absence of 50 nM α-CtxMII to differentiate the components of release mediated by the $\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChR populations. Release remaining in the presence of α-CtxMII was defined as release mediated by $\alpha4\beta2^*$ nAChRs, with the $\alpha3/\alpha6\beta2^*$ nAChR-mediated component determined by subtraction of the $\alpha4\beta2^*$ component from total release. The $\alpha3/\alpha6\beta2^*$ nAChR population seems to modulate a large portion of dopamine release regardless of the technique or tissue preparation used. The R_{max} values are derived from nonlinear regression analysis of dose-response curves in GraphPad Prism. Data represent mean ± S.E.M. of three monkeys in each treatment group.

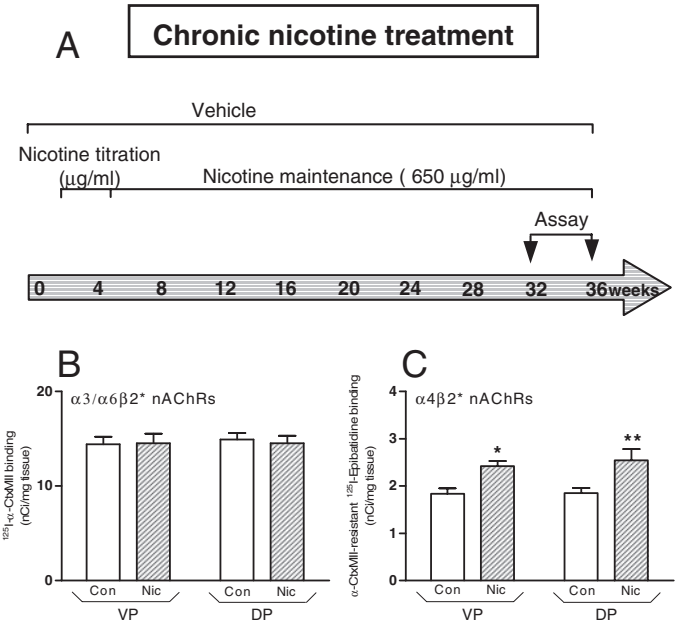

Fig. 4. Long-term nicotine treatment increases putamen $\alpha4\beta2^*$ nAChRs, with no change in the $\alpha3/\alpha6\beta2^*$ subtype. The nicotine treatment regimen is depicted in A. Nicotine (free base) was added to the drinking water starting at 50 μg/ml to a final concentration of 650 μg/ml over a 3-week period. Animals were maintained at this final nicotine dose for 8 months, after which time receptor autoradiography was performed. B, ^{125}I-α-CtxMII binding studies were done to measure $\alpha3/\alpha6\beta2^*$ nAChRs in ventral putamen (VP) and dorsal putamen (DP). C, changes in $\alpha4\beta2^*$ nAChRs were determined by measurement of ^{125}I-epibatidine binding in the presence of α-CtxMII (300 nM), with $\alpha4\beta2^*$ nAChRs defined as α-CtxMII-resistant ^{125}I-epibatidine binding sites. Data represent mean ± S.E.M. of four to seven monkeys. Significance of difference from control using a Bonferroni post hoc test, *, $p < 0.05$; **, $p < 0.01$.

ergic interneurons modulates dopamine release primarily through $\alpha3/\alpha6\beta2^*$ nAChR activation under low-frequency conditions, with only a small contribution from $\alpha4\beta2^*$ nAChRs. With increased stimulus frequency, $\alpha3/\alpha6\beta2^*$ and $\alpha4\beta2^*$ nAChR blockade also reduced dopamine release in ventral putamen, suggesting that both subtypes influenced release under these conditions. A somewhat different situation seemed to exist with burst stimulation in dorsal putamen in which nAChR antagonists did not affect electrically evoked dopamine release. These data indicate that acetylcholine exerts a complex modulatory control over dopamine release with varying stimulus frequency that is distinct in nonhuman primate ventral compared with dorsal putamen.

The primate putamen is a relatively varied structure that is topographically organized along a ventral-dorsal axis with respect to numerous afferents to and efferents from the cortex, thalamus, amygdala, substantia nigra, and other brain areas (Haber et al., 2000). Moreover, this anatomical diversity is associated with heterogeneity in striatal dopamine function (Cragg et al., 2000; McCallum et al., 2006b). The heterogeneous nature of the putamen is further illustrated by findings that there is a clear ventral-to-dorsal gradient in striatal dopaminergic cell loss with denervation (Song and Haber, 2000; McCallum et al., 2006b). The present data demonstrate anatomical diversity in the regulation of nAChR-modulated dopamine release with a differential effect of burst firing in ventral and dorsal putamen.

The cellular mechanisms responsible for these variations in nAChR-modulated responsiveness with burst firing in the ventral compared with the dorsal putamen are not known. However, they may relate to differences in the composition of the $\alpha3/\alpha6\beta2^*$ nAChR subtypes localized in these two striatal subregions. Receptor identification studies demonstrate the presence of several α-CtxMII-sensitive nAChR subtypes in monkey striatum including $\alpha6\alpha4\beta2\beta3$, $\alpha6\beta2\beta3$, and $\alpha3\beta2^*$ nAChRs (Quik and McIntosh, 2006; Bordia et al., 2007). Accumulating evidence points to differential sensitivities of these subtypes to nAChR agonists and antagonists (Salminen et al., 2004; Bordia et al., 2007). In addition to a differential expression of α-CtxMII-sensitive subtypes, the primary regulators of striatal dopamine release, there is also evidence for heterogeneity in $\alpha4\beta2^*$ nAChRs in monkey putamen, including the expression of $\alpha4\beta2$ and $\alpha4\alpha5\beta2$ nAChRs (Quik et al., 2005), which exhibit differential functional characteristics (Brown et al., 2007; Kuryatov et al., 2008). Variable proportions of these different receptor subtypes between regions could yield unique patterns of nAChR responsiveness. Alternatively or as well, there may be variable isomers of the receptors in the two regions. Heterologous expression of the $\alpha4$ and $\beta2$ subunits produces two alternate stoichiometries, $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$, which exhibit unique functional, pharmacological, and regulatory characteristics (Tapia et al., 2007; Moroni et al., 2008). In addition to the above, there may also be variations in post-translational modifications that are unique to a striatal subregion. It should be possible to discriminate between the possibilities with the development of reagents selective for these varying nAChR isomers/subtypes.

In the present study, we investigated the effects of release at approximately half-maximal stimulus strength. The results show that $\alpha3/\alpha6\beta2^*$ nAChRs make a major contribution to the control of nAChR-modulated evoked dopamine release under this condition. These results are consistent with previous work in rodents, which also show that $\alpha3/\alpha6\beta2^*$ receptor inhibition more effectively blocks non–burst-evoked dopamine release at low compared with maximal stimulus strength (Exley et al., 2008; Meyer et al., 2008). Studies are in progress to evaluate whether nAChR-modulated dopamine release is also differentially affected with stimulus strength in primate putamen.

Rodent studies have shown that nAChR blockade relieves short-term depression and enhances burst-stimulated dopamine release in dorsal striatum and nucleus accumbens (Exley et al., 2008; Perez et al., 2008a). Our studies also show that nAChR antagonism relieved short-term depression in dorsal putamen of control primates. Unexpectedly, however,

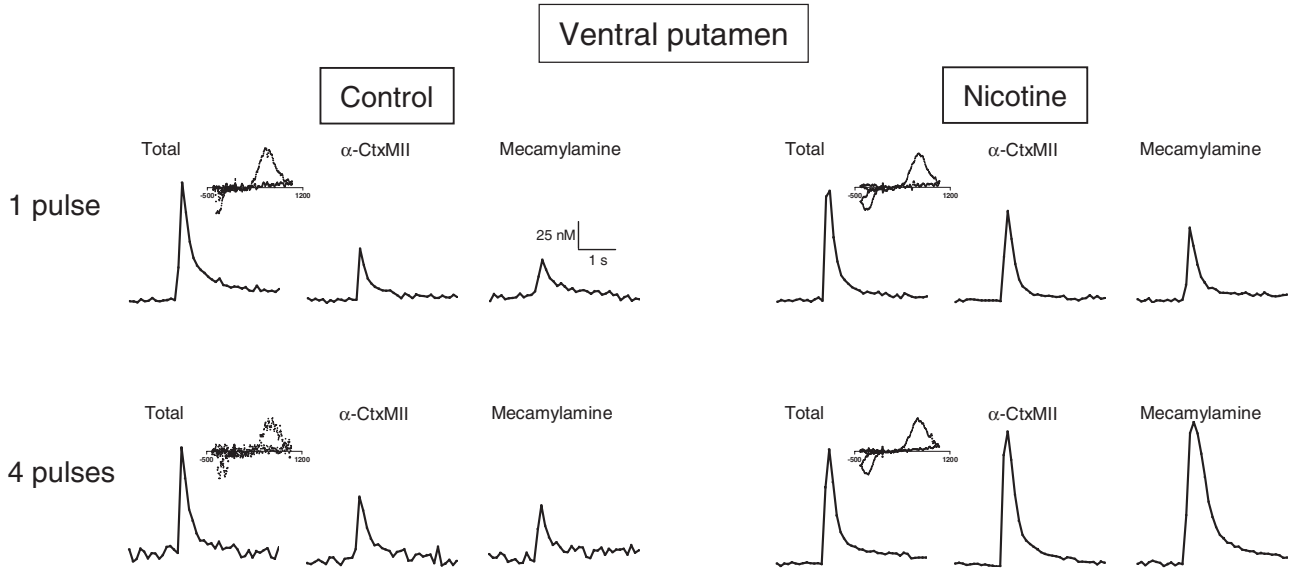

Fig. 5. Long-term nicotine treatment modulates evoked dopamine release in ventral putamen. Representative traces for stimulated dopamine release in the absence (Total) and presence of α-CtxMII (100 nM) or mecamylamine (100 μM). In controls, perfusion of the slice with α-CtxMII or mecamylamine led to a decline in single or burst pulse-stimulated release (left). This decrease was no longer observed in animals treated with nicotine (right). Insets, typical voltammograms for dopamine with an oxidation peak at 500 to 600 mV and a reduction peak of approximately −200 mV.

there was no relief of short-term depression in ventral putamen under control conditions, suggesting that there are regional variations in responsiveness in monkey putamen. Whether this represents a species or regional difference is currently not clear because effects in ventral rodent striatum remain to be evaluated. Interestingly, nicotine treatment prevented the antagonist-induced decrease in burst-stimulated dopamine release observed in control ventral putamen with single pulse stimulation. This observation suggests that nicotine treatment influences short-term depression such that antagonists inhibit single-pulse but not burst-stimulated dopamine release.

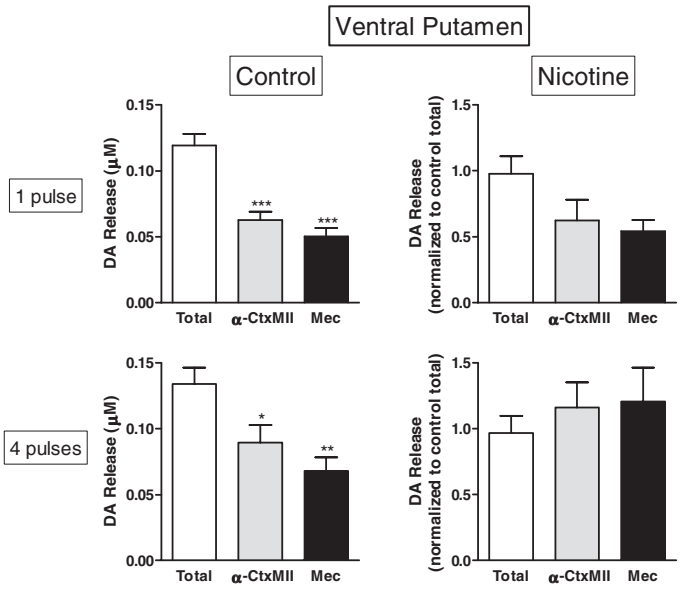

Fig. 6. Quantitative analyses of ventral putamen showing that long-term nicotine treatment modifies evoked dopamine release. Release was determined in the absence (Total) and presence of α-CtxMII (100 nM) or mecamylamine (Mec; 100 μM). In control animals, nAChR blockade decreased evoked dopamine release with both 1 and 4-pulse stimulation. Neither antagonist significantly decreased single or burst pulse stimulated release in nicotine-treated animals, although there was a trend for a decrease with single pulse stimulation. Release for the nicotine-treated animals is normalized to total stimulated release in control animals. Data represent mean ± S.E.M. of three to seven monkeys. Significance of difference from total release using a Newman-Keuls multiple comparison post hoc test, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

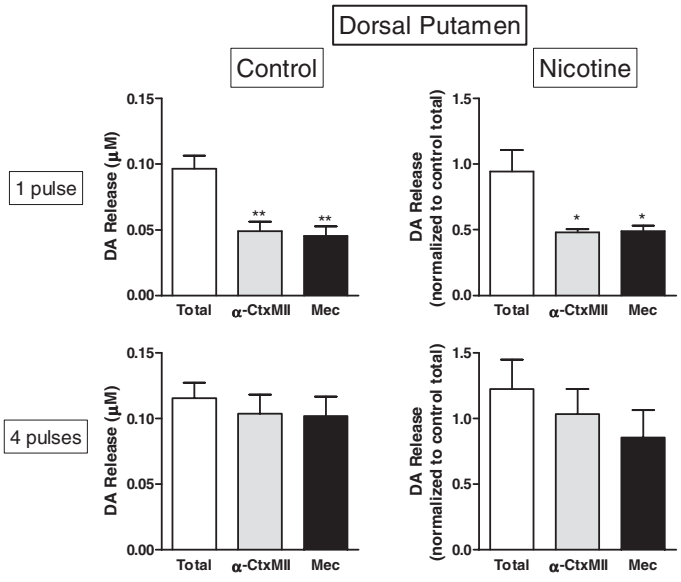

Fig. 7. Quantitative analyses of dorsal putamen showing that long-term nicotine treatment does not modulate evoked dopamine release in this region. Release was measured in the absence (Total) and presence of α-CtxMII (100 nM) and mecamylamine (Mec; 100 μM). Data for the nicotine-treated animals is normalized to total stimulated release in the control. Data represent mean ± S.E.M. of four to seven monkeys. Significance of difference from total release using a Newman-Keuls multiple comparison post hoc test, *, $p < 0.05$; **, $p < 0.01$.

Another common technique used to evaluate striatal nAChR-modulated dopaminergic function involves measurement of synaptosomal [^{3}H]dopamine release (McCallum et al., 2006a; Grady et al., 2007). In contrast to FSCV, which detects electrically evoked release of endogenous dopamine in a slice preparation with a more intact neural circuitry, synaptosomal [^{3}H]dopamine release assays measure nAChR-stimulated release of dopamine from isolated dopaminergic terminals. This latter technique thus provides a more direct measure of alterations in the release process devoid of external influences. The relative proportions of $\alpha3/\alpha6\beta2^*$ versus $\alpha4\beta2^*$ nAChR-modulated nicotine-evoked dopamine release varied somewhat between the two preparations. However, both techniques demonstrate a predominant role for $\alpha3/\alpha6\beta2^*$ nAChRs in dopamine release in the primate dorsal and ventral putamen compared with $\alpha4\beta2^*$ nAChRs under the current experimental conditions.

Accumulating evidence suggests that nAChR stimulation may be useful for the therapeutic management of addiction and neurological disorders that involve the dopaminergic system such as schizophrenia and Parkinson's disease (Picciotto et al., 2008; Quik et al., 2008). For this reason, we initiated studies to understand the consequences of long-term nAChR stimulation on striatal nAChR-modulated responsiveness. The present results show that long-term nicotine treatment altered dopamine release in ventral putamen such that α-CtxMII ($\alpha3/\alpha6\beta2^*$ nAChRs) and mecamylamine ($\alpha4\beta2^*$ and $\alpha3/\alpha6\beta2^*$ nAChRs) no longer significantly decreased dopamine release during either nonburst or burst stimulation. Such cellular adaptive mechanisms may underlie alterations in behavior that arise with long-term nicotine dosing.

The molecular mechanisms responsible for these adaptations in nAChR-modulated responsiveness with long-term nicotine treatment are probably complex. They may involve alterations in receptor desensitization that become evident only with burst stimulation. A molecular mechanism that may be responsible includes nicotine-stimulated changes in protein kinases A- or C-induced phosphorylation of specific amino acid residues on the receptor complex that modulate

TABLE 1

Long-term nicotine treatment does not alter dopamine levels or the dopamine transporter

Monkeys were administered nicotine as detailed in the protocol depicted in Fig. 4. Dopamine levels were then determined in striatal homogenates, and the dopamine transporter was assessed using [^{125}I]RTI-121 autoradiography, as described under *Materials and Methods*. Each value represents the mean ± S.E.M. of four to six animals.

Measure and Region	Control	Nicotine
Dopamine levels, ng/mg protein		
Ventral putamen	135 ± 9.80	174 ± 16.1
Dorsal putamen	144 ± 13.2	194 ± 36.0
Dopamine transporter, nCi/mg tissue		
Ventral putamen	16.5 ± 0.46	17.3 ± 0.96
Dorsal putamen	16.6 ± 0.51	18.8 ± 0.90

its ability to respond to agonists (Giniatullin et al., 2005; Picciotto et al., 2008). Another possibility is that nicotine treatment induces long-term changes in nAChR composition, for instance, by altering the proportion of $(\alpha4)_2(\beta2)_3$ and $(\alpha4)_3(\beta2)_2$ nAChRs with consequent alterations in receptor properties (Tapia et al., 2007; Moroni et al., 2008). It is also feasible that long-term nicotine treatment modifies the subunits present in the receptor complex. Evidence for such a possibility stems from our recent studies in rodents showing that nicotine treatment decreases the levels of the $\alpha6\alpha4\beta2$* subtype but increases the $\alpha6(non\alpha4)\beta2$* nAChR population (Perez et al., 2008a). Nicotine-induced alterations in the proportions of these two $\alpha6\beta2$* nAChR subtypes may explain differential functional response with long-term nicotine administration.

An intriguing observation is our finding that the effect of long-term nicotine on nAChR-modulated dopamine release occurred selectively in the ventral but not dorsal putamen. Consistent with the discussion above, such a situation could arise if nAChR subtypes/isoforms expression is distinct in these two striatal subregions. In addition, regulatory mechanisms that govern dopamine release may be different in striatal subregions. This would not be unlikely in view of the complex mosaic organization of numerous neurotransmitter systems in the striatum and the multiplicity of neuroanatomical connections, with links to sensorimotor, cognitive, and emotive functions (Gerfen, 1992; Parent and Hazrati, 1995). Further studies to identify the factors that lead to regional variations in nAChR function may be important for the development of selective targets for the treatment of addiction and/or neurological movement disorders.

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