Long-Term Nicotine Exposure Depresses Dopamine Release in Nonhuman Primate Nucleus Accumbens

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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 central nervous system changes with nicotine exposure because this may lead to more successful treatment options. Although recent work suggests that α6β2* nicotinic acetylcholine receptors (nAChRs) play a dominant role in dopaminergic function in rodent nucleus accumbens, the effects of long-term nicotine exposure remain to be determined. Here, we used cyclic voltammetry to investigate α6β2* nAChR-mediated release with long-term nicotine treatment in nonhuman primate nucleus accumbens shell. Control studies showed that nAChR-mediated dopamine release occurs predominantly through the α6β2* receptor subtype. Unexpectedly, there was a complete loss of α6β2* 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 long-term 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 α6β2* nAChR-mediated dopamine release. Dopamine uptake rates, as well as dopamine transporter and α6β2* 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 α6β2* 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 approximately 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 α4β2* nAChR subtype (* indicates the possible presence of other nicotinic subunits in the receptor complex) in the reinforcing effects of nicotine (Epping-Jordan et al., 1999; Walters et al., 2006; Picciotto et al., 2008). These include studies showing that mice lacking the β2 nAChR subunit do not self-administer nicotine and that dopaminergic responses to nicotine are decreased in α4 and β2 nAChR-null mice (Picciotto et al., 1998; Marubio et al., 2003). In addition, activation of α4* 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 α4β2* nAChRs, which possibly prevents withdrawal symptoms (Brody et al., 2006). Of importance, nicotine self-administration is reinstated in α4 and β2 knockout mice after re-expression of these subunits in the ventral tegmental area (Pons et al., 2008).

α6β2* 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 α6β2* nAChRs modulate the majority (75–100%) of nAChR-mediated release in nucleus accumbens and striatum.
of rodents and monkeys (McCallum et al., 2006; Exley et al., 2008, 2011; Perez et al., 2008, 2009). In addition, recent behavioral studies show that 682* 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 682* nAChR subtype may also play an important role in nicotine addiction.

The goal of this study was to determine the effect of long-term nicotine treatment on the 682* nAChR-mediated regulation of dopamine release in nonhuman primates. We focused on nucleus accumbens shell because 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 682* nAChRs play a key role in the control of dopamine release in accumbens shell in primates. In addition, it is the first to demonstrate that long-term nicotine exposure significantly decreases 682* nAChR-mediated dopamine release in the accumbens shell.

Materials and Methods

Animal Treatment. Adult female and male squirrel monkeys (Saimiri sciureus) weighing 0.7 to 1.0 kg were purchased from Worldwide Primates (Miami, FL) and quarantined for 1 month according to California state regulations. Animals were housed separately in a room maintained at 27 ± 3°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 Gatorade in the drinking water for 3 to 4 days. Nicotine (free base) was used was diluted Gatorade (50% in water), which was necessary to mask the bitter taste of nicotine. All animals were first given Gatorade in the drinking water for 3 to 4 days. Nicotine (free base) was then added to the Gatorade solution of the 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 Gatorade in the drinking water for 3 to 4 days. Nicotine (free base) was then added to the Gatorade solution of the nicotine-treated group at 27 ± 3°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 Gatorade in the drinking water for 3 to 4 days. Nicotine (free base) was then added to the Gatorade solution of the nicotine-treated group.

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 to 15.0 was bisected along the midline. 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 (Thermo Fisher Science, Waltham, MA), air-dried, and stored at –80°C for autoradiography. 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 125 mM NaCl, 2.5 mM KCl, 1.2 mM Na2HPO4, 2.4 mM CaCl2, 1.2 mM MgCl2, 20 mM HEPES, 11 mM glucose, and 25 mM 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 described previously (Perez et al., 2008). The electrode was positioned below the surface of the slice and its potential was 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). 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 ~200 mV). Peak oxidation currents were converted into concentrations after postexperimental calibration of the electrode with fresh solutions of 0.5 to 1 μM dopamine. Dopamine release was measured as the maximal peak response obtained after electrical stimulation.

Electrically evoked dopamine release was measured in 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, Saratoga, FL) and 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 two to four pulses (2–4 ms in duration) at 30 or 100 Hz applied every 5 min as described previously (Perez et al., 2009). It was not feasible to assess a wider range of frequencies because of 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 682* 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 N-methyl-D-aspartate receptors at high concentrations, this is most likely not a factor in our system because similar results were obtained with α-CtxMII, which is selective for 682* 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 described previously...
(McCallum et al., 2006). Tissue was homogenized in 2 ml of ice-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 addition of 100 nM [3H]dopamine (30–60 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA). Incubation with [3H]dopamine was done for 5 min, and 50-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 nM 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-s exposure to one of a range of nicotine concentrations (0.03–100 nM) or K+ (20 mM). α-CtxMII (50 nM) was perfused for 3 min before nicotine exposure to distinguish between α4β2α and α6β2α nAChR-mediated release. Twelve 18-s fractions (including basal release before and after the stimulated release) were collected, and radioactivity was determined by liquid scintillation counting.

**125I-Epitabidine Autoradiography.** Binding of 125I-epitabidine (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences) was done as previously reported (Quik et al., 2000). 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 CaCl2, and 1.0 mM MgCl2. They were incubated for 40 min with 0.015 nM 125I-epitabidine 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 that for the film blank.

**125I-α-CtxMII Autoradiography.** Binding of 125I-α-CtxMII (specific activity, 2200 Ci/mmol) was done as reported previously (Quik et al., 2001). Striatal sections were preincubated at room temperature for 15 min in binding buffer (144 mM NaCl, 1.5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 20 mM HEPES, and 0.1% bovine serum albumin, pH 7.5, plus 1 mM phenethylmethylsulfonyl fluoride). This was followed by a 1-h incubation at room temperature in binding buffer 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 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 0.1× buffer at 0°C, and then 2 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). Nicotine (100 µM) was used to determine nonspecific binding.

**Dopamine Transporter Autoradiography.** Binding to the dopamine transporter was measured using 125I-3-((4-iodophenyl)tropane-29-carboxylic acid (RTI-121) (2200 Ci/mmol; PerkinElmer Life and Analytical Sciences), as described previously (Quik et al., 2001). 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 were incubated for 2 h in buffer with 0.025% bovine serum albumin, 1 µM fluoxetine, and 50 mM [125I]RTI-121. Sections were washed at 0°C for four times for 15 min each in buffer and once in ice-cold water, air-dried, and exposed for 2 days to Kodak MR film with 3H microscale standards (GE Healthcare). Nomifensine (100 µM) was used to define nonspecific binding.

**Data Analyses.** [3H]Dopamine release was plotted as counts per minute versus fraction number using a curve-fitting algorithm of SigmaPlot 5.0 for DOS. 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 release units. Release units were normalized to the wet weight of the tissue sample on each filter.

ImageQuant (GE Healthcare) 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 femtomoles per milligram of tissue using standard curves determined from 3H standards. These were calibrated for 125I autoradiography using previously described correction factors, including exposure time, section thickness, and concentration of radioactivity (Artyomysyn 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; GraphPad Software Inc., San Diego, CA). Statistical comparisons were performed using unpaired t test analysis, one-way analysis of variance followed by a Newman-Keuls multiple comparisons test, or two-way analysis of variance followed by the Bonferroni post hoc test. p < 0.05 was considered significant. All values are expressed as the mean ± S.E.M. of the indicated number of animals. Values for each animal represent the average of 6 to 15 signals from one to two slices.

**Results**

α6β2α 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-related stimuli (Schultz, 2002; Exley and Cragg, 2008). Voltammetry studies using rodent nucleus accumbens show that α4(α6α6)β2α and α6β2α 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 investigated the role of α4(α6α6)β2α and α6β2α 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. Figure 1, top, depicts representative traces of stimulated dopamine release done in the absence (control) and presence of the α6β2α antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 µM). Quantitative analyses of dopamine release show 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 α3β2α and α6β2α nAChRs in monkey striatum, α6β2α nAChRs form the greater majority of α-CtxMII binding sites with α3β2α 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 α3nAChRs, yield results similar to those in monkeys. Thus, single-pulse stimulated dopamine release from control monkey nucleus accumbens shell appears primarily mediated by α6β2α nAChRs, and possibly α3β2α nAChRs, with very little, if any, contribution from α4(α6α6)β2α 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 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 α6β2* nAChR subtype dominates in the control of nAChR-mediated dopamine release in nucleus accumbens of nonhuman primates.

**Long-Term Nicotine Treatment Depresses Dopamine Release in Nucleus Accumbens Shell.** To date, voltammetry studies have focused on the effect of short-term 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 long-term 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 long-term 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 to 6 months. The final nicotine dose resulted in plasma cotinine levels similar to those in smokers (Table 1) (Matta et al., 2007). Animals were given nicotine in their drinking water 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 those in smokers. Values are mean ± S.E.M. of the indicated number of animals.

**Table 1**

Plasma cotinine levels in monkeys receiving long-term nicotine treatment in their drinking water

<table>
<thead>
<tr>
<th>No. of Animals</th>
<th>Nicotine Dose</th>
<th>Cotinine Level</th>
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<tr>
<td>6</td>
<td>0 μg/ml</td>
<td>0 ng/ml</td>
</tr>
<tr>
<td>10</td>
<td>650 μg/ml</td>
<td>455 ± 57.5 ng/ml</td>
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**Fig. 1.** Single-pulse- and burst-stimulated endogenous dopamine (DA) release from vehicle-treated monkey nucleus accumbens is mediated by α6β2* and not by α4β2* nAChRs. Left, representative traces of dopamine release determined in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (Mec, 100 μM). Scale bar, 10 nM. Right, quantitative analyses of dopamine release show that both α-CtxMII and mecamylamine decrease single-pulse (1p)-stimulated dopamine release to the same extent. Dopamine release stimulated via a train of four pulses at 100 Hz (4p@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 α6β2* nAChRs. The values represent the mean ± S.E.M. of six to seven monkeys. Significance of difference from control release using a Newman-Keuls post hoc test: *, p < 0.05; **, p < 0.01.

Long-term nicotine treatment greatly decreased (p < 0.001) evoked dopamine release in nucleus 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 long-term nicotine treatment on α6β2* and α4(α46)β2* nAChR-modulated dopamine release. To approach this, we again tested release in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (100 μM). Long-term 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 or 100 Hz (Fig. 3, middle and bottom panels). Long-term 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 long-term nicotine treatment depressed α6β2* nAChR-mediated dopamine release in nucleus accumbens shell, as well as release regulated by non-nAChR-mediated mechanisms.

**Long-Term Nicotine Treatment Prevents the Enhanced Paired-Pulse Ratios Observed with nAChR Blockade in Nucleus Accumbens.** As an approach to further understand how long-term 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 the absence and presence of nAChR antagonists (Rice and Cragg, 2004; Zhang and Sulzer, 2004).

Representative traces of dopamine release elicited by one or two 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 long-term 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). In 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, long-term 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 Long-Term 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, because it involves an isolated nerve terminal preparation. Release was measured in the absence and presence of α-CtxMII to distinguish between the α4(α6β2) and α6β2 nAChR-mediated components. In vehicle-treated animals (Fig. 5), the proportion of release regulated by α6β2 nAChRs (~80%) was greater than that regulated by α4(α6β2) nAChRs (~20%), similar to previous findings using the entire accumbens (McCallum et al., 2006). As a somewhat unexpected finding, there was no significant difference in α6β2 or α4(α6β2) 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 effects of long-term nicotine dosing at high- or low-affinity nAChRs. There were no significant differences in the EC50 values for either α4(α6β2) or α6β2 nAChR-stimulated dopamine release in the nicotine-treated compared with vehicle-treated animals. The EC50 values were 2.56 ± 1.40 μM (nicotine-treated) and 1.86 ± 0.29 μM (vehicle-treated) for α4(α6β2) nAChR-mediated release and 1.15 ± 0.45 μM (nicotine-treated) and 0.93 ± 0.13 μM (vehicle-treated) for α6β2 nAChR-mediated release. These data indicate that long-term nicotine treatment does not directly affect mechanisms involved in the regulation of dopamine release by nAChRs.

We also examined the effect of long-term 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.

**Long-Term 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 whether long-term 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 in vehicle- and nicotine-treated animals. Long-term nicotine treatment did not modify dopamine uptake rates (Table 2). Likewise, 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 long-term nicotine treatment arise from changes in the amount of release.
TABLE 2
Chronic nicotine treatment does not affect dopamine transport in nucleus accumbens shell

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Dopamine Transporter Densities (fmol/mg tissue)</th>
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<tbody>
<tr>
<td>Vehicle-treated</td>
<td>12.8 ± 1.52</td>
</tr>
<tr>
<td>Nicotined-treated</td>
<td>11.9 ± 1.53</td>
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Dopamine transporter densities were determined using 125I-RTI-121 autoradiography as described under Materials and Methods. To calculate dopamine uptake rate, constant 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 ± S.E.M. of 6 to 10 animals per group.

Long-Term Nicotine Treatment Increases α6β2* nAChRs but Does Not Affect α6β2* nAChR Expression.

Experiments were performed to determine the effect of long-term nicotine treatment on nAChR binding sites. To identify α6β2* nAChRs, binding of 125I-epibatidine was done in the presence of α6β2* nAChR populations. Release remaining in the presence of α6β2* nAChRs, with the α6β2* nAChR-mediated component determined by subtraction of the α6β2* component from total release. K+-stimulated [3H]dopamine release was done in the presence of 20 mM K+.

The values represent the mean ± S.E.M. of three to five monkeys.

Discussion

The major nAChRs present in the rodent and nonhuman primate nucleus accumbens are the α6β2* and α7(nona6)β2* nAChRs, with little expression of α7 nAChRs (Gotti et al., 2010). Previous voltammetric studies in rodents showed that α6β2* nAChRs primarily regulate the effects of nicotine on dopamine release in nucleus accumbens, with little contribution from α4(nona6)β2* nAChRs (Exley et al., 2008). In vehicle-treated monkeys, the selective α6β2* 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. These observations indicate that α6β2* receptors are also the major contributors to nAChR-evoked dopamine release in nucleus accumbens shell of nonhuman primates, a model offering the advantage that it more closely resembles humans at a genetic, neuroanatomical, and neurochemical level.

We next investigated the effect of long-term nicotine exposure on α6β2* nAChR-modulated dopamine release. Monkeys were exposed long-term to nicotine for several months via their 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 long-term 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 long-term nicotine dosing reduced burst-induced dopamine release, paired-pulse experiments were done. Such studies help identify whether long-term 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 long-term 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-pulse- and burst-stimulated dopamine release (Zhou et al., 2001; Exley et al., 2008). As an alternative, prolonged nAChR desensitization with long-term 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 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 whether the reduced neuronal responsiveness with long-term 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 because 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 compared with that in monkeys receiving long-term nicotine treatment. These results may imply that synaptosomes are not fully representative of the mechanism that controls 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 h before initiation of the release studies, which should allow adequate time 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 α4(α6)β2* nAChRs are up-regulated, with no change in α6β2* nAChRs. Nonspecific binding (Blank) was done in the presence of 100 μM nicotine. AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cd, caudate; Cx, cortex; Put, putamen.

### TABLE 3

<table>
<thead>
<tr>
<th>nAChR Subtype</th>
<th>Binding</th>
<th>% Vehicle-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4(α6)β2* nAChRs</td>
<td>2.82 ± 0.29</td>
<td>3.75 ± 0.32*</td>
</tr>
<tr>
<td>α6β2* nAChRs</td>
<td>0.81 ± 0.10</td>
<td>0.90 ± 0.13</td>
</tr>
</tbody>
</table>

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

### Fig. 6.
Autoradiograms depicting the effect of long-term nicotine (Nic) treatment on α4(α6)β2* and α6β2* nAChR expression. 

**α4(α6)β2**-Epibatidine binding in the presence of α-CtxMII was done to determine α4(α6)β2* nAChRs, whereas α-CtxMII binding was used as a measure of α6β2* nAChR levels. Long-term nicotine treatment increased α4(α6)β2* nAChRs, with no changes in α6β2* nAChRs. Nonspecific binding (Blank) was done in the presence of 100 μM nicotine. AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cd, caudate; Cx, cortex; Put, putamen.
type (Perez et al., 2008). Functional modifications may be linked to changes in expression of only one or another α6β2* nAChR subtype. In nucleus accumbens, this may relate to changes in α6β2γ3 nAChRs because these seem to be the primary receptor population regulating dopamine release in this brain region (Exley et al., 2012). As a somewhat unexpected finding, long-term nicotine treatment also decreased non-nAChR-mediated dopamine release. One explanation for this finding is that long-term nicotine induces long-term changes in the mesocorticolimbic pathway that alter the response of dopaminergic terminals in nucleus accumbens to an electrical stimulus. In fact, nicotine treatment up-regulates mesolimbic α6β2* 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). In addition, long-term nicotine treatment may desensitize α6β2* and/or α4β2* 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 long-term nicotine treatment attenuates the threshold for long-term depression in monkey striatal slices (Quik et al., 2006). Long-term 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 recent studies showing increases in D2 dopamine receptors with nicotine treatment (Novak et al., 2010). Finally, the observed decline in release observed with long-term nicotine exposure is not likely to be due to a decrease in dopamine content, because our previous studies showed no change in dopamine and metabolite levels in nucleus accumbens of nicotine-treated animals compared with those in 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 1 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 up-regulation 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 α4β2* nAChRs, with more recent work also indicating an involvement of α3-, β4-, and/or α5-containing nAChRs (De Biasi and Dani, 2011). Our results show that α6β2* 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 α6β2* nAChRs are also involved in nicotine dependence and withdrawal. Thus, drugs targeting α6β2* 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 α6β2* nAChRs. Moreover, they demonstrate that long-term nicotine treatment significantly decreased dopamine release stimulated by both a single pulse and a train of pulses. These findings indicate that long-term nicotine exposure has major depressive effects on dopamine release in nonhuman primate nucleus accumbens and that α6β2* nAChRs play a critical role.

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Participated in research design: Perez and Quik.
Conducted experiments: Perez and Ly.
Contributed new reagents or analytic tools: McIntosh.
Performed data analysis: Perez and Ly.
Wrote or contributed to the writing of the manuscript: Perez and Quik.

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