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* nAChR 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).

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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.

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; α-CtxMII, α-conotoxin MII; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid.
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 α6β2 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 α6β2 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 α6β2 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 α6β2 nACHRs play a key role in the control of dopamine release in accumbens shell in primates. In addition, they are the first to demonstrate that long-term nicotine exposure significantly decreases α6β2 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 randomly divided into a treatment group and a control group. Food (consisting of monkey chow fruits and vegetables) was available. Control evoked release was first assessed in physiological saline for 3 to 4 h. Nicotine (free base) was then added to the Gatorade solution of the nicotine-treated group starting at a concentration of 50 μg/ml and increased to a final concentration of 650 μg/ml over 3 weeks. Gatorade or Gatorade containing nicotine was also added to the food. One month after the final nicotine concentration was reached, 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). All animals were maintained on Gatorade or nicotine (650 μg/ml) for 3 to 6 months until they were euthanized. There was no difference in dopamine release at the different treatment time points, and therefore the data were 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 of euthanasia solution intraperitoneally (390 mg of sodium pentobarbital and 50 mg of phenytoin sodium/ml), followed by 1.5 ml/kg of the same solution administered intravenously. All studies were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996), and were approved by the Institutional Animal Care and Use Committee at SRI International (Menlo Park, CA).

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 NaH2PO4, 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 into 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, Sarasota, 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 α6β2 nACHR subtype. 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 α6β2 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 by one pulse by the amount of release stimulated with a single pulse.
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 \(^{3}H\) standards. These were calibrated for \(^{125}I\) 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**

**\(\alpha^{6}B_{2}\) 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-related stimuli (Schultz, 2002; Exley and Cragg, 2008). Voltammetry studies using rodent nucleus accumbens show that \(\alpha^{4}(\text{nono6})^{\text{B}_{2}}\) and \(\alpha^{6}B_{2}\) 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 investigated the role of \(\alpha^{4}(\text{nono6})^{\text{B}_{2}}\) and \(\alpha^{6}B_{2}\) 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. Figure 1, top, depicts representative traces of stimulated dopamine release done in the absence (control) and presence of the \(\alpha^{6}B_{2}\) antagonist \(\alpha^{6}C_{MII}\) (100 nM) or the general nAChR blocker mecamylamine (100 \(\mu\)M). Quantitative analyses of dopamine release show that single-pulse-stimulated dopamine release was decreased by \(\sim 30\%\) in the presence of \(\alpha^{6}C_{MII}\) (\(p < 0.01\)) with no further decline in the presence of mecamylamine. Although \(\alpha^{6}C_{MII}\) binds both \(\beta^{3}B_{2}\) and \(\delta^{6}B_{2}\) nAChRs in monkey striatum, \(\delta^{6}B_{2}\) nAChRs form the greater majority of \(\alpha^{6}C_{MII}\) binding sites with \(\beta^{3}B_{2}\) 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 results similar to those in monkeys. Thus, single-pulse stimulated dopamine release from control monkey nucleus accumbens shell appears primarily mediated by \(\delta^{6}B_{2}\) nAChRs, and possibly \(\beta^{3}B_{2}\) nAChRs, with very little, if any, contribution from \(\alpha^{4}(\text{nono6})^{\text{B}_{2}}\) nAChRs.

We also assessed dopamine release evoked by a train of pulses. Release was similar in the absence or presence of \(\alpha^{6}C_{MII}\) 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). The monkeys were then euthanized, and dopamine release was measured in response to a single pulse or a train of pulses delivered at 30 or 100 Hz.

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(α6)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 (Mec, 100 μM). The present findings thus show that long-term nicotine treatment depressed α6β2* nAChR-modulated dopamine release in nucleus accumbens shell, as well as release regulated by non-nAChR-modulated mechanisms.

**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 tracings 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.

**Table 1**

<p>| Plasma cotinine levels in monkeys receiving long-term nicotine treatment in their drinking water |</p>
<table>
<thead>
<tr>
<th>No. of Animals</th>
<th>Nicotine Dose</th>
<th>Cotinine Level</th>
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<td></td>
<td>μg/ml</td>
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<tr>
<td>10</td>
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<tr>
<td>10</td>
<td>650</td>
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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 those in smokers. Values are mean ± S.E.M. of the indicated number of animals.
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(6αβ2)* 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(6αβ2)* 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(6αβ2)* 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(6αβ2)* 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 1.66 ± 0.29 μM (vehicle-treated) for α4(6αβ2)* 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.

Fig. 3. Long-term nicotine (Nic) treatment abolishes the effect of α6β2* nAChR antagonism and decreases non-nAChR-mediated dopamine (DA) release in nucleus accumbens. Top panel, dopamine release in accumbal slices was determined in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (Mec, 100 μM). Quantitative analyses of dopamine release show that nAChR-modulated dopamine release occurs through α6β2* nAChRs in vehicle-treated nucleus accumbens. Long-term nicotine treatment significantly decreased control single-pulse (1p)-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 30 Hz (4p@30 Hz; center panel) or 100 Hz (4p@100 Hz; bottom 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 ± S.E.M. of six to seven monkeys. Significance of difference from control release 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 nicotine (Nic)-treated monkeys. Dopamine release was stimulated by one or two pulses at 100 Hz in the absence (control) and presence of the α6β2* antagonist α-CtxMII (100 nM) or the general nAChR blocker mecamylamine (Mec, 100 μM). Top two panels, representative 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, 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 nicotine-treated monkeys in the presence of nAChR antagonists. The values represent the mean ± S.E.M. of three to five 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.
Long-Term Nicotine Treatment Increases α4β2* 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 α4β2* nAChRs, binding of 125I-epibatidine was done in the presence of α4α1α7 nAChR populations. Release remaining in the presence of α4α1α7 was defined as release mediated by α6β2* nAChRs, with the α6β2* nAChR-mediated component determined by subtraction of the α4β2* 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 α4(αn6)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(αn6)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 accompanied by increases in the relative dopamine release in the presence of 100 µM nicotine. AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Cd, caudate; Cx, cortex; Put, putamen.

In the present study, nicotine treatment was maintained until the time of death. However, it appears unlikely 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(∆non6)β2* nAChRs were up-regulated, with no change in α6β2* nAChRs. These latter data are similar to previous findings in long-term treated monkeys in which nicotine had been withdrawn for 18 to 24 h (McCallum et al., 2006). Finally, if residual nicotine were present in the slices, we would observe decreases in low-frequency-stimulated dopamine 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 long-term nicotine treatment on dopamine release in the primate nucleus accumbens are distinct from our previous observations in monkey putamen (Perez et al., 2008, 2009). In vehicle-treated monkey putamen, α6β2* nAChRs regulate 80 to 100% of single pulse nAChR-modulated dopamine release and α4(∆non6)β2* nAChRs the remainder. However, nicotine treatment did not reduce overall 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 nucleus accumbens versus striatum may relate to differential expression of α6β2* and α4(∆non6)β2* nAChR subtypes in these brain regions. Immunoprecipitation studies show that there are different proportions of α6β4β2β3 and α6β2β3 in rat dorsal versus ventral striatum (Gotti et al., 2010). The presence of distinct nAChR subtypes may contribute to the unique regulatory mechanisms and functional roles of the accumbens compared with the striatum (McCallum et al., 2006; Gotti et al., 2010). For instance, previous work has shown that α6β4β2β3 and α6β2β3 receptors are uniquely regulated by nicotine treatment, with a decline in the former and an increase in the latter sub-

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**TABLE 3**

<table>
<thead>
<tr>
<th>nAChR Subtype</th>
<th>Vehicle-Treated</th>
<th>Nicotin-Treated</th>
<th>% Vehicle-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>α4(∆non6)β2* nAChR</td>
<td>2.82 ± 0.29</td>
<td>3.75 ± 0.32*</td>
<td>132 ± 9.09*</td>
</tr>
<tr>
<td>α6β2* nAChR</td>
<td>0.81 ± 0.10</td>
<td>0.90 ± 0.13</td>
<td>111 ± 15.5</td>
</tr>
</tbody>
</table>

* Significance of difference from vehicle-treated using a t test: p < 0.05.
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 α6β2β3 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 α6β2* 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 depressant 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 depressant effects on dopamine release in nonhuman primate nucleus accumbens and that α6β2* nAChRs play a critical role.

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Authorship Contributions

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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Long-Term Nicotine Depresses Dopamine Release


Perez XA, Bordia T, McIntosh JM, Grady SR, and Quik M (2008) Long-term nicotine treatment differentially regulates striatal α6δ4δ2 and α6δ nons δ2 nAChR expression and function. Mol Pharmacol 74:444–453.


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