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Decrease in α3*/α6* nicotinic receptors but not nicotine-evoked dopamine release in monkey brain after nigrostriatal damage

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ABBREVIATIONS: Abbreviations: α-CtxMII, α-conotoxinMII; PD, Parkinson's Disease; nAChR, nicotinic acetylcholine receptor; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; *denotes nicotinic receptors containing the indicated α and/or β subunit and also additional undefined subunits.

ABSTRACT

Nicotinic receptors (nAChRs) are decreased in the striatum of Parkinson's disease (PD) patients or in experimental models following nigrostriatal damage. Since presynaptic nAChRs on striatal dopamine terminals mediate dopamine release, receptor loss may contribute to behavioral deficits in PD. The present experiments were done to determine whether nAChR function is affected by nigrostriatal damage in nonhuman primates as this model shares many features with PD. Initial characterization of nicotine-evoked [^{3}H]dopamine release from monkey striatal synaptosomes revealed that release was calcium-dependent and inhibited by selective nAChR antagonists. Interestingly, a greater proportion (~ 70%) of release was inhibited by the α3*/α6* antagonist α-conotoxinMII (α-CtxMII) compared to rodents. Next, monkeys were lesioned with MPTP, and [^{3}H]dopamine release, dopamine transporter and nAChRs measured. As anticipated, lesioning decreased the transporter and α3*/α6* nAChRs in caudate and putamen. In contrast, α3*/α6* nAChR-evoked [^{3}H]dopamine release was reduced in caudate, but not putamen demonstrating a dissociation between nAChR sites and function. A different pattern was observed in the mesolimbic dopamine system. Dopamine transporter levels in nucleus accumbens were not reduced after MPTP, as expected, however, there was a 50% decline in α3*/α6* nAChR sites with no decrease in α3*/α6* receptor-evoked dopamine release. No declines in α-CtxMII-resistant nAChR (α4*) binding or nicotine-evoked release were observed in any region. These results show a selective preservation of α3*/α6* nAChR-mediated function in the nigrostriatal and mesolimbic dopamine systems following nigrostriatal damage. Maintenance of function in putamen, a region with selective loss of dopaminergic terminals, may be important in PD.

Parkinson's disease (PD) is characterized by motor and cognitive deficits and is manifested by reductions in dopaminergic neurons in substantia nigra and dopamine levels in striatum (Olanow, 2004). PD is also associated with decreases in striatal nicotinic acetylcholine receptors (nAChRs), including those containing α4*, α3* and/or α6*, but not α7* subunits (*denotes nicotinic receptors containing the indicated α and/or β subunit and also additional undefined subunits; Quik, 2004). Similar declines in nAChRs in Parkinsonian animal models support these findings (Quik et al., 2001, 2002, 2003; Kulak et al., 2002, Zoli et al., 2002; Champtiaux et al., 2002.)

Receptor binding and immunoprecipitation studies have revealed multiple nAChR subtypes on striatal dopaminergic terminals, including both α4* and α6* in rodents (Klink et al., 2001; Zoli et al., 2002; Champtiaux et al., 2002, 2003; Cui et al., 2003) and α3*, α4* and α6* in monkeys (Kulak et al., 2002; Quik et al., 2001, 2002, 2005). In monkey, moderate nigrostriatal damage induced by the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) preferentially affects receptors sensitive to α-CtxMII, or α3*/α6* nAChRs (refers to receptors containing α3 and/or α6 subtypes; Kulak et al., 2002; Quik et al., 2002). The function of nAChRs is of particular interest to PD because of their presence on dopamine nerve terminals. In mammalian brain, nicotine stimulates dopamine release by acting on nAChRs on striatal dopamine terminals, as well as on somatodendritic receptors in the substantia nigra (Rapier et al., 1990; Grady et al., 2001; Pidoplichko et al., 1997; Wonnacott, 1997; Wonnacott et al., 2000).

Recent studies using nAChR null mutant mice and subtype selective α-conotoxins suggest that both α6* (α-CtxMII-sensitive) and α4* (α-CtxMII-resistant) nAChRs contribute to nicotine-evoked dopamine release in striatum (Champtiaux et al., 2003; Cui et al., 2003; Salminen et al., 2004; Azam and McIntosh, 2005). While the specific nAChR populations

reduced by nigrostriatal damage have been identified, the effect of these receptor losses on nAChR function is much less clear, with only one report in rodent to date. This work showed that MPTP lesioning reduced dopamine release in mouse striatum, with the α6* and α4β2 populations being equally affected (Quik et al., 2003).

The aim of the present study was to determine the functional relevance of nAChR declines after nigrostriatal damage in a nonhuman primate model of PD. At a molecular level, the nAChR composition in human striatum may exhibit a closer resemblance to that in nonhuman primates, since striatal α-CtxMII-sensitive α3* nAChRs have been identified in monkey and human but not rodent striatum (Champtiaux et al., 2002; Whiteaker et al., 2002; Quik, 2004). We first characterized nicotine-evoked [^{3}H]dopamine release from monkey striatal synaptosomes. Subsequently, changes in dopamine release, nAChR binding and dopamine transporter levels were measured following acute administration of MPTP. Because a loss of dopamine receptors in nucleus accumbens may be observed in PD (Hornykiewicz, 1998), and is associated with other features of the disease, such as depression or anhedonia, we also measured changes in nAChR binding and function in this region. Our findings reveal a selective preservation of α-CtxMII-sensitive nAChR function following nigrostriatal damage.

Materials and Methods

Materials. The radioligands [^{3}H]dopamine (3,4-[ring-2,5,6-3H], 30–60 Ci/mmol), [^{125}I]RTI-121 (2200 Ci/mmol) and [^{125}I]epibatidine (2200 Ci/ mmol) were obtained from PerkinElmer Life Sciences (Boston, MA). α-ConotoxinMII (α-CtxMII) was synthesized as described by Cartier et al. (1996) and labeled with [^{125}I] (Whiteaker et al., 2000). HEPES was purchased from Roche Applied Science (Indianapolis, IN), and Econo-Safe and BudgetSolve scintillation cocktail from Research Products International (Mount Prospect, IL). The following compounds were obtained from Sigma Chemical Company (St. Louis, MO): α-bungarotoxin,

ascorbic acid, bovine serum albumin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), mecamylamine HCl, (-)-nicotine tartrate, nomifensine, pargyline and sucrose.

Animals. Female squirrel monkeys (*Saimiri sciureus*) were purchased from Osage Research Primates (Osage Beach, MO) or the Primate Research Laboratory at the University of South Alabama (Mobile, AL). Animals weighing between 0.5 and 0.7 kg were housed in a room with a 13:11 hr light/dark cycle. Immediately after arrival, the monkeys were quarantined and tested according to standard veterinary practice. The monkeys had free access to water and were given food once daily. Procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals guidelines and were approved by the Institutional Animal Care and Use Committee.

Drug Treatment and Behavioral Evaluation. After an acclimation period, monkeys were randomly assigned to treatment with a single saline or MPTP injection (1.75 to 2 mg/kg s.c.). Two to four weeks after saline or MPTP injection, animals were rated for parkinsonism using a modified Parkinson rating scale for the squirrel monkey (Quik et al., 2001). The disability scores ranged from 0 to a maximum of 20 for a severely parkinsonian animal. Animals were rated for parkinsonism by assessment of 1) spatial hypokinesia (reduction in use of the available cage space), 2) body bradykinesia (increased slowness in body movement), 3) manual dexterity, 4) balance and 5) freezing. If the animals were not parkinsonian (rating score < 3), they were administered a second MPTP injection. Our previous experience indicated that some animals were more resistant to the effects of MPTP, possibly due to differences in pharmacokinetics or metabolism of MPTP. The second dose of MPTP (1.75 mg/kg) was a somewhat lower dose than that originally administered as previous studies showed the animals were more susceptible to the toxic effects of MPTP upon its re-administration. A second injection to these animals led to declines in dopaminergic measures similar to that in the more sensitive animals. Parkinsonism was again assessed. The monkeys were euthanized 2-4 wk after

the final MPTP administration, at which time the effects of the lesion are maximal with little regeneration (Stanic et al., 2003; Lai et al., 2004).

Tissue preparation. Animals were euthanized in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association and conforming to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Ketamine hydrochloride (15–20 mg/kg i.m.) was administered for sedation approximately 5 min before a lethal injection of 0.22 ml/kg i.v. euthanasia solution (390 mg of sodium pentobarbital and 50 mg phenytoin sodium/ml). The brains were then immediately removed and divided along the midline. One half of each brain was placed in a mold and cut into 6 mm-thick blocks that were frozen in isopentane on dry ice and stored at -80°C for the autoradiographic studies. The other half was sliced into 2 mm sections. Brain regions, including medial caudate, ventral putamen, and nucleus accumbens, were dissected from sections between 15 and 13.5 mm anterior to bregma, according to a squirrel monkey atlas (Fig. 1; Emmers and Akert, 1963). These sub-regions of the caudate and putamen were selected since they have a greater proportion of α-CtxMII-sensitive nAChR binding sites (Quik et al., 2002). For the autoradiographic studies, 20 μm-thick sections were cut from the 6 mm blocks using a cryostat (Leica Microsystems, Inc., Deerfield, IL) cooled to -15°C. After thaw mounting onto Superfrost Plus slides (Fisher, Pittsburgh, PA), sections were air dried and stored at -80°C.

[^{3}H]Dopamine release from synaptosomes. The procedure for measuring nicotine-evoked [^{3}H]dopamine release from synaptosomal preparations of control and lesioned tissue was adapted from the method of Grady et al. (2001). Portions of medial caudate (20-40 mg wet weight), ventral putamen (20-40 mg) and nucleus accumbens (12-25 mg) were each homogenized (20 strokes by hand) using a glass-Teflon homogenizer in a 2 ml volume of ice-cold 0.32 M sucrose buffered with 5 mM HEPES (pH 7.5). In order to stay within a linear range of release, a 0.5 to 2 mg tissue aliquot was used for each sample (filter). The homogenized tissue

was centrifuged at 12,000 g for 20 min to obtain a P1 pellet. Pellets were then resuspended in 0.8 ml uptake buffer containing 128 mM NaCl, 2.4 mM KCl, 3.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.5, 10 mM glucose, 1 mM ascorbic acid, and 0.01 mM pargyline.

Synaptosomes were incubated in uptake buffer for 10 min at 37 °C, then 100 nM [^{3}H]dopamine was added (4 μCi for 0.8 ml synaptosomes) and incubation continued for an additional 5 min. An 80 μl aliquot of synaptosomes (approximately 0.5 to 2 mg tissue) was then pipetted onto 5 mm diameter A/E glass-fiber filters (Gelman, Inc, Ann Arbor, MI) mounted on polypropylene platforms. Filters were perfused with uptake buffer (plus 0.1% bovine serum albumin (BSA) and 10 μM nomifensine) for 10 min at a rate of 1 ml/min before fractions of release were collected. Release was stimulated by an 18-sec exposure to 20 mM K^+ or nicotine (0.001 to 100 μM). Some filters were perfused with 50 nM α-CtxMII or 100 μM mecamylamine for 3 min just before the nicotine exposure. For experiments with α-bungarotoxin, synaptosomes were incubated with or without 1 μM toxin for 30 min at 37°C prior to the addition of [^{3}H]dopamine. For each filter, fifteen 18-sec fractions were collected, which included fractions of basal release before and after the stimulated release. Econosafe cocktail was added to the vials and radioactivity was determined by scintillation counting on a Beckman LS6500 counter (Fullerton, CA).

[^{125}I]RTI-121 Autoradiography. Dopamine transporter binding was measured using [^{125}I]RTI-121 (2200 Ci/mmol; PerkinElmer Life Sciences, Boston, MA) as described previously (Quik et al., 2001). Frozen sections were thawed, then incubated twice for 15 min each at room temperature in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl. Next, sections were incubated for 2 hr in buffer with 0.025% BSA, 1 μM fluoxetine, and 50 pM [^{125}I]RTI-121. Nonspecific binding was determined using 100 μM nomifensine. Sections were washed four times at 0°C for 15 min each in buffer and once in ice-cold water, air dried, and exposed for 2 d

to Kodak MR film (PerkinElmer Life Sciences) with [^{125}I] microscale standards (Amersham Biosciences, Piscataway, NJ).

[^{125}I]α-CtxMII Autoradiography. α-CtxMII was iodinated (Whiteaker et al., 2000) and receptor autoradiography performed as described previously (Quik et al., 2001). Thawed sections were first incubated at room temperature for 15 min in 20 mM HEPES buffer (pH 7.5) containing 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM MgSO4, 0.1% BSA and 1 mM phenylmethylsulfonyl fluoride. This was followed by a 1 hr incubation with [^{125}I]α-CtxMII (0.5 nM) at room temperature in buffer with 0.5% BSA, 5 mM EDTA, 5 mM EGTA, and 10 μg/ml each of aprotinin, leupeptin, and pepstatin A. Slides were washed for 30 sec in 1x HEPES buffer at room temperature, 30 sec in ice-cold 1x buffer, 2 x 5 sec in 0.1x buffer (0°C), and 2 x 5 sec at 0°C in deionized water. Nonspecific [^{125}I]-α-CtxMII binding was determined using 0.1 μM epibatidine. The sections were then air-dried and exposed to Kodak MR film with [^{125}I] standards for 2 to 5 d.

[^{125}I]Epibatidine Autoradiography. Binding was performed as described previously (Perry and Kellar, 1995). Sections were thawed and incubated at room temperature for 40 min in buffer containing 50 mM Tris, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$ plus 0.015 nM [^{125}I]epibatidine (2200 Ci/mmol; PerkinElmer Life Sciences). Inhibition by α-CtxMII was performed with 0.1 μM added to the incubation buffer and incubation continued for an additional 40 min. Nonspecific binding was defined using 0.1 mM nicotine. Following incubation, sections were washed twice for 5 min each in incubation buffer at 4°C and once for 10 sec in ice-cold water. After drying, sections were exposed to Kodak MR film for 1 to 3 d with [^{125}I] standards.

Data Analysis. For dopamine release experiments, a curve-fitting algorithm of SigmaPlot 5.0 for DOS (Jandel Scientific, San Rafael, CA) was used. Release was plotted as counts per minute (cpm) versus fraction number. Basal release was then calculated by selecting

the fractions collected just before and after the peak and plotting them as a single exponential decay function. The first order equation $R_t = R_0 (e^{-kt})$ was used to determine basal release, where R_t = release at time t, R_0 = initial release, and k = rate of decline of basal release. Fractions of release that were at least 10% higher than baseline were added to achieve total cpm released. The calculated baseline release was subtracted from each fraction and the corrected cpm were then normalized to the wet weight of the tissue sample on each filter to obtain units of cpm/mg tissue. In order to obtain R_{max} (a determination of maximal response) and EC_{50} values for dose-response curves, data were fit using a nonlinear regression equation in SigmaPlot 2001 for Windows (SPSS Inc., Chicago, IL). Statistical comparisons were performed using either Student's unpaired *t* test, or two-way repeated measures ANOVA, followed by Bonferroni post-hoc tests where appropriate (GraphPad Prism, GraphPad Software Co., San Diego, CA). A level of $p < 0.05$ was considered significant.

For analysis of autoradiographic data, quantitation of optical densities associated with medial caudate, ventral putamen and nucleus accumbens was done using the ImageQuant system from Amersham Biosciences Inc. (Piscataway, NJ). The optical density values were converted to nCi/mg of tissue using standard curves generated from [^{125}I] standards simultaneously exposed to the films. Activity levels of the [^{125}I] standards ranged from 0.5 to 200 nCi/mg tissue for 20 µm-thick tissue samples as per the manufacturer's specifications (Amersham Biosciences Inc., Piscataway, NJ). Specific binding was determined by subtracting background from total values. Sample optical density readings were within the linear range of the film. The receptor binding value for a brain area for any one animal was obtained from two independent experiments, with one or two consecutive sections per experiment. All values are expressed as the mean ± SEM of the indicated number of animals. Statistical analyses were done using Student's unpaired *t* test where $p < 0.05$ was considered significant (GraphPad Prism).

Results

Characterization of nicotine-evoked [^{3}H]dopamine release from control monkey brain synaptosomes

Although stimulation-evoked release has been measured from monkey striatum *in vitro* using voltammetry (Cragg et al., 2000) and, more recently, nicotine-stimulated release was measured *in vivo* with PET (Marenco et al., 2004), nicotine-evoked [^{3}H]dopamine release from synaptosomes has not previously been studied using monkey brain, although this approach is widely used with rodent preparations (Grady et al., 2001; Wonnacott, 1997; Champtiaux et al., 2003; Quik et al., 2003). Since this technique offers the advantage that it allows for the quantitative determination of evoked-release under well-defined experimental conditions, we conducted experiments to establish its feasibility using monkey synaptosomes. Because the striatum in primates is anatomically heterogeneous (Kemel et al., 1989; Haber et al., 2000), we measured release separately in synaptosomes prepared from the caudate and putamen as well as nucleus accumbens. We focused on portions closer to the midline (medial caudate and ventral putamen) where a denser distribution of α-CtxMII-sensitive sites exists, especially in the medial caudate (Quik et al., 2001, 2002).

Tissue concentration curves using caudate, putamen and nucleus accumbens from control animals revealed a linear relationship for [^{3}H]dopamine release between approximately 0.5 and 5 mg (wet weight) tissue per sample (data not shown). With larger amounts of tissue, the release evoked by either nicotine or 20 mM K^+ showed a tendency to plateau. Therefore, tissue was kept within a range of 0.5 to 2 mg for all release experiments.

Nicotine stimulated the release of [^{3}H]dopamine from monkey synaptosomes prepared from caudate, putamen and nucleus accumbens in a concentration-dependent manner. A sample trace from a single stimulation in caudate from one monkey is shown in Fig. 2. Release evoked

by 20 mM K^+ was similar to that stimulated by a maximal concentration of nicotine (10 μM; Fig. 2).

Experiments were next done in control monkey caudate to ascertain whether nicotine-evoked [^{3}H]dopamine release was calcium-dependent and nAChR-receptor-mediated. The removal of Ca^{2+} from the release and uptake buffers resulted in no measurable dopamine release (3.2 mM Ca^{2+} = 4384 ± 1008 cpm versus 0 mM Ca^{2+} = 119 ± 72 cpm; $p < 0.05$; n=3). This clearly demonstrated that nicotine-evoked [^{3}H]dopamine release from monkey brain is a calcium-dependent process. A 3 min pretreatment of synaptosomes with 100 μM of the noncompetitive nAChR antagonist mecamylamine blocked nicotine-evoked [^{3}H]dopamine release in caudate ($p < 0.01$; Table 1), while release stimulated by 20 mM K^+ was unaffected by mecamylamine pretreatment (data not shown). Pretreatment of synaptosomes for 3 min with 50 nM α-CtxMII, an α3*/α6* nAChR antagonist, blocked 70% of [^{3}H]dopamine release ($p< 0.01$; Table 1), suggesting that dopamine release in monkey striatum is largely mediated by α3*/α6* nAChRs. A 30-min incubation of synaptosomes with 1 μM α-bungarotoxin had no effect on release stimulated by either 20 mM K^+ (not shown) or nicotine (Table 1), suggesting striatal nicotine-evoked [^{3}H]dopamine release is not mediated by α7 nAChRs, as in the rodent.

Nicotinic receptors containing α3 and/or α6 subunits mediate a large proportion of nicotine-evoked [^{3}H]dopamine release in monkey brain. In order to identify the nAChR population(s) mediating nicotine-stimulated [^{3}H]dopamine release in monkey striatum, release was performed in the presence and absence of α-CtxMII (Kulak et al., 1997; Kaiser et al., 1998; Quik et al., 2003; Salminen et al., 2004). α-CtxMII inhibition curves for nicotine-evoked [^{3}H]dopamine release in monkey caudate yielded an IC_{50} value of 4.0 nM (95% CI 0.64-21.2 nM; n = 2), with maximal inhibition at 30 nM α-CtxMII, similar to the mouse (Salminen et al.,

2004). We therefore used a 50 nM concentration of α-CtxMII in the present experiments to provide a maximal blockade of release in monkey brain. The component of dopamine release abolished by 50 nM α-CtxMII was labeled “α-CtxMII-sensitive,” while that remaining in the presence of α-CtxMII was considered “α-CtxMII-resistant”. In control monkey caudate, α-CtxMII inhibited 70% of nicotine-evoked [^{3}H]dopamine release (Table 2); thus, 70% of dopamine release was α-CtxMII-sensitive, and mediated by α3*/α6* nAChRs, while 30% was resistant to inhibition by α-CtxMII. The proportion of α-CtxMII-sensitive dopamine release was similar in putamen (70%) and nucleus accumbens (80%) (Table 2).

Declines in dopamine transporter and nicotinic receptor binding accompany behavioral deficits following damage to the nigrostriatal system. We measured striatal dopamine transporter levels, behavioral ratings of parkinsonism, and striatal [^{125}I]epibatidine binding after acute MPTP exposure in order to evaluate the behavioral and biochemical changes following nigrostriatal damage.

As a marker of dopamine terminal integrity, the dopamine transporter was measured in caudate, putamen, and nucleus accumbens using [^{125}I]RTI binding. The MPTP regimen used in this study resulted in a ~50% decline in the dopamine transporter in both caudate ($p < 0.01$; Fig. 3) and putamen ($p < 0.01$; Fig. 3), measured using [^{125}I]RTI. Mean [^{125}I]RTI binding in caudate, expressed as nCi/mg tissue, was 28.5 ± 1.9 (n = 7) in control animals and 12.1 ± 2.5 (n = 10) in MPTP-lesioned animals. In putamen, these values (in nCi/mg) were 23.0 ± 2.1 in control (n = 7) and 10.1 ± 2.3 (n = 10) in lesioned animals. These values are for medial caudate and ventral putamen, consistent with the striatal areas used for the release studies. Dopamine transporter levels in the nucleus accumbens were not decreased by MPTP treatment ($p = 0.24$; Fig. 3). Mean [^{125}I]RTI-121 binding (in nCi/mg tissue) in this region was 12.03 ± 1.27 for control animals (n = 4) and 17.88 ± 3.20 for lesioned animals (n = 7).

Following MPTP or saline treatment, we also determined the behavioral effects of nigrostriatal damage in these same animals. Parkinsonism averaged 6.3 ± 1.6 out of a maximum of 20 (n = 10) following MPTP treatment, similar to that previously obtained (Quik et al., 2001). Control monkeys showed no signs of parkinsonism.

MPTP exposure decreased total [^{125}I]epibatidine binding in the medial caudate (44%; $p < 0.01$) and ventral putamen (44%; $p < 0.01$; Fig. 3). Total [^{125}I]epibatidine binding in caudate (expressed as nCi/mg tissue) was 0.50 ± 0.05 (n = 7) for control animals and 0.29 ± 0.02 (n = 9) for MPTP-treated animals. In putamen, these values were 0.49 ± 0.05 in control animals (n = 7) and 0.28 ± 0.04 in MPTP-treated animals (n = 9). These declines were somewhat greater than previously observed (Kulak et al., 2002; Quik et al., 2002), probably due to inter-animal variability. Total [^{125}I]epibatidine binding in nucleus accumbens was unaffected after MPTP treatment (Fig. 3) (1.47 ± 0.06 nCi/mg vs 1.39 ± 0.05 nCi/mg; n = 4-5).

Reduction in total nicotine-evoked [^{3}H]dopamine release is smaller than declines in striatal dopamine transporter levels or [^{125}I]epibatidine binding after acute MPTP exposure.

In marked contrast to the consistent decreases in striatal [^{125}I]RTI and [^{125}I]epibatidine binding, nicotine-evoked [^{3}H]dopamine release was less affected, or unaffected, by MPTP administration. Representative nicotine and K^+ -evoked [^{3}H]dopamine release traces in control and MPTP lesioned monkey striatum are shown in Fig. 4. In caudate (Fig. 4, left panels) a decrease in nicotine-evoked [^{3}H]dopamine release was observed. A determination of maximal response (R_{max}) was generated from nicotine dose-response curves (see Methods). In caudate, nicotine-evoked [^{3}H]dopamine release was significantly reduced ($p < 0.01$; Fig. 3), but by a lesser extent (30%) than the dopamine transporter (44%) in this region. The mean R_{max} value for nicotine-evoked release in caudate from control animals was 4800 ± 302 cpm/mg tissue, and MPTP lesioning decreased this value to 3626 ± 136 cpm/mg.

However, in putamen, (Fig. 4, right panels) no reduction of nicotine-evoked [^{3}H]dopamine release occurred following MPTP treatment, despite a nearly 50% decrease in DAT levels (as measured by [^{125}I]RTI binding) and [^{125}I]epibatidine binding (Fig. 3). R_{max} values for dopamine release in putamen were 3620 ± 236 cpm/mg tissue for control animals and 4050 ± 97.6 cpm/mg tissue for MPTP-treated animals (Fig. 3). Nicotine-evoked [^{3}H]dopamine release was also not decreased in the nucleus accumbens following MPTP treatment, and in fact, showed a tendency for an increase, a finding not unexpected due to the lack of reduction of dopamine transporter binding in this region.

[^{3}H]Dopamine release evoked by 20 mM K^+ was examined for control and MPTP-treated monkeys (Fig. 4, middle panels). While a 17% decrease in K^+ - evoked release was observed following lesioning in caudate, this was not significant ($p = .20$; Table 3). In fact, no significant decreases in K^+ - evoked release were found following MPTP treatment in any region measured.

We investigated whether there were differences in basal [^{3}H]dopamine release in caudate, putamen and nucleus accumbens with lesioning that may account for the differential declines in nicotine-evoked release in caudate as compared to putamen. Declines in basal release correlated with the dopamine transporter in a similar manner in the caudate ($r = 0.78$; $p < 0.01$) and putamen ($r = 0.80$; $p < 0.01$) as shown in Fig. 4. No decreases in basal release were observed with MPTP treatment in nucleus accumbens (data not shown).

Reduced nicotine-evoked [^{3}H]dopamine release in caudate of lesioned monkey is due to a selective reduction of α-CtxMII-sensitive sites. In order to evaluate the effect of MPTP treatment on α3*/α6* nAChRs, [^{125}I]epibatidine binding and nicotine-evoked [^{3}H]dopamine release were performed in the presence of α-CtxMII. There were no significant declines in α-CtxMII-resistant [^{125}I]epibatidine binding sites after MPTP treatment in any region examined

(Table 4). Similarly, nigrostriatal damage resulted in no measurable reduction in α-CtxMII-resistant [^{3}H]dopamine release in striatum or nucleus accumbens (Table 4). In fact, a 20% increase in α-CtxMII-resistant release from nucleus accumbens was observed in lesioned animals compared to controls ($p < 0.01$; n = 6-7).

In contrast to the lack of effect of MPTP treatment on α-CtxMII-resistant binding, there was a decrease in α3*/α6* nAChRs in both caudate and putamen, determined using either [^{125}I]α-CtxMII binding or α-CtxMII inhibition of [^{125}I]epibatidine binding. [^{125}I]α-CtxMII binding was significantly decreased in caudate (72%; $p < 0.01$) and putamen (56%; $p < 0.01$) (Fig. 5). Correspondingly, α-CtxMII-sensitive [^{125}I]epibatidine binding was significantly reduced by ~50% in both caudate ($p < 0.05$; Fig 5) and putamen ($p < 0.05$; Fig. 5) following nigrostriatal damage.

Interestingly, a significant decrease in α3*/α6* nAChR binding was also found in the nucleus accumbens of MPTP-treated monkeys. The α-CtxMII-sensitive component of [^{125}I]epibatidine binding was reduced by 62% (Fig. 5). Mean binding levels (nCi/mg) were 0.31 ± 0.03 in control animals (n =11) and 0.13 ± 0.04 in lesioned animals (n = 5; $p < 0.01$). As well, [^{125}I]α-CtxMII binding was reduced by 60% after lesioning (Fig. 5) (0.52 ± 0.04 nCi/mg vs 0.21 ± 0.03 nCi/mg, $p < 0.01$; n = 3-4).

Decreases in α3*/α6* nAChR binding were accompanied by a reduction in α-CtxMII-sensitive nicotine-evoked [^{3}H]dopamine release only in caudate, where the R_{max} for nicotine-stimulated release was reduced by 43% ($p < 0.01$; Fig. 5). The R_{max} for striatal dopamine release in untreated animals was 3261 ± 241 cpm and the R_{max} for release in lesioned animals was 1862 ± 96 cpm. Despite reductions in α3*/α6* nAChR binding in both putamen and nucleus accumbens, no decreases in α-CtxMII-sensitive nAChR function were observed in these regions (Fig. 5).

Nicotine is significantly more potent in stimulating α-CtxMII-sensitive dopamine release than α-CtxMII-resistant release in monkey brain. EC_{50} values for α-CtxMII-sensitive and -resistant release were generated from the dose-response curves from control and MPTP-lesioned monkeys (Fig 6; Table 5). Regardless of treatment group or region, the α-CtxMII-sensitive component of dopamine release consistently exhibited a two-to-five-fold higher affinity for nicotine than the α-CtxMII-resistant component. MPTP treatment had no significant effects on EC_{50} values for either the α-CtxMII-resistant or the α-CtxMII-sensitive component of dopamine release (Table 5). The EC_{50} values (μM) for nicotine-evoked release in control monkey brain were approximately 0.5 μM in each brain region measured (Table 5). These values are similar to those for nicotine-evoked dopamine release from mouse striatal synaptosomes (Salminen et al., 2004).

The analyses of the dose-response curves by two-way ANOVA were in agreement with previous comparisons of the R_{max} values with reductions in dopamine release following MPTP treatment only in caudate. In this region, both total release (two-way ANOVA by treatment: $F_{(1,130)} = 6.5$; $p < 0.05$; Fig. 6) and α-CtxMII-sensitive release ($F_{(1,127)} = 6.7$; $p < 0.01$) were significantly affected by nigrostriatal damage, but dopamine release in putamen ($F_{(1,132)} = 0.60$; $p = 0.44$) and nucleus accumbens ($F_{(10,103)} = 1.29$; $p = 0.26$) was not reduced by MPTP treatment.

Discussion

This investigation is the first report of nicotine-evoked [^{3}H]dopamine release using striatal synaptosomes from nonhuman primates, a species selected for our studies because of its usefulness as a model for PD. Our data demonstrate robust dopamine release mediated by both

α-CtxMII-sensitive (α3*/α6*) and -resistant (α4*) nAChR subtypes, similar to previous measurements from rodent striatum. However, distinct from the rodent, a substantially greater proportion (~70%) of release was mediated through the α3*/α6* nAChR population. Furthermore after nigrostriatal damage, we show that there is a decline in α3*/α6* nAChR-evoked [^{3}H]dopamine release in caudate but not putamen, although lesioning decreased α3*/α6* nAChRs in both caudate and putamen. A dissociation between the number of α3*/α6* nAChR sites and the amount of nicotine-evoked dopamine release was also seen in the nucleus accumbens, with a decline in α3*/α6* receptor binding but no decrease in α3*/α6* receptor-evoked release. These results demonstrate that α3*/α6* nAChR-mediated dopamine release can function at control levels in the presence of a partial receptor loss in selected dopaminergic pathways.

Stimulation-evoked release has previously been measured from control monkey striatum *in vitro* using voltammetry (Cragg et al., 2000) and, more recently, nicotine-stimulated release was measured *in vivo* with PET (Marenco et al., 2004). However, nicotine-evoked [^{3}H]dopamine release has not yet been investigated in control monkey brain or after nigrostriatal damage. Control studies, reported here, show that nicotine elicited a robust, calcium-dependent increase in dopamine release in both striatum and nucleus accumbens with EC_{50} values of ~0.5 μM, similar to that in mouse (Salminen et al., 2004). The pharmacology of dopamine release was comparable to that in the rodent, with a complete block with the nonselective nAChR antagonist mecamylamine, a partial block with the α3*/α6* antagonist α-CtxMII and no effect of the α7 receptor antagonist α-bungarotoxin. These combined data suggest the involvement of non-α7 nAChRs in release, including α3*, α4* and/or α6* subtypes.

Although there are many similarities in release characteristics in monkey and mouse brain, one important distinction is the ratio of α-CtxMII-sensitive versus α-CtxMII-resistant

nAChR-mediated dopamine release. In rodents, ~30% of striatal dopamine release is sensitive to inhibition by α-CtxMII (Kulak et al., 1997; Quik et al., 2003; Salminen et al., 2004). In contrast, in both monkey striatum and nucleus accumbens ~70% of nicotine-evoked [^{3}H]dopamine release was blocked by the toxin. This species difference in release may be related to the greater proportion of α-CtxMII-sensitive nAChRs measured by binding assays in monkey as compared to rodent brain (Whiteaker et al., 2000; Quik et al., 2001, 2003). Although the molecular basis for this interspecies heterogeneity in nAChR subtype proportions remains unknown, it likely reflects species differences in nAChR composition. α-CtxMII interacts with both α3* and α6* nAChRs (McIntosh et al., 2004). However, studies using α3 and α6 null mutant mice have shown that primarily α6* but not α3* nAChR subtypes are found in mouse striatum (Zoli et al., 2002; Whiteaker et al., 2002; Champtiaux et al., 2002). In contrast, recent immunoprecipitation and radioligand binding studies demonstrate that both α3* and α6* subtypes are located in monkey striatum (Quik et al., 2005). Thus, the presence of α3* nAChR may contribute, at least in part, to the greater proportion of α-CtxMII-sensitive receptors in monkey striatum.

Our previous work has shown that striatal nAChR binding sites in monkeys were differentially affected by moderate nigrostriatal damage, with a selective decline in α3*/α6* nAChRs and no change in subtypes containing other subunits such as α4 (Quik et al., 2001). The present results (summarized in Table 6) show that, in caudate, only the α-CtxMII-sensitive component (α3*/α6* receptors) of nicotine-stimulated [^{3}H]dopamine release was decreased following nigrostriatal damage. This was consistent with the receptor decline and with the dopamine transporter decline (Quik et al., 2001). In addition, the present data show there was no significant decrease in α-CtxMII-resistant function (α4* nAChRs) in agreement with the lack of effect of moderate MPTP treatment on α-CtxMII-resistant binding sites (Quik et al., 2001). The small, although non-significant decrease in K^+-evoked [^{3}H]dopamine release in caudate may

suggest that function in only a subset of striatal dopaminergic terminals is affected by moderate nigrostriatal damage.

Similar to the caudate, neither α-CtxMII-resistant binding sites nor evoked-dopamine release mediated by these sites were significantly decreased in the putamen after nigrostriatal damage. As in the caudate, α3*/α6* nAChRs, as well as the dopamine transporter, were decreased ~50% in the putamen with moderate nigrostriatal damage. Unexpectedly, however, α3*/α6* receptor-mediated dopamine release in putamen was not reduced with nigrostriatal damage. Thus, the functional consequences of lesioning are quite distinct in the caudate and putamen, despite similar receptor changes in the two regions after MPTP treatment,

The regulation of nAChR expression in the mesolimbic system appears distinct from that in the nigrostriatal system, as evidenced by the differential pattern of changes obtained in nucleus accumbens in α3*/α6* nAChR sites and function after MPTP treatment (summarized in Table 6). The nucleus accumbens receives dopaminergic projections from the ventral tegmental area, a region more resistant to MPTP treatment than the substantia nigra, with no decline in the dopamine transporter (Hung and Lee, 1996). Consistent with such a finding, no decrease was observed in α-CtxMII-resistant nAChR number or function, and in fact, release was increased. Unexpectedly, however, we observed a decline in α3*/α6* nAChR binding, measured using either [^{125}I]α-CtxMII or α-CtxMII-sensitive [^{125}I]epibatidine binding. One possible interpretation of these findings is that nicotinic α3*/α6* nAChRs in the nucleus accumbens are present on a very small proportion of the dopamine terminals that are selectively vulnerable to MPTP treatment. Another possibility is that nucleus accumbens α3*/α6* nAChRs are downregulated by striatal inputs that are lost with nigrostriatal damage. Despite the decline in α3*/α6* nAChRs, α-CtxMII-sensitive nicotine release remained intact in nucleus accumbens providing another example of functional sparing of α3*/α6* nAChRs similar to that seen in putamen.

Lesion-induced changes previously measured in mouse brain (Quik et al., 2003) had a different pattern than seen in any region measured in monkey brain. In mouse striatum both α-CtxMII-sensitive (α6*) and -resistant (α4*) components of dopamine release were reduced to the same extent with a moderate lesion (Quik et al., 2003). In contrast, the present data show that only α-CtxMII-sensitive and not α-CtxMII-resistant nAChR function is affected by moderate nigrostriatal damage in nonhuman primates. These variations most likely relate to species differences in nicotinic receptor distribution, number or regulation and/or the mechanisms that mediate nigrostriatal damage in the two species. These data suggest that species heterogeneity in striatal dopamine function must be considered when extrapolating responses from rodent to primate brain.

An extensive literature indicates that the basal ganglia are anatomically heterogeneous and composed of distinct nigral neuronal populations that project to divergent striatal areas (Parent et al., 1983; Fearnley and Lees, 1991; Haber et al., 2000). Moreover, various neurotransmitter receptors, transporters and enzymes are organized in distinct ventromedial to dorsolateral gradients in the striatum (Kemel et al., 1989; Haber et al., 2000). Thus, differential effects in nAChR-evoked dopamine release in the caudate and putamen may be possible. While the dopamine transporter and α3*/α6* nAChRs were decreased in both caudate and putamen by nigrostriatal damage, and a similar percentage decline in α3*/α6* mediated release was found in caudate, function in the putamen was unaffected. A different pattern was seen in nucleus accumbens, where there was no change in dopamine transporter, a decrease in α3*/α6* nAChRs and, as in putamen, no change in α3*/α6* nAChR function. In fact, a slight increase in function was observed in nucleus accumbens. These data indicate that function can be maintained in the presence of a receptor decline in both putamen and nucleus accumbens. Although the mechanism(s) by which this enhanced evoked-release in selected regions may occur is not presently known, possibilities include: 1) spare receptors or a larger intracellular pool of

receptors in some regions which can be mobilized to the nerve terminal surface under conditions of damage; 2) enhanced coupling of nicotinic receptors to the dopamine exocytotic process; 3) a change in release properties through decreased auto-inhibition by the D2 receptor in the presence of lower synaptic dopamine levels; and/or 4) compensatory increases in the vesicular monoamine transporter such that quantal release from vesicles is enhanced. There may also be selective recovery of dopaminergic nerve terminals in the putamen, although compensatory sprouting and other regenerative changes generally tend to occur over a longer time frame (Stanic et al., 2003; Lai et al., 2004).

Interestingly, PD is associated with differential declines in the dopamine transporter and dopamine levels by region in striatum. There is an uneven pattern of dopaminergic nerve terminal damage with a more severe decline in dopamine levels and the dopamine transporter in putamen (80-90%) than caudate (~50%) (Kish et al. 1988; Brooks et al. 1990). The present data suggest that mechanisms develop with nigrostriatal damage to maintain nicotine-evoked dopamine release, in the form of enhanced nicotinic receptor-evoked function, possibly because of the critical role of dopamine in movement and posture control.

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Footnotes

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Figure Legends

Fig. 1. Schematic representation of regions used to measure evoked-[^{3}H]dopamine release from monkey brain. Medial portion of caudate (MC), the ventral portion of putamen (VP) and the nucleus accumbens (NAC).

Fig. 2. Nicotine- and K^+- evoked [^{3}H]dopamine release from monkey caudate. Release stimulated by 0.1 μM, 1 μM and 10 μM nicotine (top panel), or 20 mM K^+ (bottom panel) are expressed as counts per minute (18-sec fractions). Data shown are from one control monkey and are representative of 16 experiments.

Fig. 3. Dissociation between [^{125}I]epibatidine ([^{125}I]EPI) binding and nicotine (NIC)-evoked [^{3}H]dopamine (DA) release in putamen but not caudate or nucleus accumbens with nigrostriatal damage. Left panels show that dopamine transporter levels, measured by [^{125}I]RTI binding are reduced by 50% in caudate and putamen but not in nucleus accumbens following nigrostriatal damage. Middle panels show that there are corresponding changes in total [^{125}I]EPI binding in caudate and putamen. In contrast, the right panels show that maximal nicotine-evoked [^{3}H]dopamine release (R_{max} values generated from dose-response curves) is decreased in caudate (28%) but not in putamen or nucleus accumbens following MPTP treatment. (**$p < 0.01$ relative to control by unpaired t-test). Each value represents mean ± SEM of 4 to 9 monkeys.

Fig. 4. Basal and evoked release in control and MPTP-treated monkey striatum. Top panels: representative, single-stimulation traces evoked by 10 μM nicotine from one control monkey and one MPTP-treated monkey for caudate (left) and putamen (right). Middle panels: single-stimulation traces for release evoked by 20 mM K^+ in one control and one MPTP-lesioned monkey. Basal release was slightly decreased with moderate MPTP lesioning. Bottom panels:

correlations between basal release and dopamine transporter levels are significant in caudate and putamen ($p < 0.01$).

Fig. 5. Declines in α3*/α6* nAChR binding and nicotine-evoked [^{3}H]dopamine release in caudate but not putamen and nucleus accumbens. Left panels show that [^{125}I]α-CtxMII binding is significantly decreased in all regions following nigrostriatal damage. Middle panels show a similar pattern for α-CtxMII-sensitive [^{125}I]epibatidine binding. Right panels show a selective reduction in nicotine-evoked, α-CtxMII-sensitive [^{3}H]dopamine release in caudate, while the R_{max} for dopamine release in putamen and nucleus accumbens is unaffected by MPTP treatment. (* $p < 0.05$ and ** $p < 0.01$ relative to control using an unpaired t-test). Each value represents mean ± SEM of 3 to 9 monkeys.

Fig. 6. Dose-response curves for nicotine-evoked [^{3}H]dopamine release in control and MPTP-treated animals. As shown in Table 5, EC_{50} values were not changed by MPTP treatment. However, EC_{50} values for α-Ctx-MII resistant release are consistently higher than those for the α-CtxMII-sensitive component. A decrease in the R_{max} for total release with MPTP lesioning was observed in caudate (see Figure 3), but not putamen or nucleus accumbens (left panels). No differences were observed for α-CtxMII-resistant release in any region measured (middle panels). MPTP lesioning caused a significant decline in the α-CtxMII-sensitive component of dopamine release only in caudate (see Figure 5). Experiments were performed with 8 monkeys per group.

Table 1. Effects of nAChR antagonists on nicotine-evoked [^{3}H]dopamine release

Drug	Conc. (μM)	Nicotine-evoked [^{3}H]dopamine release		
		Control (cpm/mg tissue)	+ Antagonist (cpm/mg tissue)	% Control
Mecamylamine	100	6515 ± 194	50 ± 0.1**	<1
α-ConotoxinMII	0.05	5016 ± 574	1512 ± 281**	30
α-Bungarotoxin	1.0	4302 ± 698	4063 ± 519	94

Mean counts per minute (± SEM) are from experiments performed in monkey caudate (n = 3 to 8 monkeys) and represent a single stimulation with 10 or 30 μM nicotine. (**$p < 0.01$ relative to control using an unpaired t-test).

Table 2. $\alpha3^*/\alpha6^*$ nAChR-mediated nicotine-evoked [^{3}H]dopamine release in monkey brain

Region	R_{max} values (cpm)			% α-CtxMII -sensitive
	Total release	α-CtxMII-resistant release	α-CtxMII-sensitive release	
Caudate	4800 ± 302	1483 ± 135	3261 ± 241**	68%
Putamen	3620 ± 236	1040 ± 113	2530 ± 172**	70%
Nucleus accumbens	5755 ± 300	1913 ± 131	4605 ± 401*	80%

Note that α-CtxMII-sensitive nicotine-evoked dopamine release represents ~70% of the total dopamine release in control monkey striatum compared to ~30% in the rodent. In nucleus accumbens, 80% of dopamine release was mediated via $\alpha3^*/\alpha6^*$ nAChR. R_{max} values (± SEM), expressed as cpm were obtained from nonlinear curve fits of dose-response curves (n = 6-8 monkeys per group). (*$p < 0.05$ and ** $p < 0.01$ relative to α-CtxMII-resistant release using an unpaired t-test).

Table 3. Dopamine release evoked by 20 mM K^+ in control and MPTP-lesioned monkey brain

Region	20 mM K^+ - evoked [^{3}H]dopamine release (± SEM)	
	Control (n = 6-7)	MPTP (n = 8)
Caudate	6256.0 ± 456.9	5214.0 ± 609.7
Putamen	5248.4 ± 599.1	4515.0 ± 908.3
Nucleus accumbens	8302.9 ± 1879.6	9029.6 ± 1277.4

Mean (± SEM) values for K^+ - evoked release are shown for control and MPTP-treated monkeys. No differences in K^+-evoked release were observed between treatment groups in any region measured.

Table 4. Effect of nigrostriatal damage on α-CtxMII-resistant [^{125}I]epibatidine binding and nicotine-evoked [^{3}H]dopamine release

Region	α-CtxMII-resistant [^{125}I]epibatidine binding (nCi/mg)		α-CtxMII-resistant nicotine-evoked [^{3}H]dopamine release (cpm/mg)	
	Control	MPTP	Control	MPTP
Caudate	0.29 ± 0.04	0.21 ± 0.01	1483 ± 135	1643 ± 85
Putamen	0.27 ± 0.03	0.20 ± 0.03	1040 ± 113	1330 ± 120
Nucleus accumbens	1.16 ± 0.09	1.29 ± 0.05	1913 ± 131	2380 ± 36**

[^{125}I]epibatidine binding was performed in the presence of 0.1 μM α-CtxMII. The R_{max} for α-CtxMII-resistant nicotine-evoked [^{3}H]dopamine release was obtained using 50 nM α-CtxMII. Although there was a decline in α-CtxMII-sensitive nicotine-evoked [^{3}H]dopamine release in caudate after nigrostriatal damage (see Fig. 5), α-CtxMII-resistant release was not reduced by lesioning. Means (± SEM) represent values from 4-8 animals per group. (** $p < 0.01$ to corresponding control using an unpaired t-test)

Table 5. EC_{50} values for α-CtxMII-resistant and -sensitive [^{3}H]dopamine release in control and MPTP-lesioned monkeys

		EC_{50} values for nicotine (μM)	
Region	Component	Control	MPTP
Caudate	Total	0.53 ± 0.14	0.60 ± 0.08
	α-CtxMII-resistant	1.62 ± 0.50	1.19 ± 0.20
	α-CtxMII-sensitive	0.31 ± 0.10*	0.45 ± 0.10*
Putamen	Total	0.54 ± 0.14	0.74 ± 0.07
	α-CtxMII-resistant	1.53 ± 0.56	1.15 ± 0.44
	α-CtxMII-sensitive	0.32 ± 0.10#	0.44 ± 0.07
Nucleus accumbens	Total	0.83 ± 0.19	0.77 ± 0.15
	α-CtxMII-resistant	2.02 ± 0.54	1.36 ± 0.09
	α-CtxMII-sensitive	0.58 ± 0.24*	0.81 ± 0.22*

Note that α-CtxMII-sensitive sites exhibit a 2- to 5-fold higher affinity for nicotine-evoked dopamine release. EC_{50} values (mean ± SEM) are from 5-8 animals per group. (* $p < 0.05$ relative to corresponding EC_{50} value for α-CtxMII-resistant component by unpaired t-test. # $p = 0.054$).

Table 6. Summary of alterations in the dopamine transporter, nAChR subtypes and nAChR-subtype-evoked dopamine release in the striatum and nucleus accumbens after nigrostriatal damage

Region	DA transporter	α3*/α6* subtype		α4* subtype	
		Receptor number	Evoked release	Receptor number	Evoked release
Caudate	53*	40*	57*	72	111
Putamen	50*	47*	**102**	74	128
N. Accumbens	166	39**	**126**	111	167**

All values are expressed as percent of the unlesioned controls. Note the selective functional preservation of α3*/α6* receptor-evoked dopamine release in monkey putamen and nucleus accumbens after nigrostriatal damage (bold). Values for the α3*/α6* subtype represent the average of the α-CtxMII-sensitive and $[^{125}I]$α-CtxMII sites, since these two measures were very similar (see Fig. 5). Significantly different from unlesioned monkeys: *$p < 0.05$; **$p < 0.01$.

Fig. 1.

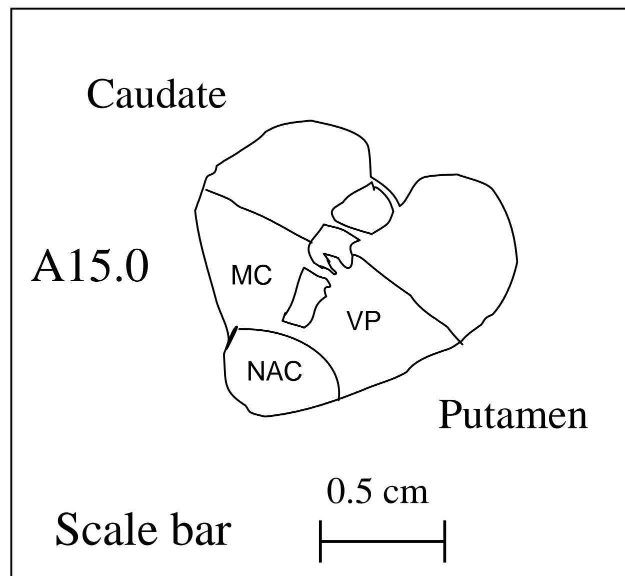

Fig. 2.

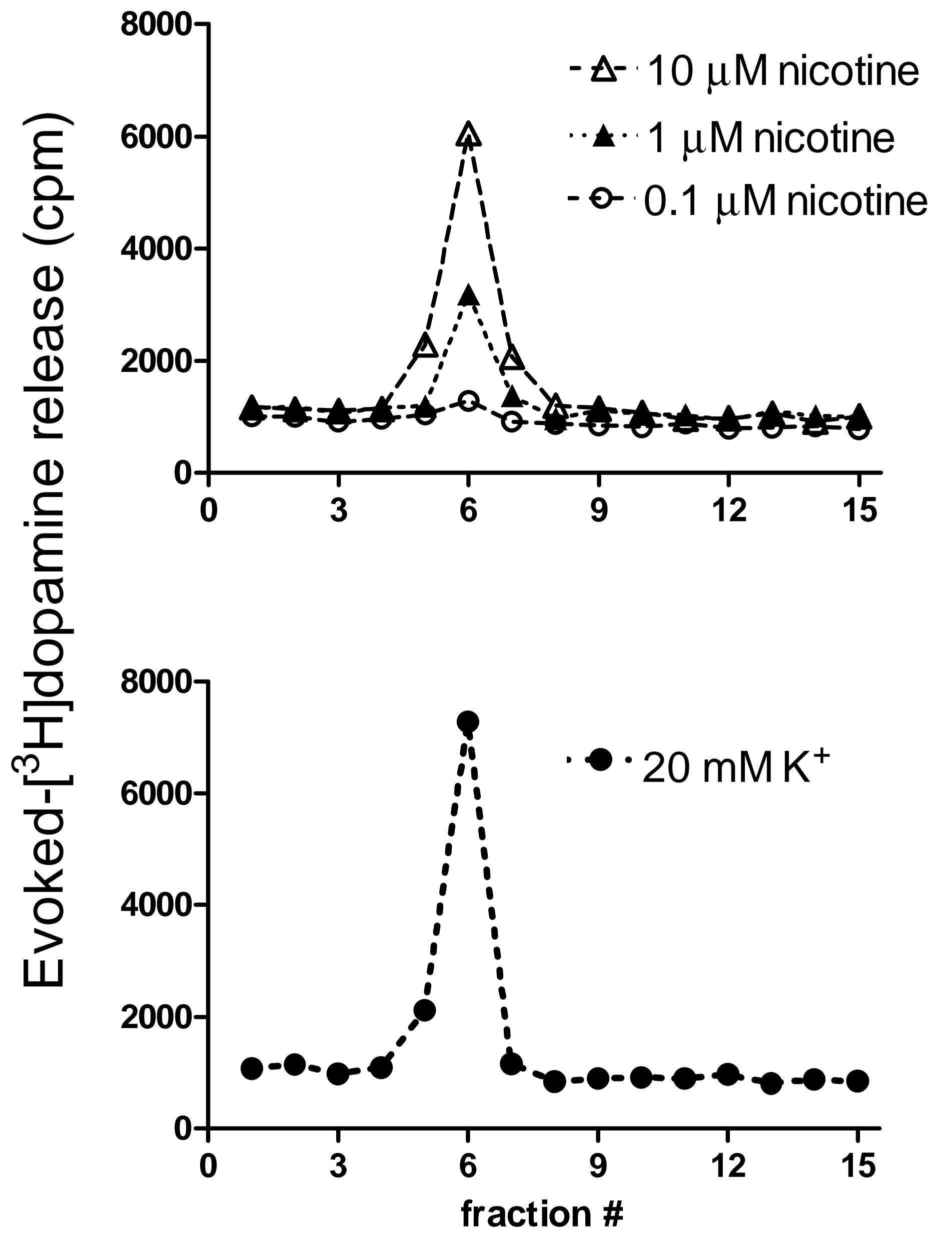

Fig. 3.

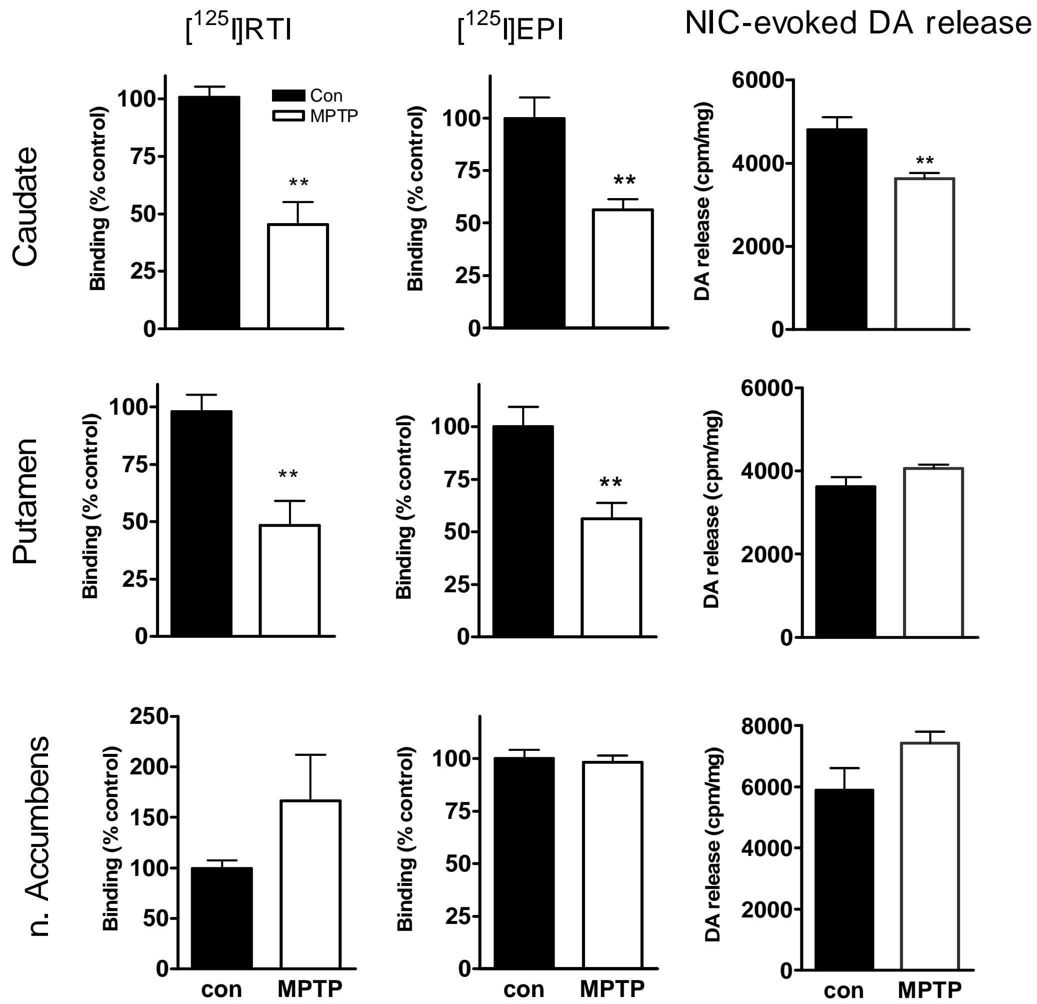

Fig. 4.

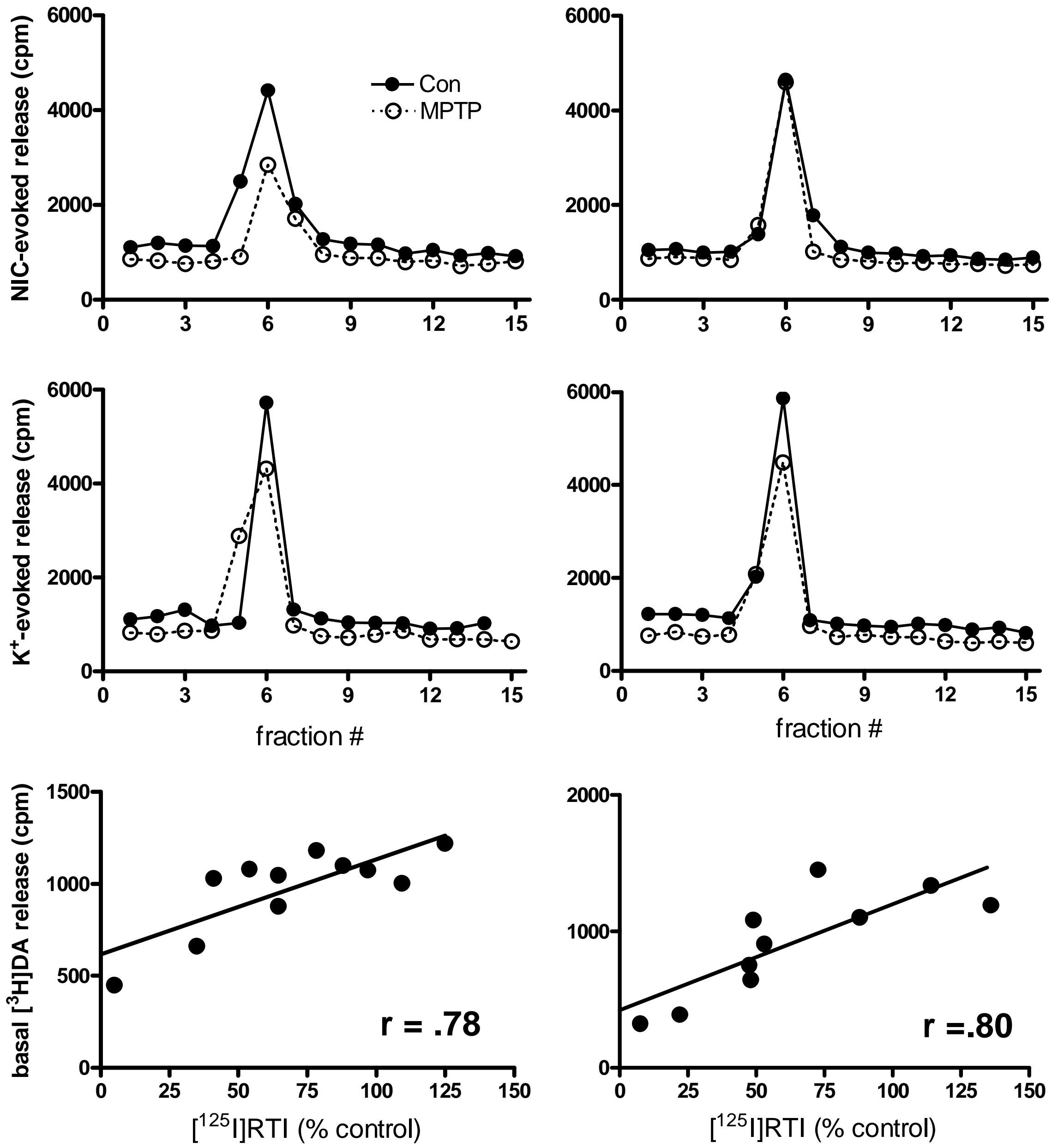

Fig. 5.

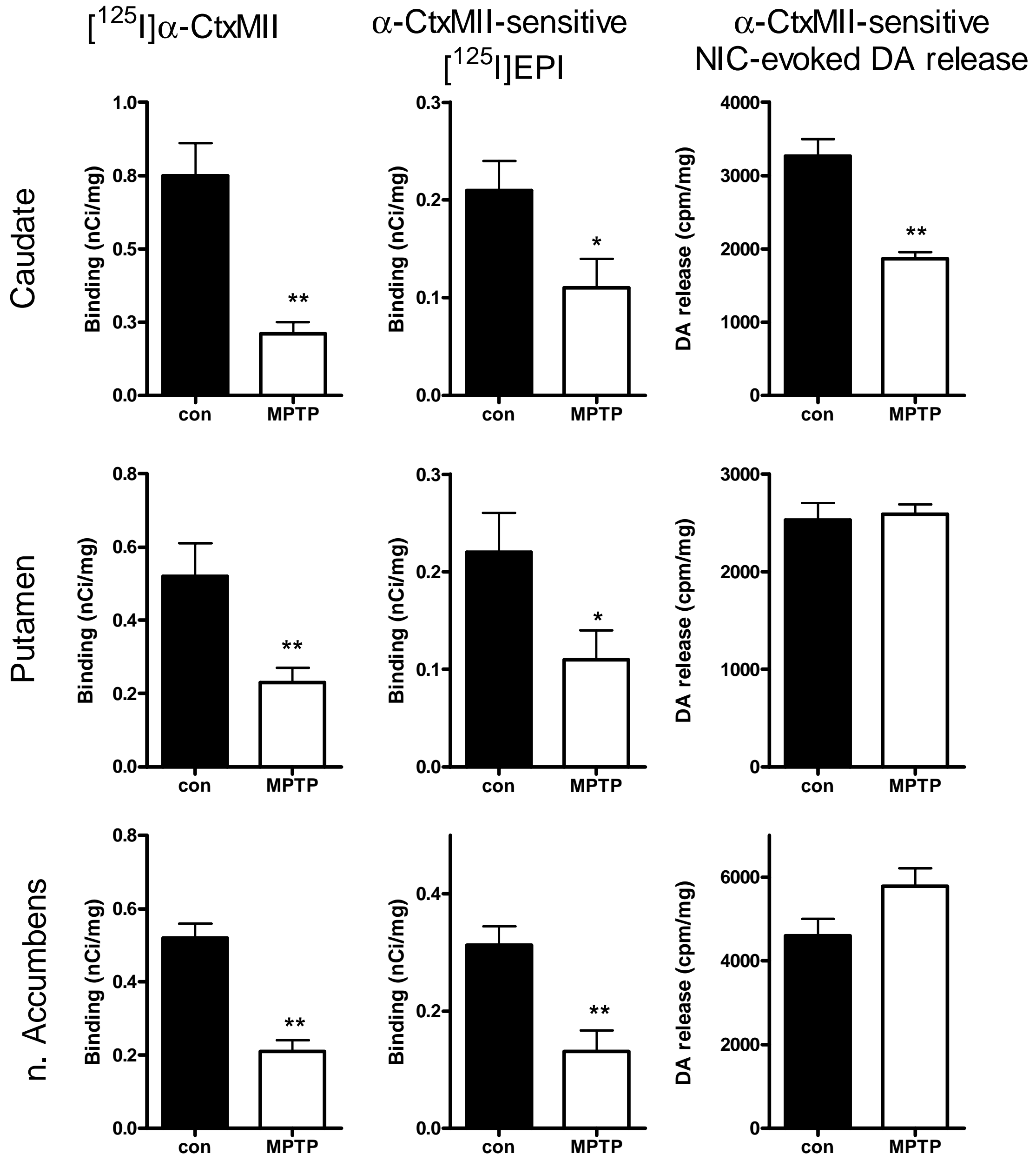

Fig.6.