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Differential Declines in Striatal Nicotinic Receptor Subtype Function after Nigrostriatal Damage in Mice

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ABSTRACT

Nigrostriatal damage leads to a reduction in striatal nicotinic acetylcholine receptors (nAChRs) in rodents, monkeys, and patients with Parkinson's disease. The present studies were undertaken to investigate whether these nAChR declines are associated with alterations in striatal nAChR function and, if so, to identify the receptor subtypes involved. To induce nigrostriatal damage, mice were injected with the selective dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). We measured [^{125}I]3β-(4-iodophenyl)tropane-2β-carboxylic acid isopropyl ester (RTI-121, dopamine transporter), ^{125}I-α-conotoxin MII (putative α6-containing sites in the central nervous system), ^{125}I-epibatidine (multiple sites), 5-[^{125}I]iodo-3-[2(*S*)-azetidinylmethoxy]pyridine-2HCl ([^{125}I]A85380; β2-containing sites), and ^{125}I-α-bungarotoxin (α7-containing sites) binding in brains from control and MPTP-treated mice, as well as nAChR function by [^{3}H]dopamine release, [^{3}H]GABA release, and [^{86}Rb^{+}] efflux. After MPTP treatment, declines were observed in striatal dopamine transporter levels, both binding and functional measures of striatal α-conotoxin MII-sensitive nAChRs, and selected measures of striatal α-conotoxin MII-resistant nAChRs. In contrast, ^{125}I-α-bungarotoxin binding sites were not altered after nigrostriatal damage. The changes in striatal nAChRs were selective, with no declines in cortex, thalamus, or septum. Those striatal binding and functional measures of nAChRs that decreased with MPTP treatment correlated with dopamine transporter declines, an observation suggesting that the binding and functional changes in nAChRs are limited to dopaminergic terminals. The present results are the first to demonstrate differential alterations in nAChR subtype function after nigrostriatal damage, with a close correspondence between changes in receptor binding sites and function. These data suggest that the declines in nAChR sites observed in Parkinson's disease brains may be of functional significance.

Nigrostriatal degeneration is associated with significant declines in nAChRs in all species studied so far, including man. In Parkinson's disease, a neurological disorder characterized by selective damage to dopaminergic nigral neurons (Ball, 2001), radiolabeled epibatidine, cytisine, and nicotine binding sites are decreased in both the striatum and substantia nigra, suggesting an involvement of α4* nAChRs (Perry et al., 1995; Court et al., 2000). Work in animal models also showed that nigrostriatal damage significantly reduced binding of several nAChR radioligands, including [^{3}H]acetylcholine, [^{3}H]nicotine, [^{3}H]epibatidine, [^{125}I]A85380, and ^{125}I-α-conotoxin MII (Schwartz et al., 1984; Clarke and Pert, 1985; Quik et al., 2001, 2002; Kulak et al., 2002a,b; Zoli et al., 2002). These receptor studies, as well as immunoprecipitation experiments (Zoli et al., 2002), suggest that α4β2* and α6β2*, as well as other subtypes, are affected by nigrostriatal damage in rats and monkeys. Furthermore, in monkeys, α-conotoxin MII-sensitive nAChRs (putative α6* nAChRs in the central nervous system; Champtiaux et al., 2002; Whiteaker et al., 2002; Zoli et al., 2002) seem selectively vulnerable to nigrostriatal damage, whereas α4* nAChRs are decreased only after a severe lesion (Quik et al., 2001, 2002; Kulak et al., 2002a,b). Thus, converging data point to deficits in nAChRs with nigrostriatal damage, with an involvement of α4*, α6*, and possibly other nAChR subtypes.

Nigrostriatal damage is linked to movement abnormalities in Parkinson's disease (Ball, 2001). As noted above, neurode-

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ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; [^{125}I]A85380, 5-[^{125}I]iodo-3-[2(*S*)-azetidinylmethoxy]pyridine-2HCl; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; RTI-121, 3β-(4-iodophenyl)tropane-2β-carboxylic acid isopropyl ester; *, nicotinic receptors containing the indicated α and/or β subunit and possibly additional undefined subunits; dopa, 3,4-dihydroxyphenylalanine; BSA, bovine serum albumin.

generation also decreases nAChRs. If nAChR stimulation modulates motor activity, lesion-induced receptor declines may contribute to behavioral deficits. Thus, nicotinic receptor drugs may be beneficial in restoring activity closer to normal levels. Indeed, administration of nicotine or nicotinic agonists seems to ameliorate motor deficits after nigrostriatal degeneration in both rodents and monkeys (Janson et al., 1988; Schneider et al., 1998; Domino et al., 1999; le Novere et al., 1999). In humans, cigarette smoking, the nicotine patch, or nicotine gum also alleviate some of the motor dysfunction observed with Parkinson's disease (see Quik and Kulak, 2002).

Striatal nAChR-stimulated dopamine release represents an excellent functional index of nicotinic-dopaminergic interactions at the cellular level (Wonnacott, 1997; MacDermott et al., 1999). Since the initial studies of Westfall (1974), numerous investigators have shown that nicotine and nicotinic agonists stimulate [^{3}H]dopamine release both in vitro and in vivo (Grady et al., 1992, 1994; Marshall et al., 1997). Although the link between nAChR activation and dopaminergic function is well studied in normal animals, no work has been done to explore this relationship after nigrostriatal damage. However, such studies are critical to understand the significance of receptor changes after denervation, as occurs in Parkinson's disease.

Based on this premise, we examined the relationship between receptor declines and function after MPTP-induced nigrostriatal degeneration and investigated whether select nicotinic receptor subtypes were affected. To approach this, we measured ^{125}I-α-conotoxin MII (α6* sites), ^{125}I-epibatidine (multiple sites), [^{125}I]A85380 (β2* sites), and ^{125}I-α-bungarotoxin (α7* sites) binding in control and MPTP-treated mice. Functional changes were evaluated by measuring nicotine-stimulated release of [^{3}H]dopamine and [^{3}H]GABA and efflux of [$^{86}Rb^+$], as well as K^+-evoked [^{3}H]dopamine release in the striatum. We provide evidence that there is a close correspondence between receptor sites and function and that both α-conotoxin MII-sensitive and -resistant nAChR function is reduced after nigrostriatal damage in the mouse.

Materials and Methods

Animals

Eight- to 10-week-old male C57BL/6 mice were purchased from Simonsen Laboratories (Gilroy, CA) and randomly divided into different treatment groups after 4 days of acclimatization. Mice were placed in a temperature-controlled room with an 11-h/13-h dark/light cycle. They were housed in groups of 3 to 4 per cage and had free access to food and water. MPTP was injected according to one of the following treatment regimens: 20 mg/kg i.p. twice daily over a 3-day period or a single 30 to 35 mg/kg s.c. dose; similar results were obtained with either treatment. Control mice received saline using a similar injection regimen. For the receptor studies, mice were killed by cervical dislocation 7 days after the last MPTP or saline injection. For functional studies, mice were shipped to Colorado 3 days after the final MPTP or saline injection and killed 7 to 11 days after lesioning. All experimental procedures were approved by the Institutional Animal Care and Use Committees and conform to the National Institute of Health *Guidelines for the Care and Use of Laboratory Animals*.

Receptor Autoradiography

For the autoradiographic studies, the brains were quick frozen in isopentane on dry ice and stored at −80°C. When required, brains were sectioned (14 μm) at −20°C using a Leica cryostat. The sections were thaw-mounted onto poly(L-lysine)-coated slides, dried, and stored at −80°C.

[^{125}I]RTI-121 Binding. 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). Thawed sections were first incubated twice for 15 min each at room temperature in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl (buffer A). This was followed by a 2-h incubation in buffer A plus 0.025% bovine serum albumin (BSA), 1 μM fluoxetine, and 100 pM [^{125}I]RTI-121. Nonspecific binding was determined in the presence of nomifensine (100 μM). Sections were washed four times for 15 min each time at 0°C in buffer A and once in ice-cold water, air dried, and exposed for 2 days to Kodak MR film (PerkinElmer Life Sciences) with ^{125}I microscale standards (Amersham Biosciences, Piscataway, NJ).

^{125}I-α-Conotoxin MII Autoradiography. For quantitative autoradiography using ^{125}I-α-conotoxin MII (Quik et al., 2001), sections were first incubated in 20 mM HEPES, pH 7.5, 144 mM NaCl, 1.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, and 0.1% BSA (buffer B) plus 1 mM phenylmethylsulfonyl fluoride at room temperature for 15 min. This was followed by a 1-h incubation at room temperature in buffer B plus 0.2% BSA, 5 mM EDTA, 5 mM EGTA, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, and 0.5 nM ^{125}I-α-conotoxin MII. Nonspecific binding was defined in the presence of 0.1 μM epibatidine. After incubation with ^{125}I-α-conotoxin MII, the slides were rinsed for 30 s in buffer B at room temperature, followed by another 30-s wash in ice-cold buffer B (0°C), two washes for 5 s in 0.1× buffer B (0°C), and two 5-s washes at 0°C in water. Sections were then air dried and exposed to Kodak MR film for 2 to 4 days together with known ^{125}I standards.

^{125}I-Epibatidine Autoradiography. Binding was performed as described previously (Perry and Kellar, 1995; Quik et al., 2000). Sections were thawed and incubated at room temperature for 40 min in 50 mM Tris, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (buffer C) plus 0.03 nM ^{125}I-epibatidine (2200 Ci/mmol; PerkinElmer Life Sciences). Nicotine (0.1 mM) was used to define nonspecific binding. After incubation, sections were washed twice for 5 min each in buffer C at 4°C and once for 10 s in ice-cold water. After drying, sections were exposed to Kodak MR film for 1 to 3 days with ^{125}I standards.

^{125}I-α-Bungarotoxin Binding. Sections were initially incubated for 30 min at room temperature in 50 mM Tris HCl, pH 7.0, and 0.1% BSA (buffer D) as described previously (Quik et al., 2000). The sections were then incubated for 1 h in buffer D plus 3.0 nM ^{125}I-α-bungarotoxin (145 Ci/mmol; PerkinElmer Life Sciences). After four 15-min rinses in buffer D at 4°C, the sections were rinsed once in ice-cold water, air dried, and exposed to Kodak MR film for 5 to 7 days, along with ^{125}I standards.

[^{125}I]A85380 Autoradiography. Thawed sections were incubated at room temperature for 1 h in 50 mM Tris, pH 7.0, 120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, and 1.0 mM $MgCl_2$ (buffer C) plus 95 pM [^{125}I]A85380 (1450 Ci/mmol) as described previously (Kulak et al., 2002b). Nonspecific binding was determined using 0.1 mM nicotine. After incubation, sections were washed twice for 5 min in buffer C at 4°C and once for 10 s in ice-cold water. Air-dried sections were exposed for 1 to 3 days to Kodak MR film, simultaneously with known ^{125}I standards.

Quantitation of the Autoradiographic Images and Data Analysis. Computer-assisted densitometry (ImageQuant; Amersham Biosciences) was used to quantitate radioligand binding. Optical densities of the film images were determined by subtracting background from tissue values. The optical density values were converted to femtomoles per milligram of tissue by comparison with

standard curves generated from ^{125}I standards exposed to film with the tissue sections. Absorbances for tissue sections were within the linear range of the film. Each result for any one animal was determined by averaging the values from 12 striata from two separate experiments. The data represent the mean ± S.E.M. of the indicated number of mice. For statistical analysis, either a Student's t test or a one-way analysis of variance followed by Newman-Keuls multiple comparison test was used, where $p < 0.05$ was considered significant (Prism; GraphPad Software, San Diego, CA).

Functional Studies

Synaptosomal Preparation. Brains were removed, placed on ice, and each striatum and thalamus was dissected and placed into 0.5 ml of 0.32 M sucrose buffered with 5 mM HEPES, pH 7.5. Each region was homogenized (16–20 strokes by hand), diluted to 2 ml with buffered sucrose, and divided into three (thalamus) or five (striatum) aliquots. These were centrifuged for 20 min at 12,000g.

Protein Determination. Protein was assayed by the method of Lowry et al. (1951) using bovine serum albumin as standard. One aliquot of each synaptosomal preparation was assayed, as well as an aliquot of each membrane preparation used to determine [^{125}I]RTI-121 binding.

[^{3}H]GABA Release. Pellets containing the crude synaptosomal preparation from one aliquot of striatum and one of thalamus from each mouse were assayed for [^{3}H]GABA release by the method of Lu et al. (1998) with perfusion buffer slightly modified to yield a somewhat greater release (119 mM NaCl, 3.6 mM KCl, 1.2 mM $MgSO_4$, 3.2 mM $CaCl_2$, 10 mM CsCl, 5 mM HEPES, pH 7.5, 10 mM glucose, and 0.1% BSA). An aliquot containing 10% of the total striatal or thalamic synaptosomal preparation from one mouse was assayed on each filter. Release was stimulated by a 12-s exposure to 30 μM nicotine.

[^{86}Rb^{+}] Efflux. Aliquots of the striatal and thalamic synaptosomal preparations were assayed for [^{86}Rb^{+}] efflux according to the method of Marks et al. (1999). Efflux was stimulated by a 3-s exposure to 10 μM nicotine.

[^{3}H]Dopamine Release. The method of Grady et al. (1992, 2001) was used for measuring [^{3}H]dopamine release from lesioned mice, with minor modifications. Basal release, nicotine-stimulated release, and [^{3}H]dopamine transported by the synaptosomes were linear in the range of 5 to 20 μg of protein per sample. A protein concentration within this range was used, therefore, to allow for an appropriate comparison of results from control and MPTP-treated animals.

For the release assay, an aliquot of the striatal synaptosomal preparation from one mouse (~50–200 μg of protein, equivalent to 6–12% of the total striatal preparation) was resuspended in 0.8 ml of uptake buffer (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). The synaptosomes were incubated for 10 min at 37°C; then, 4 μCi of [^{3}H]dopamine [3,4-[ring-2,5,6]-^{3}H] at 30 to 60 Ci/mmol (PerkinElmer Life Sciences) was added, and the incubation continued for 5 min. Aliquots of labeled synaptosomes were distributed onto eight filters (80 μl containing 5–20 μg of protein/filter) and each was perfused at 1 ml/min with perfusion buffer (uptake buffer with 0.1% BSA and 10 μM nomifensine added) for 10 min before collecting fractions. Release was stimulated with an 18-s exposure to 20 mM K^{+} (2 filters) or to 10 μM nicotine (6 filters). Three of the filters stimulated with nicotine were perfused with 50 nM α-conotoxin MII for 3 min just before the nicotine exposure. For all filters, 15 fractions (18 s) were collected, which included fractions of basal release before and after the stimulated release.

[^{125}I]RTI-121 Binding. Dopamine transporter determinations were also performed on membranes from aliquots of each striatal synaptosomal preparation used for the functional assays. Synaptosomal preparations were resuspended and lysed by homogenization in 3 ml of distilled water followed by incubation for 15 min at 22°C. The resulting membrane preparation was collected by centrifugation at 12,000g (20 min, 4°C) then washed twice by resuspension in ice-cold distilled water and recentrifugation. Membranes were then incubated at 22°C for 3 h in perfusion buffer (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, and 0.1% BSA to maintain compatibility with the conditions used in the functional assays), supplemented with 1 μM fluoxetine and 100 pM [^{125}I]RTI-121. Total and nonspecific binding (in the presence of nomifensine, 100 μM) were determined in triplicate for each sample, using a 96-well plate format. Binding reactions were terminated by filtration onto polyethylenimine-soaked [0.5% (w/v) in perfusion buffer] GF/F glass fiber filters (Gelman Sciences, Ann Arbor, MI) using an Inotech cell harvester (Inotech Biosystems Intl., Rockville, MD). The filters were then washed six times using ice-cold perfusion buffer. Bound radioactivity was measured at 85% efficiency using a PerkinElmer Cobra gamma counter. Protein concentrations, determined for each sample, were used to express specific binding in terms of femtomoles per milligram of protein.

Data Analysis. For release and efflux studies, the fractions preceding and after the stimulated release were used to calculate basal release using the first-order equation $R_t = R_0\ (e^{-kt})$, where R_t is release at time t, R_0 is initial basal release, and k is the rate of decline of basal release. Theoretical basal release for fractions with stimulated release was calculated and subtracted to give the amount of stimulated release in each fraction. Those fractions with significant stimulated release were summed. Data for [^{3}H]GABA release and [^{86}Rb^{+}] efflux were normalized to basal release. Data for [^{3}H]dopamine release were normalized to protein on the filter.

Results

Nigrostriatal Damage Decreases Specific nAChR Populations in Mouse Striatum. To evaluate the magnitude of nigrostriatal damage after MPTP treatment, we measured the dopamine transporter (Figs. 1 and 2), a presynaptic marker of dopaminergic nerve terminal integrity (Quik et al., 2001). A qualitative comparison of lesion-induced changes in nicotinic receptor binding in brain sections from mice with a partial lesion (MPTP1) and from animals with more severe (MPTP2) nigrostriatal damage is depicted in Fig. 1. In these experiments, the dopamine transporter cut-off value to define the severe group was 30% of control.

A quantitative comparison (Fig. 2) of control to all MPTP-treated mice shows that ^{125}I-α-conotoxin MII binding is decreased in parallel with the dopamine transporter, with a decline to 38 ± 5.5% (n = 22) of control for ^{125}I-α-conotoxin MII sites and 33.0 ± 3.4% (n = 22) of control for the dopamine transporter. In contrast, ^{125}I-epibatidine sites and [^{125}I]A85380 sites are reduced to 78 ± 1.6 and 73 ± 1.1% (n = 22) of control, respectively, whereas ^{125}I-α-bungarotoxin binding is unchanged (96 ± 2.6%, n = 22).

Nigrostriatal Damage Selectively Decreases nAChRs in Striatum and Not Other Brain Regions. To determine whether the nAChRs in other brain regions were also affected after MPTP treatment, receptor binding was measured in the cortex and the septal area both at the same rostrocaudal level as the striatum. The results in Table 1 show that the declines were specific to the striatum and not merely the result of nonselective damage to the brain after systemic MPTP administration.

Loss of α-Conotoxin MII-Sensitive and Resistant Sites after Nigrostriatal Damage. ^{125}I-Epibatidine (0.03 nM) binding was measured on striatal sections in the absence and presence of 10^{-7} M α-conotoxin MII, a concentration that

maximally inhibits binding to α-conotoxin MII-sensitive sites (M. Quik and J. Sum, unpublished observations). The results depicted in Fig. 3 show that in unlesioned mice, mean ^{125}I-epibatidine binding was 6.44 ± 0.01 fmol/mg (n = 8). Of these sites, 5.06 ± 0.16 fmol/mg (n = 8) were bound in the presence of α-conotoxin MII (α-conotoxin MII-resistant), indicating that 1.38 fmol/mg of striatal sites are α-conotoxin MII-sensitive. In MPTP-lesioned mice, there was a decrease in total epibatidine binding to 5.04 ± 0.20 fmol/mg (n = 7). Of these sites, 4.16 ± 0.14 fmol/mg (n = 8) remained bound in the presence of α-conotoxin MII (α-conotoxin MII-resistant), whereas the difference of 0.88 fmol/mg represents α-conotoxin MII-sensitive sites. Thus, in mice, MPTP treatment decreases both α-conotoxin MII-resistant sites (from 5.06 to 4.16 fmol/mg) and α-conotoxin MII-sensitive sites (from 1.38 to 0.88 fmol/mg). The magnitude of the declines was not significantly different between the 2 groups ($p > 0.05$).

MPTP Treatment Decreases Striatal Nicotine- and K$^+$-Evoked Dopaminergic Function. To determine the relationship between nAChR sites and receptor-mediated function in the striatum after nigrostriatal damage, we evaluated several measures of nicotine- and K$^+$-evoked function in striatum (Fig. 4). The results show there is a marked reduction in evoked release that is most pronounced in the more severely affected animal. In addition, there is a decline in basal release that corresponds to the decline in the dopamine transporter, as might be expected if these two measures occur in the same cellular compartment. The means ± S.E.M. for groups of control (n = 19) and MPTP-lesioned mice (n = 26) for several measures of nicotine- and K$^+$-evoked function after nigrostriatal damage are depicted in Fig. 5. For this series of experiments, the mean dopamine transporter values (Fig. 5, top) for the MPTP-treated mice (All MPTP) was 47 ± 3% (n = 26) of control, with a range between 11 and 73% of control. To evaluate the effects of more severe nigrostriatal damage, the data for MPTP-treated mice that had dopamine transporter values <45% of control were analyzed separately; the mean dopamine transporter values for these mice was 32 ± 3% (n = 12) of control.

We evaluated the effect of MPTP treatment on the total releasable pool of [^{3}H]dopamine by measuring striatal K$^+$-

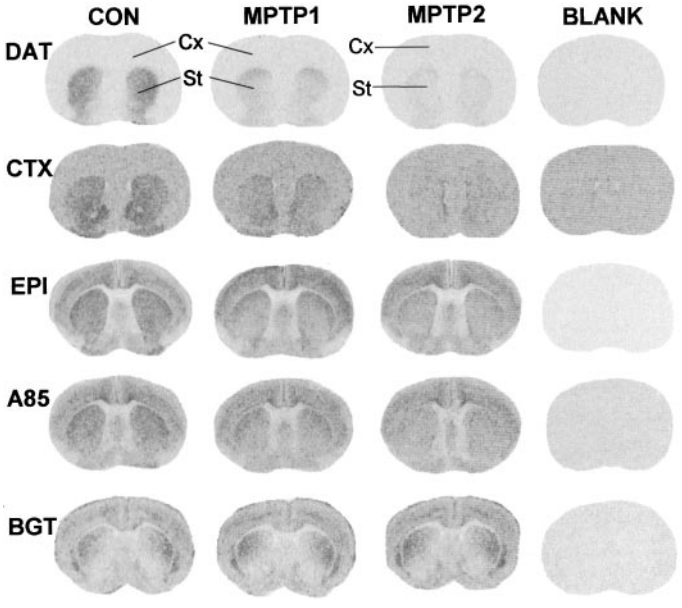

Fig. 1. Autoradiographs depicting the effects of nigrostriatal damage in mouse striatum using [^{125}I]RT1-121 (DAT) to measure the dopamine transporter, ^{125}I-α-conotoxin MII (CTX) to measure putative α6* sites, ^{125}I-epibatidine (EPI) to measure multiple nAChRs, [^{125}I]A85380 (A85) to measure β2* sites, and ^{125}I-α-bungarotoxin (BGT) to measure α7* nAChRs. Mice were treated with saline (CON) or MPTP. Representative sections are shown from a group of mice with a partial (MPTP1) and another from animals with a more complete (MPTP2) nigrostriatal lesion. Note the declines in ^{125}I-α-conotoxin MII binding after MPTP treatment parallel the decreases in the dopamine transporter. Reductions in ^{125}I-epibatidine binding are similar to those in [^{125}I]A85380 binding; however, these are much smaller than those in the dopamine transporter and ^{125}I-α-conotoxin MII binding. No change was observed in ^{125}I-α-bungarotoxin binding after lesioning. Nonspecific binding (BLANK) for each radioligand is shown in the last column. Cx, cortex; St, striatum.

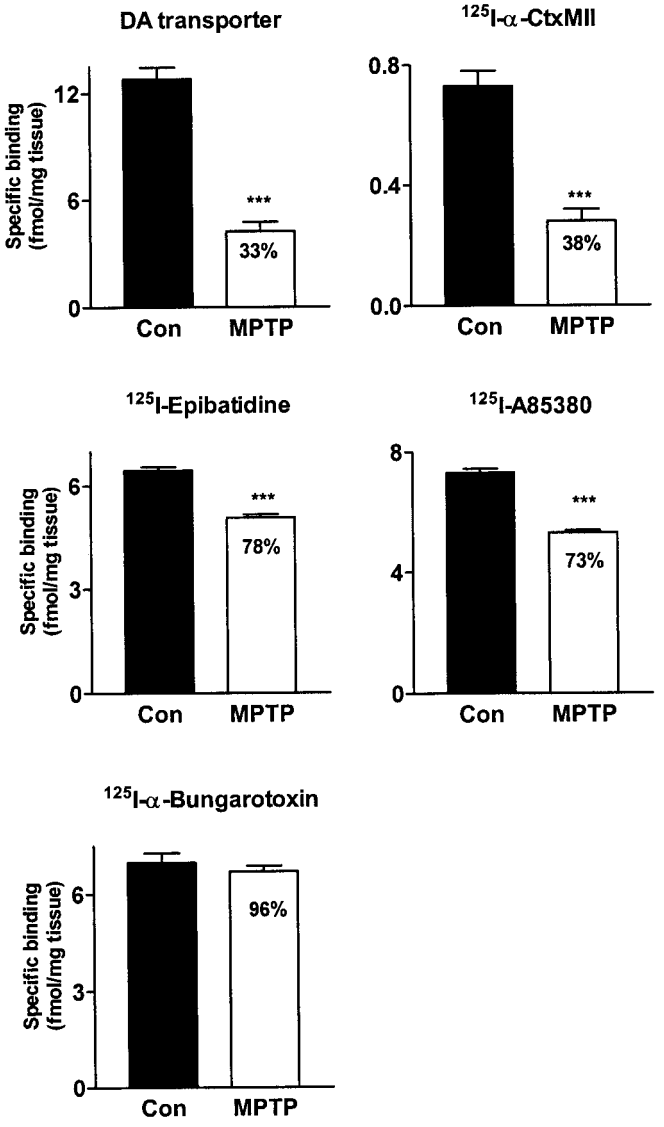

Fig. 2. Quantitative comparison of the distribution of nAChRs in control and MPTP-lesioned striatum. Note the corresponding and relatively large declines in the dopamine transporter and ^{125}I-α-conotoxin MII (α6*) sites with MPTP-induced nigrostriatal damage, suggesting that these receptors are located presynaptically. This is in contrast to the much smaller, although significant, reductions in ^{125}I-epibatidine (multiple sites) and [^{125}I]A85380 (β2* sites) binding after lesioning. No change was detected in α7* sites (^{125}I-α-bungarotoxin) after nigrostriatal damage. Each bar represents the mean ± S.E.M. of 11 control and 22 MPTP-treated mice (includes data from all lesioned animals). Significance of difference from control, ***, $p < 0.001$.

evoked [^{3}H]dopamine release (Fig. 5, middle). K^+-induced release was significantly decreased to 57 ± 7% (n = 18) of control in the all MPTP-treated mice group, and to 36 ± 4% (n = 10) in the more severely affected subgroup of animals. Similar decreases were observed in nicotine-evoked release, which was 67 ± 8% (n = 18) of control for all MPTP-treated mice and 40 ± 5% (n = 10) for the more severe group (Fig. 5, middle). Both these sets of values significantly correlated with declines in the dopamine transporter (see Fig. 8). The corresponding reduction in these two measures of evoked dopamine release suggests that all dopaminergic terminals are similarly affected by MPTP treatment.

Nigrostriatal Damage Results in a Decline in Both α-Conotoxin MII-Sensitive and α-Conotoxin MII-Resistant Nicotine-Evoked Dopamine Release. To determine whether MPTP-induced declines in receptor function were restricted to select nAChR subtypes, release was measured in the absence and presence of α-conotoxin MII, a ligand selective for α6* nAChRs in mouse brain (Champtiaux et al., 2002; Whiteaker et al., 2002; Zoli et al., 2002). The data in Fig. 6 show that there were significant decreases in α-conotoxin MII-resistant release in the all MPTP- and severe MPTP-treated groups of mice (61 ± 8% of control, n = 18, and 35 ± 5% of control, n = 10, respectively). A decline was also observed in the α-conotoxin MII-sensitive component of release (78 ± 10% of control n = 18 for the all MPTP-treated group, and 49 ± 6% of control n = 10 for the severe MPTP group).

MPTP-Induced Nigrostriatal Damage Selectively Modulates Striatal Dopaminergic Function. Striatal nicotine-evoked [$^{86}Rb^+$] efflux (Fig. 5 bottom, Table 2) is mediated by multiple striatal nAChRs present on both dopaminergic and nondopaminergic afferents, as well as possibly a contribution from those on striatal neuronal cell bodies and dendrites. There is a much smaller reduction in this measure of nAChR function with values of 89 ± 4% (n = 15) of control

TABLE 1

Selective declines in striatal ^{125}I-α-conotoxin MII, ^{125}I-epibatidine, and [^{125}I]A85380 receptor sites after MPTP treatment

In striatum, there were significant declines in ^{125}I-α-conotoxin MII, ^{125}I-epibatidine, and [^{125}I]A85380 binding with lesioning, but no change in ^{125}I-α-bungarotoxin sites. In contrast, there were no alterations in binding of any of the nAChR radioligands in the cortex or septal area of animals after MPTP treatment. ^{125}I-α-Conotoxin MII sites were determined at 0.5 nM of the radioligand, ^{125}I-epibatidine at 0.03 nM, [^{125}I]A85380 at 0.095 nM, and ^{125}I-α-bungarotoxin at 3.0 nM. These concentrations were selected to yield an optimal ratio of specific to nonspecific binding or were based on availability (^{125}I-epibatidine). Because these concentrations are not maximal, it is not possible to directly compare the density of sites measured with the different radioligands. Each value represents the mean ± S.E.M. of five to six animals.

Region	Radioligand	Control	MPTP	Percentage of Control
		fmol/mg of tissue		
Striatum	[^{125}I]RTI-121	10.93 ± 0.64	1.63 ± 0.15***	15
	^{125}I-α-Conotoxin MII	0.77 ± 0.06	0.18 ± 0.03***	23
	^{125}I-Epibatidine	6.43 ± 0.09	4.70 ± 0.11***	73
	[^{125}I]A85380	7.84 ± 0.06	5.28 ± 0.06***	67
	^{125}I-α-Bungarotoxin	6.94 ± 0.28	6.38 ± 0.07	92
Cortex	[^{125}I]RTI-121	N.D.	N.D.	
	^{125}I-α-Conotoxin MII	N.D.	N.D.	
	^{125}I-Epibatidine	5.26 ± 0.16	5.34 ± 0.13	102
	[^{125}I]A85380	6.34 ± 0.06	6.23 ± 0.11	98
	^{125}I-α-Bungarotoxin	6.59 ± 0.14	6.38 ± 0.28	97
Septal Area	[^{125}I]RTI-121	N.D.	N.D.	
	^{125}I-α-Conotoxin MII	N.D.	N.D.	
	^{125}I-Epibatidine	2.85 ± 0.17	2.93 ± 0.06	103
	[^{125}I]A85380	3.84 ± 0.11	3.73 ± 0.06	97
	^{125}I-α-Bungarotoxin	2.29 ± 0.07	2.36 ± 0.14	103

N.D., not detected.
***, $P < 0.001$ compared with control.

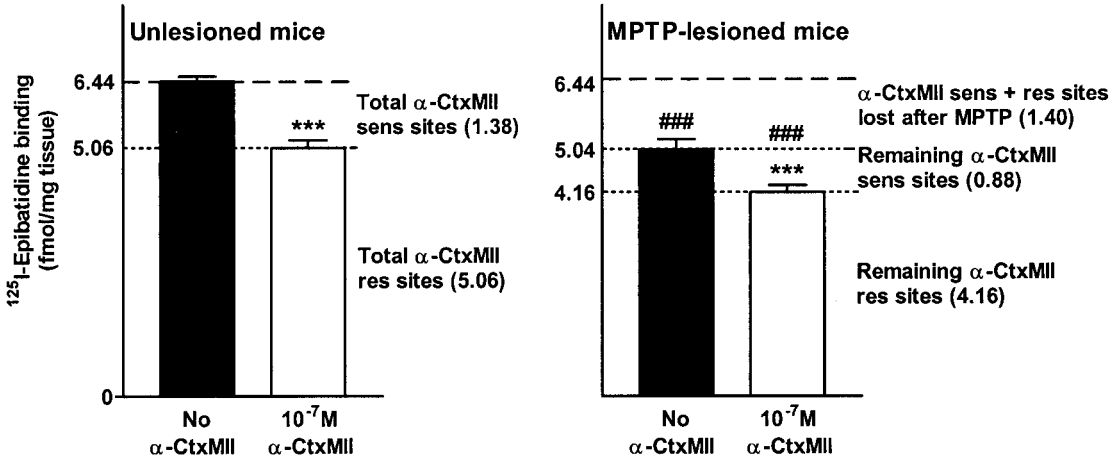

Fig. 3. α-Conotoxin MII (10^{-7} M) inhibition of ^{125}I-epibatidine (0.03 nM) binding in control and MPTP-treated striatum. After MPTP treatment, there is a loss of both α-conotoxin MII-resistant and -sensitive sites, suggesting that both populations are present on nigrostriatal dopaminergic neurons in the mouse. Dopamine transporter levels in MPTP-treated mice were 53 ± 4.8% of control (n = 8) in this experiment. Each bar represents the mean ± S.E.M. of 7 to 8 animals. ***, $p < 0.001$, significantly different from α-conotoxin MII; ###, $p < 0.001$, significantly different from the corresponding group of unlesioned mice. The numbers in parentheses indicate femtomoles per milligram of tissue.

in the all MPTP-treated group and 80 ± 4% (n = 6) in the severely affected mice. These data suggest that the deficit in nAChR function after nigrostriatal damage is largely limited to a loss of receptors on dopaminergic terminals.

Striatal nicotine-evoked [^{3}H]GABA release is a functional response linked to GABAergic neurons that are not directly influenced by MPTP treatment. No change in release is observed (Fig. 5, bottom; Table 2), supporting the assertion that MPTP-induced effects are limited to nigrostriatal dopaminergic terminals.

As another index of selectivity, we measured nicotine-evoked [$^{86}Rb^+$] efflux and nicotine-evoked [^{3}H]GABA release in thalamus in the absence and presence of nigrostriatal damage (Table 2). Neither measure in thalamus was significantly affected by MPTP treatment.

Correlation between nAChR Alterations and the Dopamine Transporter with Nigrostriatal Damage. Correlational analysis was used to evaluate the relationship between changes in the different nAChRs after MPTP treatment and the dopamine transporter, a marker of nigrostriatal damage (Fig. 7). A significant correlation was observed between the transporter and α-conotoxin MII-sensitive receptors ($r = 0.75, p < 0.001$, df = 1, 31), indicating that the decline in these sites corresponds to that in the dopamine transporter. Moreover, for this comparison, the y-intercept fell near the origin (0.12 ± 0.05), suggesting that most α-conotoxin MII-sensitive sites are located on presynaptic dopaminergic terminals.

There was also a significant correlation between dopamine transporter declines and alterations in either striatal ^{125}I-epibatidine sites ($r = 0.85$, $p < 0.001$, df = 1, 31) or [^{125}I]A85380 sites ($r = 0.81$, $p < 0.001$, df = 1, 31), showing that these sites are decreased in parallel with the dopamine transporter. However, for these latter two nAChR populations, the y-intercept was removed from the origin (to 4.5 ± 0.13 and 4.7 ± 0.20, respectively), suggesting that only a subpopulation of sites identified by either ^{125}I-epibatidine (~30%) or [^{125}I]A85380 (~35%) is susceptible to nigrostriatal damage.

^{125}I-α-Bungarotoxin sites were unaffected by MPTP treatment ($r = -0.13, p > 0.05$, df = 1, 16). These findings suggest that ^{125}I-α-bungarotoxin sites are localized on other presynaptic inputs to the striatum (glutamatergic, serotonergic, other) and/or on postsynaptic GABAergic or cholinergic neurons not affected by nigrostriatal damage.

Correlation Between Changes in Nicotine- and K$^+$-Evoked Dopaminergic Function and the Dopamine Transporter with Nigrostriatal Damage. An analysis similar to that described in the preceding section was used to assess the relationship between declines in nAChR-mediated function and the dopamine transporter in the synaptosomal preparation (Fig. 8). A significant correlation was obtained with MPTP treatment between the dopamine transporter- and K$^+$-evoked dopamine release as percentage of control ($r = 0.85$, $p < 0.001$, df = 1, 16), suggesting that these two measures are similarly changed after lesioning. In this instance, the y-intercept was not significantly different from the origin (−6.8 ± 10.3) indicating a close relationship between changes in the dopamine transporter and K$^+$-evoked dopamine release. There was also a significant correlation between dopamine transporter declines and alterations in nicotine-evoked dopamine release ($r = 0.83$, $p < 0.001$, df = 1, 16), again with the y-intercept not significantly different from the origin (−11.4 ± 14.2).

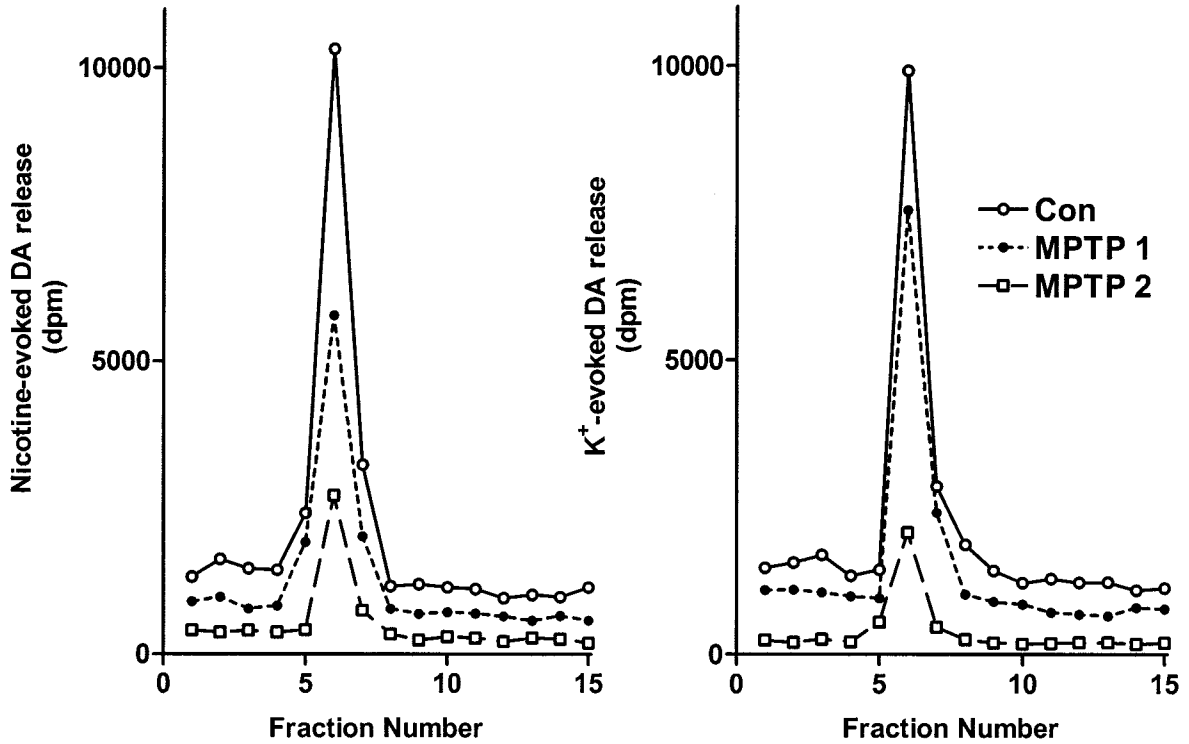

Fig. 4. Effect of MPTP treatment on nicotine (10 μM)- and K$^+$ (20 mM)-evoked dopamine release from striatal synaptosomes. Raw data are from single filters from a representative control mouse (Con), from a mouse with a dopamine transporter value 66% of control (MPTP 1), and from a mouse with a dopamine transporter value 11% of control (MPTP 2). Note the decline in both nicotine- and K$^+$-evoked dopamine release, as well as in baseline release. Eighteen-second fractions were collected.

The relationship between MPTP-induced changes in the dopamine transporter and either α-conotoxin MII-resistant ($r = 0.82$, $p < 0.001$, df = 1, 16; y-intercept = −12.3 ± 13.6) or α-conotoxin MII-sensitive ($r = 0.77$, $p < 0.001$, df = 1, 16; y-intercept = −11.1 ± 19.5) nicotine-evoked dopamine release was similar to that described above for nicotine-evoked dopamine release. These data suggest that these nAChR subpopulations are altered in a corresponding fashion by nigrostriatal damage.

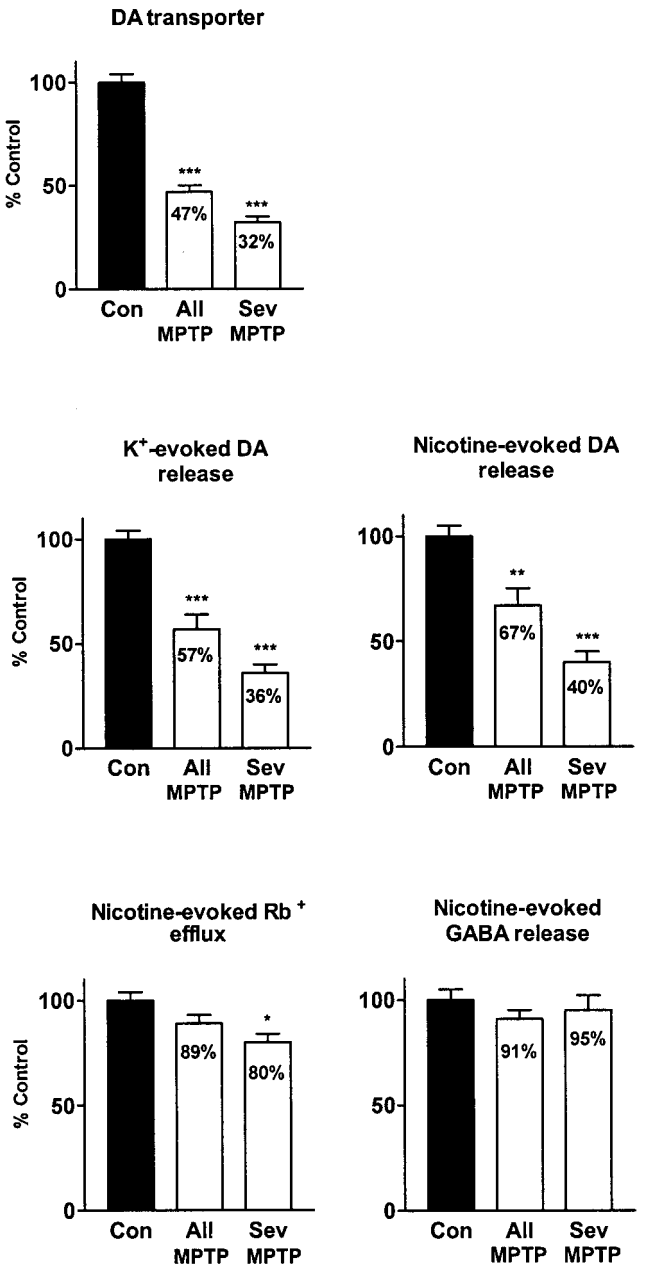

Fig. 5. Selective declines in striatal dopaminergic function after MPTP treatment. The first bar represents data from control animals (Con), the second from all MPTP-treated mice (All MPTP), and the third from MPTP-treated mice with dopamine transporter values <45% of control (Sev MPTP). Note the parallel declines in the striatal dopamine transporter and K^+- and nicotine-evoked dopamine release. In contrast, there is only a small, although significant, decline in nicotine-evoked Rb^+ efflux, with no change in nAChR-mediated GABA release. Each bar represents the mean ± S.E.M. of 6 to 18 mice. Significance of difference from control, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Declines in nicotine-evoked [$^{86}Rb^+$] efflux also correlated positively with changes in the dopamine transporter. However, in this case, the y-intercept was significantly different from the origin (53.5 ± 9.7), suggesting that only a subpopulation of sites mediating [$^{86}Rb^+$] efflux is localized to dopaminergic terminals. Nicotine-evoked GABA release was unaffected by MPTP treatment ($r = 0.15$, $p > 0.05$, df = 1, 14; y-intercept = 85.7 ± 10.6), suggesting that striatal GABAergic terminals are not affected by nigrostriatal damage.

All Dopaminergic Terminals seem Equally Affected after MPTP-Induced Neuronal Damage. To determine whether there may be a selective vulnerability of dopaminergic terminals expressing nAChRs to MPTP treatment, we compared the ratio of K^+- to nicotine-evoked dopamine release with varying degrees of nigrostriatal damage. Because the correlation coefficient ($r = 0.18$, $p > 0.05$, df = 1,16) was not significantly different from zero, the results show that the ratio of release by the two stimulants was similar regardless of the extent of nigrostriatal damage (Fig. 9 top). In addition, the ratio did not differ from that determined in control mice. These results suggest that MPTP affects dopaminergic terminals indiscriminately.

α-Conotoxin MII-Sensitive and α-conotoxin MII-Resistant nAChR-Mediated Dopamine Release Is Reduced to a Similar Extent after MPTP Treatment. We also evaluated the ratio of α-conotoxin MII-sensitive to -resistant nicotine-evoked dopamine release with varying degrees of nigrostriatal damage. The data (Fig. 9, bottom) show that there is no significant correlation ($r = 0.45$, $p > 0.05$, df = 1, 16) between the ratio of these two release components and changes in the dopamine transporter, suggesting that there is a corresponding change in α-conotoxin MII-sensitive and -resistant sites after lesioning.

Discussion

Differential Changes in nAChR Subtypes in Striatum after Nigrostriatal Damage. In the present study, we determined the effects of MPTP-induced nigrostriatal damage on nAChR sites and function in mouse striatum. The main findings are that there are distinct alterations in dif-

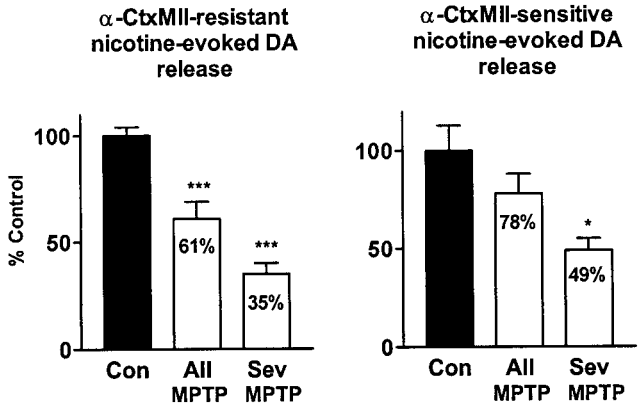

Fig. 6. Similar declines in α-conotoxin MII-resistant and -sensitive nicotine-evoked dopamine release. The first bar represents data from control animals (Con), the second from all MPTP-treated mice (All MPTP), and the third from MPTP-treated mice with dopamine transporter values <45% of control (Sev MPTP). Note the corresponding decreases in the α-conotoxin MII-resistant and -sensitive components of nicotine-evoked dopamine release. Each bar represents the mean ± S.E.M. of 10 to 18 mice. Significance of difference from control, *, $p < 0.05$; ***, $p < 0.001$.

ferent nAChR subtypes after lesioning, with declines in both α-conotoxin MII-sensitive and -resistant sites, but no change in α7* nAChRs. These changes parallel those in striatal nAChR responsiveness, with a close correspondence between nAChR function and receptor sites after nigrostriatal damage.

Nigrostriatal damage decreased ^{125}I-α-conotoxin MII sites in parallel with the dopamine transporter, a marker localized to dopaminergic neurons (Miller et al., 1999). The coincident declines suggest that striatal ^{125}I-α-conotoxin MII sites are primarily localized to presynaptic dopaminergic terminals in mice, a finding consistent with that in monkeys (Quik et al., 2001). By contrast, ^{125}I-epibatidine and [^{125}I]A85380 binding sites, although altered in a similar manner after MPTP treatment, were decreased to a much smaller extent than the ^{125}I-α-conotoxin MII sites. These receptor changes are consistent with our understanding of the nAChR subtypes with which the different radioligands are thought to interact. ^{125}I-

TABLE 2

Selective declines in nicotine-evoked dopamine release and [^{86}Rb$^+$] efflux after MPTP treatment

Nicotine-evoked dopamine release, [^{86}Rb$^+$] efflux, and [^{3}H]GABA release from synaptosomes was performed as described. A comparison of functional changes in striatum and thalamus in control and MPTP-treated mice showed that there were significant declines in nicotine-evoked [^{3}H]dopamine release and [^{86}Rb$^+$] efflux in the striatum but not the thalamus, with no change in [^{3}H]GABA release in either region. Dopamine transporter levels in the striatum of MPTP-treated mice were 38 ± 3% (n = 6) of control. Each value represents the mean ± S.E.M. of five to six animals.

Region	Nicotine-Stimulated Release/Efflux	Control	MPTP	Percentage of Control
		dpm	*dpm*	
Striatum	[^{3}H]dopamine release[a]	491 ± 33	269 ± 29***	55 ± 6
	[^{86}Rb$^+$] efflux[b]	22.7 ± 1.11	17.8 ± 1.18*	79 ± 5
	[^{3}H]GABA release[c]	4.20 ± 0.33	4.49 ± 0.18	107 ± 4
Thalamus	[^{3}H]dopamine release[a]	N.A.	N.A.	N.A.
	[^{86}Rb$^+$] efflux[b]	47.1 ± 2.98	48.6 ± 1.89	104 ± 4
	[^{3}H]GABA release[C]	2.62 ± 0.27	2.38 ± 0.11	91 ± 4

N.A., not assayed.
*$P < 0.05$; ***$P < 0.001$ compared with control.
[a] By 10 μ M nicotine/μ g of synaptosomal protein.
[b] By 10 μ M nicotine/dpm in baseline.
[c] By 30 μM nicotine/dpm in baseline.

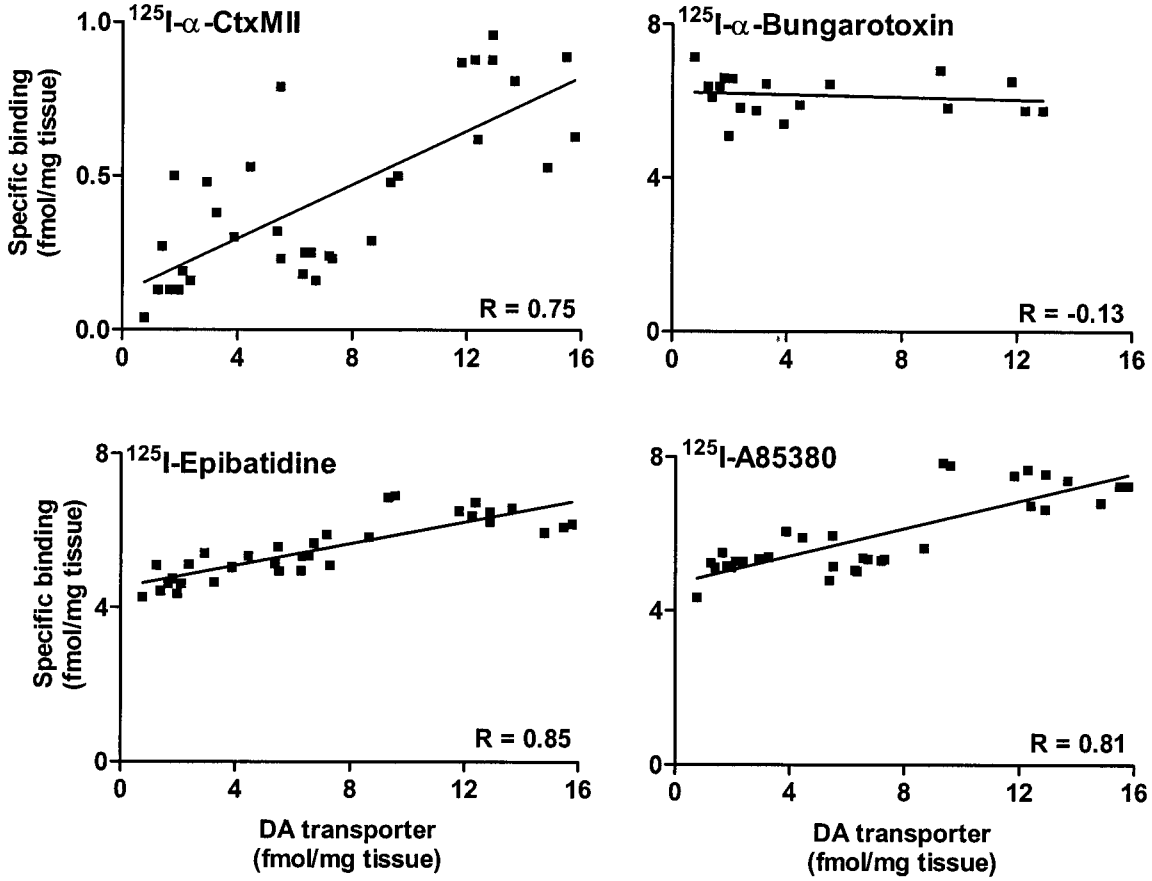

Fig. 7. Correlation between the dopamine transporter and ^{125}I-α-conotoxin MII, ^{125}I-epibatidine, [^{125}I]A85380, and ^{125}I-α-bungarotoxin sites in control and MPTP-lesioned mouse striatum. The correlations between the dopamine transporter and ^{125}I-α-conotoxin MII, ^{125}I-epibatidine, and [^{125}I]A85380 are all significant at $p < 0.001$. Note that the y-intercept is significantly above the origin for ^{125}I-epibatidine and [^{125}I]A85380, suggesting that changes in the transporter are linked to only a subpopulation of nAChRs labeled by these radioligands. No correlation is obtained with ^{125}I-α-bungarotoxin binding. The symbols represent the data from 11 control and 22 MPTP-lesioned mice, except for ^{125}I-α-bungarotoxin binding where they represent the data from five control and 13 MPTP-lesioned animals.

Epibatidine, although selective for nAChRs, labels multiple populations possibly composed of $\alpha 2$–$\alpha 6$ and $\beta 2$–$\beta 4$ subunits (Perry et al., 1995). Similarly, [^{125}I]A85380 has little apparent selectivity for α subtypes, although it does exhibit specificity for $\beta 2$* nAChRs (Kulak et al., 2002b; Perry et al., 2002). Because ^{125}I-α-conotoxin MII interacts with nAChR subsets containing the $\alpha 6\beta 2$* receptors, they comprise only a portion of the sites labeled by ^{125}I-epibatidine or [^{125}I]A85380 (Grady et al., 2001; Champtiaux et al., 2002; Whiteaker et al., 2002; Zoli et al., 2002). Extrapolation to the y-axis of the data correlating changes in receptor binding with nigrostriatal damage suggests that only about 30% of ^{125}I-epibatidine and 35% of the [^{125}I]A85380 sites are present on nigrostriatal dopaminergic afferents. This is in contrast to striatal ^{125}I-α-conotoxin MII sites that seem to reside primarily on dopaminergic terminals. The large subset of ^{125}I-epibatidine and [^{125}I]A85380 sites unaffected by MPTP treatment may be present on striatal GABAergic neurons, cholinergic interneurons, incoming glutamatergic terminals from the cortex, serotonergic afferents from the raphe nucleus, and/or other neurotransmitter inputs to the striatum (Schwartz et al., 1984; Smith and Kieval, 2000; Zhou et al., 2001). Interestingly, the $\alpha 7$* sites were completely unaffected by the lesion, suggesting they are not localized to dopaminergic terminals but are present primarily on these latter elements.

Is the reduction in striatal ^{125}I-epibatidine sites in mice primarily caused by a decline in α-conotoxin MII-sensitive

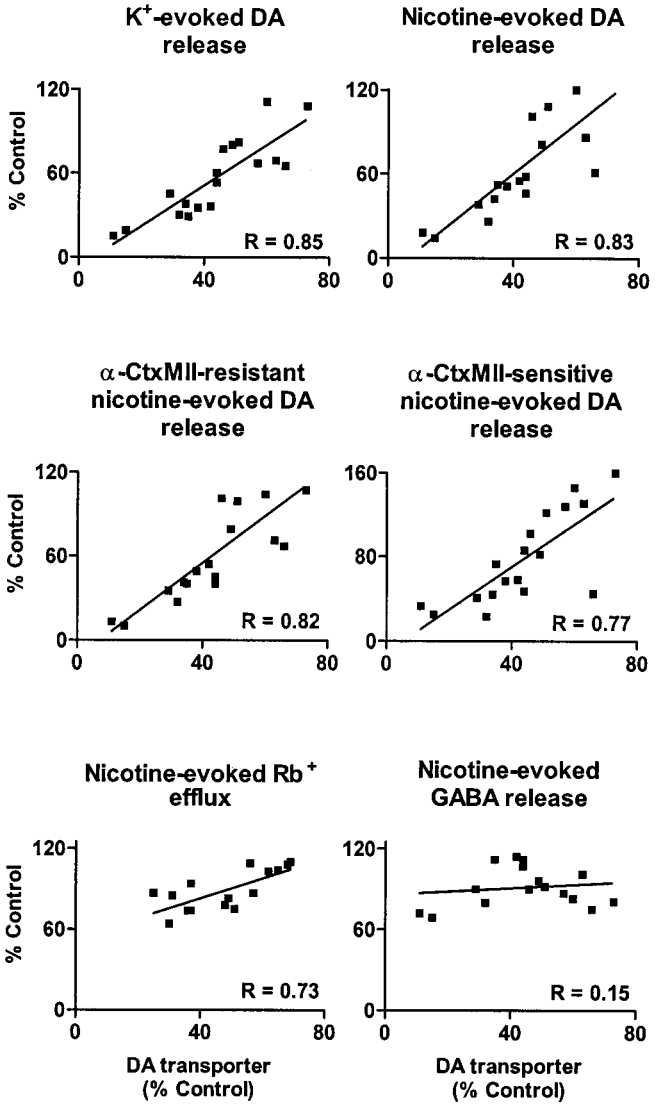

Fig. 8. Correlation between the dopamine transporter and K^+- and nicotine-evoked function in control and MPTP-lesioned mouse striatum. Note the significant correlation between the dopamine transporter and all functional measures ($p < 0.01$), except nicotine-evoked GABA release. The y-intercept is significantly above the origin for nicotine-evoked Rb^+ efflux, suggesting that changes in the transporter are linked to only a subpopulation of these functional nAChRs. The symbols represent the data from 15 to 18 MPTP-lesioned mice.

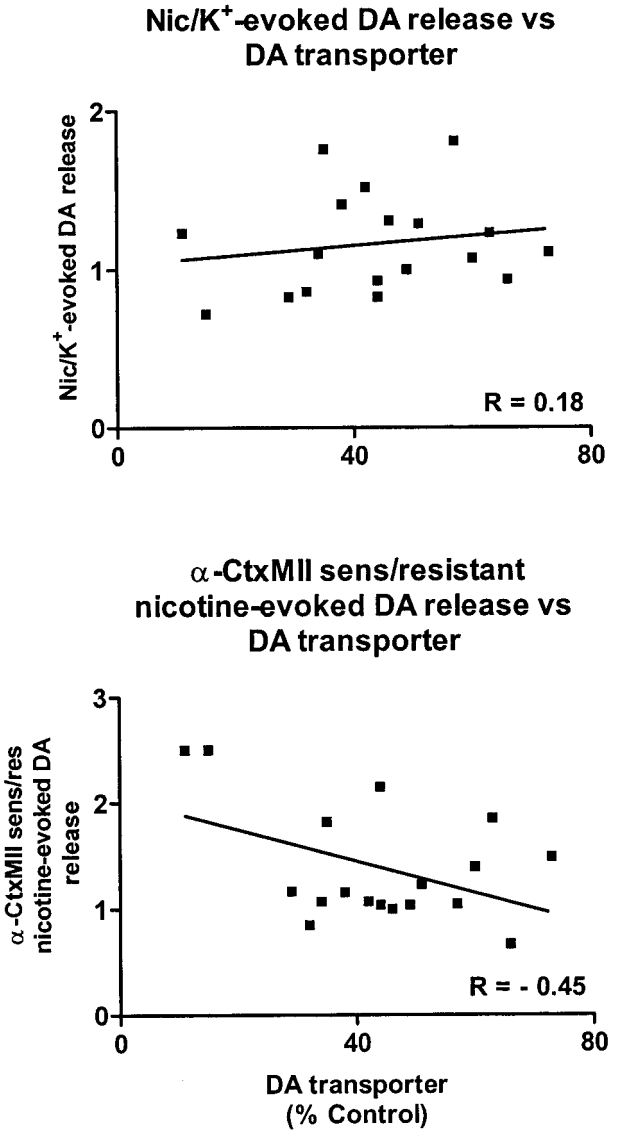

Fig. 9. Characteristics of nAChR function after nigrostriatal damage. The ratio of nicotine- to K^+-evoked dopamine release (expressed as change from control, no change = 1) with increasing nigrostriatal damage is depicted in the top. The data show there are parallel changes in these two functional measures, suggesting that all dopaminergic terminals are similarly affected by MPTP treatment. The lower provides a comparison of the ratio of α-conotoxin MII-sensitive to resistant nicotine-evoked dopamine release (again as change from control, no change = 1) with increasing nigrostriatal damage. There are corresponding changes in the α-conotoxin MII-sensitive and -resistant component, suggesting that the two responses are similarly affected after MPTP treatment. The symbols represent the data from 18 MPTP-lesioned mice.

nAChRs, as in the monkey, or were other subtypes also affected with denervation (Quik et al., 2001; Kulak et al., 2002a)? The results of the ^{125}I-epibatidine competition studies with α-conotoxin MII suggest both α-conotoxin MII-sensitive and -resistant nAChRs are decreased with nigrostriatal damage. Analyses of striatal nAChR changes after nigrostriatal damage were somewhat complex because nAChRs may have both pre- and postsynaptic locations on dopaminergic and nondopaminergic neurons. Because the declines in α-conotoxin MII-sensitive sites after MPTP treatment mirrored those in the dopamine transporter, we assumed these sites were primarily presynaptic and dopaminergic, with a similar loss in α-conotoxin MII-sensitive and -resistant receptors after MPTP treatment. These results are somewhat different from those in MPTP-treated monkeys, in which α-conotoxin MII-sensitive sites are more vulnerable to nigrostriatal damage than the resistant ones, with an almost complete loss of the toxin-sensitive sites before the resistant population is affected (Quik et al., 2001; Kulak et al., 2002a). In the monkey, the toxin-sensitive sites also comprise a much larger proportion of striatal ^{125}I-epibatidine sites (~50% versus ~20% in mice). There thus seem to be substantial species differences in dopaminergic terminal nAChR subtype expression; therefore, nAChR subtype vulnerability to nigrostriatal damage must be considered when extrapolating results from animal studies to the human condition.

The differential changes in nAChR subtypes seen between brain regions in mice 7 days after MPTP treatment suggest that declines in striatal receptor subtypes are not merely the result of nonspecific damage to neuronal tissue. The lack of change in nAChRs in the septum and cortex further demonstrate that MPTP-induced alterations are not caused by nonspecific neuronal damage. On the other hand, there is an extensive organization of striatal inputs and outputs to and from a multitude of brain regions; thus, receptor alterations in the cortex and septal area with nigrostriatal degeneration are not inconceivable (Smith and Kieval, 2000).

Selective Changes in Striatal nAChR Function after Nigrostriatal Damage. Neuronal damage is linked to numerous compensatory changes in an attempt to maintain cellular homeostasis. To add to the level of complexity, biological responses may be associated with an initial receptor or other molecular reserve, such that receptor losses do not affect the overall functional response. We therefore sought to determine the relationship between changes in nAChR subtypes and receptor-mediated activity after nigrostriatal damage. Significant reductions were observed in nicotine-evoked [^{3}H]dopamine release that parallel those in the dopamine transporter, suggesting that nAChR-mediated function is closely coupled to dopamine nerve terminal integrity. In contrast, nicotine-evoked [^{86}Rb$^+$] efflux, which represents release mediated by multiple striatal nAChRs present on both dopaminergic and other neurotransmitter afferents, and possibly striatal GABAergic and cholinergic postsynaptic neurons (Schwartz et al., 1984; Smith and Kieval, 2000; Zhou et al., 2001), is reduced to a much smaller extent. Striatal nicotine-evoked GABA release from GABAergic neurons not directly influenced by MPTP treatment was not changed. Altogether, these results indicate that lesioned-induced deficits in nAChR function most likely reflect a loss of receptors primarily on dopaminergic terminals.

Our binding studies showed that multiple nAChRs are present in the striatum, with lesion-induced declines in both α-conotoxin MII-sensitive and -resistant sites. The results of the functional studies also indicate that release mediated by both these populations of sites is decreased. Interestingly, the trend to a larger deficit in the dopamine transporter (47% of control) than α-conotoxin MII-resistant (61% of control) or α-conotoxin MII-sensitive (76% of control) mediated function after nigrostriatal damage may suggest that there is a small nicotinic receptor reserve and/or that there is (are) some compensatory change(s) in receptor characteristics or signaling steps to yield an enhanced response from undamaged dopaminergic terminals after lesioning. Indeed, Fig. 8 indicates that there is no reduction in release parameters until ~20% of the dopamine transporter is lost.

Nigrostriatal Afferents seem Similarly Sensitive to MPTP Treatment. Unique patterns of innervation and/or neuronal cell types exist in different areas of the basal ganglia (Desban et al., 1993; Damier et al., 1999), which may result in distinct neuronal environments with varying sensitivities to the neurotoxin effect of MPTP. However, a comparison of the ratio of K^+- to nicotine-evoked dopamine release with the dopamine transporter deficits showed that this ratio was unchanged with varying nigrostriatal damage. This result suggests that all dopaminergic terminals are similarly affected by MPTP treatment. The ratio of α-conotoxin MII-sensitive to -resistant nicotine-evoked dopamine release was also similar with differing nigrostriatal damage. These combined data suggest that nigrostriatal dopaminergic terminals in mice, regardless of whether they contain nicotinic receptors or which type of receptors are present, are equally sensitive to the destructive effects of MPTP.

Not only might these nicotinic receptor populations be involved in the motor deficits after nigrostriatal damage, but they may also play a role in putative neuroprotective effects of nicotine. Extensive studies have shown that nicotine exerts a neuroprotective action against a variety of insults in different culture systems (Quik and Kulak, 2002), including MPP^+-induced nigral neuron-induced degeneration in mesencephalic cultures (Jeyarasasingam et al., 2002). Furthermore, nicotine partially prevents nigrostriatal damage in some animal models of Parkinson's disease (Janson et al., 1992; Costa et al., 2001; Ryan et al., 2001). This may extend to the human condition, as epidemiological studies demonstrate a decreased incidence of Parkinson's disease in smokers possibly mediated by the nicotine in tobacco products (Morens et al., 1995).

Acknowledgments

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