Published in final edited form as:

J Neurochem. 2008 June ; 105(5): 1861–1872. doi:10.1111/j.1471-4159.2008.05268.x.

Pre-synaptic dopaminergic compensation after moderate nigrostriatal damage in non-human primates

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Abstract

Despite a dramatic loss of nigrostriatal dopaminergic neurons in Parkinson's disease, clinical symptoms only arise with 70–80% reduction of striatal dopamine. The mechanisms responsible for this functional compensation are currently under debate. Although initial studies showed an enhanced pre-synaptic dopaminergic function with nigrostriatal degeneration, more recent work suggests that functional compensation is not dopamine-mediated. To address this issue, we used cyclic voltammetry to directly measure endogenous dopamine release from striatal slices of control monkeys and animals with a moderate or severe MPTP-induced dopaminergic lesion. The moderately lesioned monkeys were asymptomatic, while the severely lesioned animals were parkinsonian. In monkeys with a moderate lesion, a 300% increase was obtained in endogenous striatal dopamine release. In contrast, in striatal slices from severely lesioned animals, a small % of evoked dopamine signals were similar in amplitude to control while the greater majority were undetectable. These findings suggest that pre-synaptic dopaminergic compensation develops in residual dopaminergic terminals with moderate lesioning, but that this response is lost with severe nigrostriatal damage. Such an interpretation is supported by the results of dopamine turnover studies. This enhanced pre-synaptic dopaminergic activity may be important in maintaining normal motor function during the initial stages of Parkinson's disease.

Keywords

compensation; dopamine release; MPTP; non-human primate; Parkinson's disease; voltammetry

Parkinson's disease (PD) is the second most prominent neurodegenerative disorder among the aging population. It is characterized by an irreversible loss of dopaminergic neurons in the nigrostriatal pathway that gives rise to various motor deficits, including tremor, rigidity and bradykinesia (Singh *et al.* 2007). These symptoms, however, only begin to develop when striatal dopamine is reduced 70–80%. An understanding of the mechanisms underlying this phenomenon is therefore important as it may yield insight about improved therapies for PD.

Although the nature of this functional compensation has been the subject of intense research for many years, results have been conflicting. Initial work had suggested that pre-synaptic dopaminergic mechanisms played a key role in the early asymptomatic stages of the disease. This idea, originally proposed by Hornykiewicz (Hornykiewicz 1966, 1975), was further supported by studies showing an increase in dopamine release, dopamine turnover,

dopamine uptake and tyrosine hydroxylase activity/protein after nigrostriatal damage (Bernheimer *et al.* 1973; Zigmond *et al.* 1984, 1990; Onn *et al.* 1986; Snyder *et al.* 1990; Pifl and Hornykiewicz 2006).

However, more recent studies suggest that non-dopaminergic mechanisms mediate the functional compensation that develops with PD. Garriss and coworkers reported decreases in evoked dopamine release and uptake that correlated with the degree of nigrostriatal damage, supporting the hypothesis of stabilization of the dopamine system through volume transmission without a functional dopaminergic pre-synaptic compensation (Garriss *et al.* 1997; Bergstrom and Garriss 2003). Bezard *et al.* also showed a decrease in endogenous dopamine release from severely lesioned mice, suggesting that increased release does not constitute a pre-synaptic compensatory mechanism (Bezard *et al.* 2000). Moreover, primate studies suggest that enhanced activity of the subthalamic nucleus and internal globus pallidus, and increases in D2 receptors act as adaptive mechanisms in the early stages of disease progression (Bezard *et al.* 2003; Obeso *et al.* 2004). Thus, this work generally favored a primary role for postsynaptic mechanisms in the functional compensation that accompanies PD. An alternate possibility is that both pre- and postsynaptic mechanisms play a role in compensation after nigrostriatal damage, but that the involvement of one or other mechanism is more prominent under specific conditions.

To address this idea, we initiated a study in moderately and severely lesioned non-human primates because this model closely resembles PD in terms of symptomatic features and neurochemical changes. To determine effects on function, we assessed both endogenous dopamine release in striatal slices and K⁺-evoked [³H]dopamine release from striatal synaptosomes. We also evaluated changes in measures reflecting intra- and extracellular nigrostriatal dopamine turnover, defined as the ratio of dihydroxyphenylacetic acid (DO-PAC)/dopamine and homovanillic acid (HVA)/dopamine, respectively. The results suggest that enhanced dopaminergic activity may be a particularly relevant compensatory mechanism in the early presymptomatic phase of PD. Moreover, they show that this mechanism is lost with more severe nigrostriatal damage and the onset of parkinsonian symptoms in a non-human primate model.

Materials and methods

Animal treatments and behavioral studies

Adult female squirrel monkeys (*Saimiri sciureus*) weighing between 0.5 and 0.7 kg were purchased from Osage Research Primates (Osage Beach, MO, USA) or from Worldwide Primates (Miami, FL, USA). Immediately after arrival, monkeys were quarantined for 1 month according to California State regulations. They were housed in a room with a 13-h: 11-h light/dark cycle, and given food once daily with water *ad libitum*. After quarantine, the monkeys were injected subcutaneously with saline ($n = 4$) or MPTP ($n = 9$). Moderately lesioned monkeys ($n = 6$) were administered 1.75 mg/kg MPTP once only. Severely lesioned animals ($n = 3$) received 2.0 mg/kg MPTP. Parkinsonism was evaluated 3 weeks after MPTP treatment on the following categories: spatial hypokinesia, bradykinesia, left- and right-handed dexterity, balance, freezing and action tremor for a total score ranging from 0 to 28. Scores of 0–3 fell within the normal range, of 4–8 were considered moderately parkinsonian and of 9–28 considered severe (Quirk *et al.* 2007). If the scores of the animals designated for the severely lesioned group fell within the normal range, MPTP treatment (1.8–2.0 mg/kg) was repeated on a monthly basis up to 5 times for cumulative doses of 2.0 to 9.3 mg/kg.

Monkeys were killed after behavioral assessment according to the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. They were injected

with 1.5 mL euthanasia solution intraperitoneally (390 mg sodium pentobarbital and 50 mg phenytoin sodium/mL), followed by 1.5 mL/kg of the same solution administered intravenously. Ketamine was not used as previous work had shown it interferes with nicotinic receptor function (Flood and Krasowski 2000; Coates and Flood 2001; Tassonyi *et al.* 2002). All studies were performed according to the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the Parkinson's Institute.

Brain slice preparation

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

The other half of the brain was cut into 2 mm sections. Five mg tissue aliquots of medial and lateral caudate, and ventral and dorsal putamen were dissected from the slice at level A 13.0–14.0 for measurement of dopamine, HVA and DOPAC levels. Ten milligrams of tissue aliquots of ventral putamen were dissected from the slice at level A 12.0–13.0 for measurement of [^3H]dopamine release. The slice at level A 11.0–12.0 was used for determination of endogenous dopamine release using cyclic voltammetry. It was bisected at the level of the internal capsule and the portion containing the putamen placed in ice-cold, pre-oxygenated (95% O_2 / 5% CO_2) artificial cerebrospinal fluid (aCSF) consisting of 125 mM NaCl, 2.5 mM KCl, 1.2 mM NaH_2PO_4 , 2.4 mM CaCl_2 , 1.2 mM MgCl_2 , 20 mM HEPES, 11 mM glucose, 25 mM NaHCO_3 , pH 7.4. Striatal slices (400 μm thick) were prepared using a vibratome (Leica VT1000S) and incubated at room temperature (25°C) for 2 h in oxygenated aCSF. Thirty min before recording, the slice was transferred to a submersion recording chamber (Campden Instruments Ltd., Lafayette, IN, USA), perfused at 1 mL/min with 30°C , oxygenated aCSF, and allowed to equilibrate.

Electrochemical measurement of dopamine release

For the fast scan cyclic voltammetry experiments, carbon fiber microelectrodes were constructed as previously described with minor modifications (Perez *et al.* 2007). A single 7 μm carbon fiber (Thornel T650/35, Cytec industries Inc., West Paterson, NJ, USA) was aspirated through a 1.2 mm o.d. $\times$ 0.68 mm i.d. borosilicate glass capillary (WPI, Sarasota, FL, USA). An automatic P-97 horizontal puller (Sutter instruments, Novato, CA, USA) was used to pull each capillary to produce two electrodes so that the fiber protrudes at the pulled end. The exposed fiber was then trimmed to a tip length of 100–150 μm using a non-inverting microscope at $40\times$ magnification. Electrodes were then dipped in epoxy (Polysciences Inc., Warrington, PA, USA) for 7 min followed by a quick rinse in acetone (10 s) and heated overnight at 80°C to allow the epoxy to harden. Each electrode was filled with 150 mM KCl solution to make electrical contact between the carbon fiber and the silver wire connected to the electrode headstage.

The electrode potential was linearly scanned from 0 to -400 to 1000 to -400 to 0 mV versus an Ag/AgCl reference electrode at a scan rate of 300 mV/ms, as previously described (Zhang *et al.* 2004). This triangular wave was repeated every 100 ms at a sampling frequency of 50 Hz. Current was recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in the V-clamp mode with a low-pass Bessel filter setting at 5 kHz. Triangular wave generation and data acquisition were controlled by

pClamp 9.0 software (Axon instruments). Electrical stimulation was applied using a bipolar tungsten stimulating electrode (Plastics One, Roanoke, VA, USA) connected to a linear stimulus isolator (WPI, Saratoga, FL, USA) and triggered by a Master-8 pulse generator (A.M.P.I., Jerusalem, Israel). Dopamine release was evoked by a single, rectangular electrical pulse (8 V in amplitude and 4 ms in duration) applied every 5 min. All electrode placements were aided by the use of micromanipulators (Narishige International, East Medow, NY, USA) and a stereomicroscope (Nikon SMZ645, Technical Instruments, Burlingame, CA, USA). Background current was digitally subtracted and the peak oxidation currents were converted into concentration after post-experimental calibration of the carbon fiber electrode with a fresh solution of 1 μ M dopamine. Dopamine uptake can be fit to one-phase exponential decay in regions where dopamine release is in the low nanomolar range (Cragg *et al.* 2001; John *et al.* 2006). All our data fit well with this model and it was therefore used to calculate the uptake rate constant (*k*) values for both control and MPTP-treated animals.

[³H]dopamine release from striatal synaptosomes

A crude synaptosomal fraction was prepared as previously described (McCallum *et al.* 2005). Briefly, tissue was homogenized in 2 mL ice-cold 0.32 M sucrose buffered with HEPES (pH 7.5). A P1 pellet was obtained by centrifuging the homogenate at 12 000 g for 20 min. Resuspended synaptosomes were incubated in 37°C uptake buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH₂PO₄, 3.2 mM CaCl₂, 1.2 mM MgSO₄, 25 mM HEPES, pH 7.5, 10 mM glucose, 25 mM NaHCO₃, 1 mM ascorbic acid and 0.01 mM pargyline) for 10 min before adding 100 nM [³H]dopamine (3,4-[ring-2,5,6-³H]; 30-60 Ci/mmol, Perkin-Elmer Life Sciences, Boston, MA, USA). Incubation with [³H]dopamine (4 μ Ci for 0.8 mL synaptosomes) was done for 5 min, then 80 μ L aliquots of synaptosomes (0.5 to 1 mg tissue) were pipetted onto 5 mm diameter A/E glass-fiber filters (Gelman, Inc, Ann Arbor, MI, USA) mounted on polypropylene platforms. Synaptosomes were perfused for 10 min with perfusion buffer (uptake buffer plus 0.1% bovine serum albumin and 10 μ M nomifensine) at a rate of 1 mL/min before fraction collection began. Release of [³H]dopamine was evoked by an 18-s exposure to 20 mM K⁺ buffer. Fifteen 18-s fractions (including basal release before and after the stimulated release) were collected, and radioactivity determined by liquid scintillation counting.

Release was plotted as counts per minute (cpm) versus fraction number using a curve-fitting algorithm of SigmaPlot 5.0 for DOS (Jandel Scientific, San Rafael, CA, USA). Basal release was calculated by selecting fractions before and after the peak and plotting them as a single exponential decay function. Baseline release was subtracted from each fraction and fractions of release that were at least 10% higher than baseline were added to achieve total cpm released. The corrected cpm were then normalized to the wet weight of the tissue sample on each filter to obtain units of cpm/ mg tissue.

Dopamine transporter autoradiography

Binding to the dopamine transporter was measured using [¹²⁵I]RTI-121 (2200 Ci/mmol; Perkin-Elmer Life Sciences), as previously described (McCallum *et al.* 2006). Thawed sections were pre-incubated twice for 15 min each at room temperature (25°C) in 50 mM Tris-HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl, and then incubated for 2 h in buffer with 0.025% bovine serum albumin, 1 μ M fluoxetine and 50 pM [¹²⁵I]RTI-121. Sections were washed at 0°C for 4 $\times$ 15 min each in buffer and once in ice-cold water, air dried, and exposed for 2 d to Kodak MR film (Perkin-Elmer Life Sciences) with [¹²⁵I] microscale standards (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Nomifensine (100 μ M) was used to define non-specific binding.

Dopamine and metabolite measurements

Striatal tissue samples (5 mg) were placed in a 0.25 mL volume of ice-cold 0.4 M perchloric acid then sonicated and centrifuged at 14 000 g for 12 min. The supernatants were used for measurement of dopamine and its metabolites, DOPAC and HVA using HPLC with electrochemical detection (Coulchem II detector; ESA, Chelmsford, MA, USA) (McCallum *et al.* 2006). The mobile phase was delivered at a flow rate of 1 mL/min and consisted of a mixture of 90 mM sodium acetate, 35 mM citric acid, 130 μ M EDTA and 230 μ M 1-octanesulfonic acid. The elution order and retention times were as follows: DOPAC (7.0 min), dopamine (9.5 min) and HVA (18.3 min). Protein was determined using the method of Lowry (Lowry *et al.* 1951).

Statistical analyses

All statistics and one-phase exponential decay curve calculations were conducted using GraphPad Prism (Graph Pad Software Co., San Diego, CA, USA). All exponential decay curves to calculate uptake rate constant values fit the data with $r^2 \geq 0.90$. Statistical comparisons were performed using either Student's *t*-test or one or two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test (GraphPad prism). A value of $p < 0.05$ was considered significant. All values are expressed as the mean $\pm$ SEM.

Results

Effect of MPTP lesioning on parkinsonism and the striatal dopamine transporter

Monkeys treated with a single dose of the dopaminergic neurotoxin MPTP (1.75 mg/kg) exhibited parkinsonian scores within the normal range. Dopamine transporter autoradiography using ^{125}I -RTI-121 binding (Table 1) was evaluated in the striatum of the lesioned monkeys as a biochemical measure of MPTP-induced nigrostriatal damage. Although asymptomatic, these moderately lesioned animals exhibited significant decreases in the dopamine transporter with values reduced to 80% ($p < 0.05$) of control in the medial caudate and 73% of control ($p < 0.05$) in the ventral putamen. As shown previously (McCallum *et al.* 2006), larger declines were observed in the dorsolateral striatum, with dopamine transporter values reduced to 45% ($p < 0.01$) and 47% ($p < 0.01$) of control in the lateral caudate and dorsal putamen, respectively.

The more severely lesioned monkeys receiving 2 mg/kg MPTP from 1 to 5 times exhibited parkinsonian symptoms, including bradykinesia, hypokinesia, decreased manual dexterity, loss of balance, freezing and/or tremor with an average parkinsonian rating of 15.5 ± 4.40 ($n = 3$). This was associated with more severe declines in dopamine transporter expression to 27% ($p < 0.001$) and 23% ($p < 0.001$) of control for medial caudate and ventral putamen, respectively, while the dorsolateral regions were more severely decreased to 8% ($p < 0.001$) and 14% ($p < 0.001$) of control for lateral caudate and dorsal putamen, respectively (Table 1).

Declines in dopamine and metabolite levels after moderate and severe MPTP lesions

Striatal dopamine and its metabolites were measured in the different subregions of the striatum for control and MPTP-treated animals as an additional marker of nigrostriatal integrity since this measure has been more commonly used in previous studies (Stachowiak *et al.* 1987; Elsworth *et al.* 1989; Snyder *et al.* 1990; Garriss *et al.* 1997; Bezard *et al.* 2000, 2001c; Bergstrom and Garriss 2003). Control dopamine levels were 95.9 ± 6.83 , 98.8 ± 11.7 , 96.2 ± 7.83 and 118 ± 8.63 ng/mg protein for medial caudate, ventral putamen, lateral caudate and dorsal putamen, respectively. Levels for DOPAC in the control animals were 11.5 ± 3.94 , 10.4 ± 2.27 , 17.1 ± 6.68 and 10.4 ± 3.47 ng/mg protein for medial caudate, ventral putamen, lateral caudate and dorsal putamen, respectively. Control levels of HVA,

the major dopamine metabolite in primates, were 39.7 ± 2.60 , 68.1 ± 5.09 , 42.6 ± 2.93 and 64.3 ± 1.83 ng/mg protein for medial caudate, ventral putamen, lateral caudate and dorsal putamen, respectively.

A significant reduction was observed in dopamine levels after moderate nigrostriatal damage, with a more pronounced decline in the ventral and dorsal putamen than in the medial and lateral caudate (Table 2). Levels of DOPAC, one of the major dopamine metabolites, were decreased in all striatum subregions, with significant declines in the lateral caudate ($p < 0.05$) and dorsal putamen ($p < 0.01$). Although the levels of HVA, the other major metabolite, were decreased throughout the striatum, this did not reach statistical significance after a moderate lesion (Table 2).

As expected, severe MPTP-induced nigrostriatal damage resulted in greater decreases in dopamine levels across all subregions of the striatum. Dopamine levels were decreased to 11% and 7% of control in medial caudate and ventral putamen, respectively, while there was an almost complete loss of dopamine (~1% of control) in the lateral caudate and dorsal putamen (Table 2). Metabolite levels were also significantly decreased across the striatum, with DOPAC levels generally more affected than those for HVA.

Characterization of evoked dopamine release in monkey ventral putamen using cyclic voltammetry

Since the putamen is the portion of the striatum that receives major inputs from the somatosensory, motor and premotor cortices (Wichmann and DeLong 1996, 2003) and is also most affected in Parkinson's disease (Hornykiewicz 2001), we selected this region for our functional assays. We focused on the ventral putamen (Fig. 1a) since nigrostriatal damage is less severe and responses more likely to be observed with severe neurodegeneration. To elicit endogenous dopamine release, a single electrical stimulus pulse was applied through a bipolar stimulating electrode positioned ~ 150 μ m from the recording carbon fiber electrode. Fig. 1b shows a representative trace for stimulated dopamine release from a control monkey. The mean evoked dopamine concentration was 32 ± 9.6 nM ($n = 4$ monkeys). The identity of the released neurotransmitter was confirmed by the characteristic oxidation (500–600 mV) and reduction (~200 mV) peak potentials observed in the cyclic voltammogram obtained for each peak (Fig. 1b inset). Since dopamine release is known to be regulated by Ca^{2+} -dependent mechanisms (Chen and Rice 2001; Zhou *et al.* 2002), we tested the Ca^{2+} -dependence using Ca^{2+} -free aCSF. Dopamine release was first monitored in Ca^{2+} -containing aCSF for 1 h (Fig. 1b) and the perfusion buffer then switched to Ca^{2+} -free aCSF. Release was completely abolished under these conditions (Fig. 1c).

Dopamine release is significantly increased in striatal slices from moderate but not severely lesioned monkeys

We next assessed the effects of nigrostriatal damage on endogenous dopamine release in monkey striatal slices using cyclic voltammetry. For all three treatment groups, mean concentrations of dopamine release were calculated from the detected release after each stimulus for each animal. Evoked dopamine release from control monkey striatal slices was robust and reproducible (32 ± 9.6 nM; $n = 4$) (Figs 2a and 3). There was a significant increase ($p < 0.05$) in evoked dopamine release of ~300% in the moderately lesioned group (96 ± 18 nM, $n = 6$) compared to control (Figs 2b and 3).

In contrast to the consistent, reproducible increase in dopamine release in ventromedial putamen of moderately lesioned animals, stimulation induced only sporadically evoked dopamine signals in the severely lesioned monkeys (Table 3). These signals were similar in magnitude to those in striatal slices from control animals (Fig. 2c). The mean concentration

of dopamine release after each of these sporadic peaks corresponded to 26 ± 2.4 nM dopamine signals (Fig. 2c). For one of the animals, dopamine release was not detectable (Table 3). This animal displayed the most severe parkinsonian symptoms and showed the greatest decrease in dopamine transporter and dopamine and metabolite levels in the severely lesioned group (Table 3). To evaluate mean striatal dopamine release per animal, a zero value was used to indicate no signal. This yielded a mean dopamine release of 1.3 ± 0.8 nM for the severely lesioned group, which was significantly decreased ($p < 0.05$) to 4% of control (Fig. 3). These results suggest that the pre-synaptic compensatory mechanisms in the release process that arise with moderate nigrostriatal damage are lost with continued neurodegeneration.

Effect of nigrostriatal damage on total and fractional K^+ -evoked [3H]dopamine release from striatal synaptosomes

An alternate, commonly used approach to evaluate pre-synaptic dopaminergic function involves measurement of synaptosomal [3H]dopamine release in response to a depolarizing concentration of K^+ (20 mM) (McCallum *et al.* 2005, 2006; Quik *et al.* 2006). This approach offers the advantage that it provides a measure of release in an isolated synaptosomal preparation devoid of influences from other regulatory neuronal inputs. We measured [3H]dopamine release from synaptosomes prepared from ventral putamen of control and lesioned monkeys (Fig. 4a). The results show that K^+ -evoked [3H]dopamine release was maintained at control levels in the moderately lesioned group, while release was decreased to 57% of control after a severe lesion ($p < 0.05$).

The above results reflect release averaged across the striatum, without considering the dopaminergic neuronal loss that occurs with MPTP treatment. As an approach to evaluate dopamine release from terminals remaining after nigrostriatal damage, fractional [3H]dopamine release was determined. This was done by dividing the total evoked release by the dopamine content, as previously described (Stachowiak *et al.* 1987; Snyder *et al.* 1990). The results in Fig. 4b show a significant increase in fractional dopamine release with both moderate ($p < 0.01$) and severe ($p < 0.001$) nigrostriatal damage. The results with the moderate lesion are thus similar to those obtained using cyclic voltammetry. However, differential results were obtained for the severely lesioned group using the two approaches with a decreased release using cyclic voltammetry but an apparent increase in [3H]dopamine release when calculated as fractional dopamine release from synaptosomes. In these latter studies, the level of dopamine in the remaining striatal tissues is quite low in the severely lesioned brains. This finding, coupled with the development of compensatory mechanisms, may be responsible for the large increase in fractional release calculated for the severely lesioned brains. It is also possible that alternate molecular mechanisms that develop with a severe compared to a moderate lesion are evident using only one approach and/or may be due to technical differences between the two techniques, as discussed later.

Dopamine uptake rate constants after moderate and severe nigrostriatal damage

Dopamine levels are not only controlled through the release process but also by the rate of dopamine uptake back into the terminal. To determine uptake rate constants in slices from control and MPTP-treated animals, the dopamine peaks obtained after stimulation were fitted to one-phase exponential decay analysis, as previously described (Wightman and Zimmerman 1990; Cragg *et al.* 2001; John *et al.* 2006). This method is used to model uptake rate constants when dopamine release is in the low nanomolar range, as under the current experimental conditions, since uptake is unsaturated and thus follows first order kinetics. Fig. 5a shows a representative dopamine peak with a one-phase exponential fit overlaid on the clearance phase of the peak. All data fit with r^2 -values of 0.90 or greater. Comparison of the mean uptake rate constant, k , between the treatment groups demonstrated no difference

in uptake between the control and moderate MPTP-lesioned groups with values of $2.19 \pm 0.31/s$ and $2.36 \pm 0.28/s$ for each group, respectively (Fig. 5b). These data suggest that uptake into terminals is similar to control, as might be expected since residual terminal integrity may still be intact after a moderate lesion. These results appear to conflict with the decline in dopamine transporter levels with moderate damage described in the preceding sections. However, this discrepancy most likely relates to the different outcomes evaluated by these two techniques, with the dopamine transporter providing a measure of the loss of dopamine terminals with lesioning whereas uptake measured using cyclic voltammetry reflects function of surviving terminals.

In contrast to the results in moderately lesioned monkeys, dopamine peaks in the severely lesioned group were broader than those observed in control animals (Fig. 2c). This is most likely due to the significant decrease in dopamine uptake rate ($0.29 \pm 0.05/s$) in striatum of severely lesioned animals compared to controls ($p < 0.05$) (Fig. 5b).

Dopamine turnover is altered with moderate and severe MPTP-induced nigrostriatal damage

As an alternate measure of neuronal function, we assessed dopamine turnover in striatum of control, moderate and severely lesioned monkeys. Turnover was initially calculated as the ratio of both metabolites (DOPAC + HVA) to dopamine (Fig. 6a). Turnover evaluated in this manner was increased in all subregions of the striatum, with a significant main effect of lesioning in the moderately [$F(1,28) = 14.8$; $p < 0.001$] and severely lesioned groups [$F(1,16)=100.8$; $p < 0.001$] (Fig. 6b).

We also evaluated dopamine turnover as the DOPAC/ dopamine and HVA/dopamine ratios, as these measures are thought to more closely reflect effects on intra- and extra-cellular dopamine metabolism (Roffler-Tarlov *et al.* 1971; Elsworth and Roth 1997). For the moderately lesioned monkeys, HVA/dopamine ratios were significantly increased [$F(1,28) = 12.7$; $p < 0.01$] in both subregions of the putamen and caudate (Fig. 6c). A significant increase was also observed in the severely lesioned group [$F(1,28) = 49.7$; $p < 0.001$] (Fig. 6d).

Turnover evaluated using the DOPAC/dopamine ratios was significantly increased in the moderately lesioned group [$F(1,28) = 30.1$; $p < 0.001$] (Fig. 6e). In contrast, DOPAC/ dopamine ratios were significantly decreased in ventral and dorsal putamen, and lateral caudate of the severely lesioned group [$F(1,16) = 5.96$; $p < 0.05$] (Fig. 6f).

Discussion

This is the first report of electrically evoked dopamine release from striatal slices derived from non-human primates with moderate and severe nigrostriatal damage. Dopamine release was measured using carbon fiber voltammetry, a technique with excellent temporal and spatial resolution that allows for real-time measurement of neurotransmitter release (Michael and Wightman 1999; Troyer *et al.* 2002). Our data demonstrate that moderate nigrostriatal damage without overt parkinsonism results in a robust increase in endogenous dopamine release. In contrast, the more severe MPTP treatment, which resulted in marked parkinsonism, led to a dramatic decline in endogenous dopamine release. These results suggest pre-synaptic dopaminergic compensation develops with moderate but not severe nigrostriatal damage, an idea further supported by the results of dopamine turnover studies. The DOPAC/dopamine ratio, which has been linked to intra- and extra-neuronal metabolism, was increased with moderate but decreased with severe striatal damage. In contrast, the HVA/dopamine ratio, a marker of extraneuronal dopamine metabolism, was increased with both MPTP lesions. Altogether, these data suggest that pre-synaptic

dopamine-mediated compensatory mechanisms develop with the onset of nigrostriatal dopaminergic denervation but are lost as neurodegeneration progresses.

The preservation of dopamine uptake by residual terminals, coupled with the enhanced neurotransmission in the moderately lesioned animals, may result in elevated striatal dopamine levels and allow for dopamine diffusion to denervated areas away from the release site, further contributing to dopaminergic compensation. This idea is consistent with previous observations which indicate that 'passive stabilization' of the dopamine system represents an active mechanism that maintains dopamine homeostasis with denervation (Garris *et al.* 1997; Bezard *et al.* 2000; Bergstrom and Garris 2003). This critical role of diffusion in the conservation of dopamine homeostasis in the intact and denervated striatum is supported by numerous studies involving animals with varying striatal dopamine depletion (Zigmond *et al.* 1990; Garris *et al.* 1997; Zoli *et al.* 1998; Bergstrom *et al.* 2001; Bergstrom and Garris 2003) and also in a progressive MPTP-induced neurodegeneration mouse model with a 74% dopamine depletion (Bezard *et al.* 2000). Although passive diffusion appears to contribute to pre-synaptic dopaminergic compensation in both rodents and monkeys, the enhanced endogenous dopamine release without changes in uptake observed in the present study was not identified in striatum of lesioned rodents (Bezard *et al.* 2000; Bergstrom and Garris 2003). This disparity may relate to species differences, variations in the degree of lesioning, and/ or differences in the striatal region used for assessment of release.

Our voltammetry results demonstrating an increase in dopamine release in slices from primates with moderate MPTP-induced lesions are consistent with the observed increase in fractional K⁺-evoked [³H]-dopamine release from synaptosomes. This fairly straightforward situation in striatum of monkeys with moderate nigrostriatal damage contrasts with results in severely lesioned animals which proved somewhat more complex. For instance, although there was a significant decrease in endogenous dopamine release from striatal slices measured using voltammetry and exogenous [³H]dopamine release from striatal synaptosomes, the decline in stimulated [³H]dopamine release was not as pronounced as the decrease in evoked endogenous dopamine release observed with voltammetry. This discrepancy may relate in part to the use of pargyline in the [³H]dopamine release assays but not in the voltammetry studies. By inhibiting monoamine oxidase, pargyline may allow [³H]dopamine to be free in the cytoplasm rather than restricted to vesicles. If potassium evoked release includes cytoplasmic pools, it could raise the amount of [³H]dopamine released under these conditions. An exogenous dopamine source for the synaptosomal [³H]dopamine release assays may also result in differential effects between the two assays. The [³H]dopamine transported into residual terminals may result in a greater amount of intracellular [³H]-dopamine than what would be present endogenously after a severe nigrostriatal lesion. This would lead to a larger releasable synaptosomal [³H]dopamine pool than anticipated, with a resultant greater release of [³H]dopamine. Similarly the large increase in fractional [³H]dopamine release in the severely lesioned animals may be due to complexities linked to the use of an exogenous dopamine source.

A point of note is the lack of enhanced endogenous dopamine release in striatum of severely lesioned primates. These data contrast with previous reports in 6-hydroxydopamine-lesioned rats which showed a compensatory increase in fractional release even after 85–90% decreases in tissue dopamine levels (Stachowiak *et al.* 1987; Snyder *et al.* 1990). One possibility is that the primates used for the present study displayed severe bilateral behavioral impairments, which are not reproduced in rodents with a unilateral lesion. As well, the extent of lesioning in our severely lesioned monkeys was greater than that observed in the rats and may be beyond the threshold for development of compensatory release mechanisms. Such an interpretation is consistent with a previous electrochemical study in

which there was a significant reduction in dopamine release in severely lesioned primates with dopamine depletion comparable to those observed in our animals (Gerhardt *et al.* 1996).

For the present study, we used a non-human primate model since it offers the advantage that parkinsonism in monkeys with nigrostriatal damage shares many features with PD, and that molecular changes with nigrostriatal damage are very similar in monkeys and humans. Dopamine transporter autoradiography was used as one measure to assess the extent of nigrostriatal damage. This marker is selectively expressed on dopaminergic terminals and thus represents a measure of dopaminergic nerve terminal integrity. As expected, the decrease in dopamine transporter expression was less pronounced with the moderate than with the severe lesion. In addition, MPTP treatment led to a greater dopamine transporter density loss in dorsolateral striatum compared to ventromedial striatum in agreement with findings in PD and other parkinsonian animal models (Alexander *et al.* 1992; Hornykiewicz 2001; McCallum *et al.* 2006). We also measured dopamine and metabolite levels as an additional marker of dopaminergic damage. In agreement with other studies, significant decreases were observed with greater declines in the severely lesioned group as compared to the more moderately lesioned group (Elsworth *et al.* 1989; Pifl and Hornykiewicz 2006). As previously reported, dopamine and metabolite levels were generally more affected by MPTP treatment than the dopamine transporter (McCallum *et al.* 2006; Quik *et al.* 2006).

In PD and in animal models of nigrostriatal damage, dopamine turnover is a parameter frequently used as an index of dopaminergic function (Bernheimer *et al.* 1973; Zigmond *et al.* 1984; Bezard *et al.* 2000, 2001c; Pifl and Hornykiewicz 2006). Increases in dopamine turnover following nigrostriatal damage are thought to reflect a compensatory upregulation in dopaminergic activity that allows for normal function despite significant neurodegeneration (Bernheimer *et al.* 1973; Zigmond *et al.* 1984; Bezard *et al.* 2000, 2001c; Pifl and Hornykiewicz 2006). Our results further support these findings since we also observed significant increases in overall dopamine turnover calculated as the (DOPAC +HVA)/dopamine ratio in both moderately and severely lesioned animals. We next re-analyzed these data to discern whether the increase in turnover was mainly a result of increased intracellular dopamine metabolism (i.e., increased synthesis) or increased extracellular dopamine metabolism (i.e., increased synthesis/release or reduced uptake). To approach this, we evaluated the DOPAC/dopamine and HVA/dopamine ratios. We observed increases in DOPAC/dopamine and HVA/dopamine ratios after moderate nigrostriatal damage. These results suggest that increased turnover after a moderate lesion is mainly because of dopamine synthesis and release. Such an interpretation is further supported by the observation that dopamine uptake rate constants were not affected with moderate lesioning, indicating that the enhanced increase in the extracellular dopamine signal after electrical stimulation cannot be attributed to decreased dopamine uptake by the remaining terminals.

In contrast, dopamine turnover was somewhat differently affected in the severely as compared to the moderately lesioned animals. We still observed a significant increase in turnover calculated as the HVA/dopamine ratio, however, the DOPAC/dopamine ratio was decreased with the severe lesion in contrast to previous studies (Zigmond *et al.* 1984; Pifl and Hornykiewicz 2006). This difference may relate to species variation, since DOPAC and not HVA is the main dopamine metabolite in rodents (Zigmond *et al.* 1984), or to the prolonged use of L-dopa and dopamine agonist in the primate studies (Pifl and Hornykiewicz 2006). The high (DOPAC+HVA)/dopamine ratio after severe lesioning appears in large part because of the elevated HVA levels. Since HVA arises from dopamine, these data suggest that extrasynaptic dopamine levels are elevated with severe damage thus representing another form of dopaminergic compensation.

Altogether, the present study agrees with a body of literature suggesting that pre-synaptic dopamine-mediated compensation delays the appearance of parkinsonian symptoms with moderate nigrostriatal damage. This increased dopamine release from spared dopamine terminals could help maintain dopamine homeostasis at the early stages of neuronal degeneration. With further degenerative changes, the continued loss of dopamine nerve terminal integrity may no longer support increased dopaminergic function. In addition to pre-synaptic changes, alterations in postsynaptic mechanisms most likely help maintain normal motor function. This may involve adaptive changes in any of the numerous input and output connections within the basal ganglia and other brain circuitries outside the basal ganglia (Zigmond 1997; Bezard *et al.* 2001a, 2003). Dopamine depletion alters expression of multiple neurotransmitters, neuropeptides and receptors present in the different compartments of the striatopallidal and striatonigral output pathways (Langer and Graybiel 1989; Obeso *et al.* 2002, 2004; Bezard *et al.* 2003; Widnell 2005; Galvan and Wichmann 2007; Huot and Parent 2007; Pisani *et al.* 2007). For instance, nigrostriatal damage has been associated with increases in peptidomelanin mRNA, enkephalin, D2 receptors and subthalamic nucleus activity in the indirect output pathway (Bezard *et al.* 1999, 2001b,c; Morissette *et al.* 1999; Betarbet and Greenamyre 2004) while changes in the direct output pathway include increased substance P expression in the matrix and the internal globus pallidus, decreased substance P expression in striosomes and changes in D1 receptors (Bezard *et al.* 1999; Betarbet and Greenamyre 2004). A combination of pre- and post-synaptic compensatory mechanisms most likely accounts for the delayed development of the motor symptoms characteristic of PD.

Acknowledgments

This work is supported by NIH grants NS42091 and NS47162 to MQ and a fellowship from the Tobacco Related Disease Research program to XAP. The authors thank Dr Dino Di Monte for providing the tissue and the dopamine/metabolite concentrations for some of the monkeys.

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Abbreviations used

DAT	dopamine transporter
DOPAC	dihydroxyphenylacetic acid
HVA	homovanillic acid
PD	Parkinson's disease
RTI-121	2 β -carboxylic acid isopropyl ester-3 β -(4-iodophenyl) tropane

Fig. 1. Electrically evoked dopamine release in control non-human primate putamen. (a) Representative diagram showing the different subregions of monkey striatum at level A12.5. Evoked dopamine release was measured in the ventromedial portion of the putamen. DP, dorsal putamen; LC, lateral caudate; MC, medial caudate; VP, ventral putamen. (b) Release was elicited by a single stimulation pulse at the time indicated by the arrows. Shown is a representative trace from a single control slice perfused with aCSF containing 2.4 mM Ca^{2+} . Inset. Typical voltammogram for dopamine with an oxidation peak at 500–600 mV and a reduction peak at approximately -200 mV. Recordings were made over a 1 h period to ensure stability of the signal. (c) No response was observed in the presence of Ca^{2+} -free aCSF. Results are representative of four other experiments.

Fig. 2.

Enhanced striatal dopamine release with moderate but reduced with severe MPTP-induced nigrostriatal damage in monkeys assessed using cyclic voltammetry. Endogenous dopamine release from striatal slices was evoked by a single pulse stimulus applied every 5 min.

Representative consecutive dopamine traces are shown for a control (a), moderately (b) and severely lesioned monkey (c). Evoked release was robust and reproducible in control and moderately lesioned animals. In contrast, release occurred sporadically with severe denervation. Importantly, whenever dopamine release was detected in the severely lesioned animals, peak dopamine release was close to that observed in controls, while dopamine clearance was significantly decreased compared to control as evidenced from the broader peak. Insets. Typical voltammograms for dopamine with an oxidation peak at 500–600 mV and a reduction peak around -200 mV.

Fig. 3.

Electrically evoked endogenous striatal dopamine release from striatal slices in control, moderately and severely lesioned monkeys. Evoked-dopamine release was elicited by a single pulse stimulation applied every 5 min. For all groups, the data shown corresponds to the mean of the peak signals detected per stimulus during a time span of 2 h. Moderate nigrostriatal damage results in a 300% increase in evoked dopamine release from striatal slices compared to control. To evaluate mean evoked release in striatum of severely lesioned monkeys, a value of zero was used when no signal was obtained yielding an overall release of < 5%. Data represent means $\pm$ SEM of 3–6 monkeys per group. * $p < 0.05$ indicates significance of difference from control.

Fig. 4.

[³H]-dopamine release from striatal synaptosomes of control, moderately and severely lesioned monkeys. (a) Total K⁺-evoked [³H]dopamine release was measured from striatal synaptosomes of control and lesioned monkeys. No significant change in the total amount of dopamine release was observed after a moderate lesion while a significant decrease was observed in the severely lesioned group. (b) Fractional K⁺-evoked [³H]dopamine release, which represents release normalized to dopamine levels, was significantly enhanced after moderate and severe dopaminergic damage. These results with the moderately lesioned group resembled those observed with voltammetry. However, the elevated fractional release obtained for the severely lesioned group are opposed to those obtained using cyclic voltammetry, possibly because of the methodological differences between the two release assays. Data represent means $\pm$ SEM of 4–10 monkeys per group. ** $p < 0.01$; *** $p < 0.001$ indicate significance of difference from control.

Fig. 5.

Uptake rate constants in control and MPTP-treated monkeys. (a) A representative dopamine release trace from one control animal. Uptake rate constant values were calculated by fitting the clearance portion of the curve to one-phase exponential decay. The open squares represent raw data points. The solid line shows the decay curve fit. (b) Uptake rate constant values were not significantly different in slices from moderately lesioned animals compared to controls; however, uptake was significantly decreased in the severely lesioned group. Values represent mean $\pm$ SEM of three to six monkeys per group. * $p < 0.05$ indicates significance of difference from control.

Fig. 6.

Dopamine turnover in control and MPTP-lesioned monkeys. Dopamine turnover calculated as the (DOPAC+HVA)/dopamine ratio was significantly increased in (a) moderately and (b) severely lesioned animals. Extracellular turnover calculated as the HVA/dopamine ratio was also significantly elevated in striatum of both (c) moderately and (d) severely lesioned monkeys. However, intracellular turnover calculated as the DOPAC/dopamine ratio was increased in regions with moderate damage but decreased in severely lesioned regions (e and f). Values represent mean $\pm$ SEM of four control, six moderately and three severely lesioned monkeys. Turnover in control animals is expressed as 100%. * p < 0.05; ** p < 0.01; *** p < 0.001 indicate significance of difference from control. DP, dorsal putamen; LC, lateral caudate; MC, medial caudate; VP, ventral putamen.

Table 1

Nigrostriatal damage decreases dopamine transporter binding in monkey striatum

Group	Dopamine transporter binding (nCi/mg tissue)			
	Medial caudate	Ventral putamen	Lateral caudate	Dorsal putamen
Control	26.3 ± 1.47	28.4 ± 0.68	23.5 ± 1.66	26.1 ± 1.69
Moderate	21.1 ± 1.08 *	20.8 ± 2.48 *	10.7 ± 2.55 **	12.3 ± 2.49 **
Severe	7.24 ± 0.21 ***	6.55 ± 1.33 ***	1.84 ± 0.72 ***	3.66 ± 0.65 ***

Monkeys were treated with MPTP to achieve either moderate or severe nigrostriatal damage. Alterations in striatal dopamine transporter expression were assessed using ¹²⁵I-RTI-121 autoradiography. Quantitative analyses of each subregion showed that there was a greater decline in the transporter in the dorsolateral compared to ventromedial striatum with MPTP treatment. Each value represents the mean ± SEM of four control, six moderately and three severely lesioned monkeys.

*
 $p < 0.05$;

**
 $p < 0.01$;

 $p < 0.001$ indicate significance of difference from control.

Table 2

Decline in dopamine and metabolite levels in striatum after MPTP treatment

Region		Levels (% control)		
		Control	Moderate	Severe
Medial caudate	Dopamine	100 ± 2.96	56.9 ± 8.18**	11.3 ± 3.66***
	DOPAC	100 ± 7.54	87.3 ± 11.9	35.0 ± 12.4*
	HVA	100 ± 4.66	65.9 ± 10.3	29.2 ± 4.99***
Ventral putamen	Dopamine	100 ± 10.2	18.7 ± 11.7**	6.64 ± 2.52**
	DOPAC	100 ± 28.4	28.8 ± 18.4	2.65 ± 1.87*
	HVA	100 ± 11.4	72.6 ± 27.7	27.7 ± 7.48**
Lateral caudate	Dopamine	100 ± 15.7	42.8 ± 8.21**	1.31 ± 0.50***
	DOPAC	100 ± 25.8	42.5 ± 8.18*	0.00 ± 0.00*
	HVA	100 ± 9.03	67.7 ± 10.1	19.2 ± 4.92**
Dorsal putamen	Dopamine	100 ± 11.5	12.0 ± 5.74***	0.96 ± 0.36***
	DOPAC	100 ± 26.9	9.73 ± 4.74**	5.88 ± 4.16*
	HVA	100 ± 5.13	70.0 ± 21.5	20.4 ± 5.82***

Each value represents the mean percent control ± SEM of four control, six moderately, and three severely lesioned monkeys.

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$ indicate significance from control.

Table 3

Comparison of behavioral, biochemical and functional measures in severely lesioned monkeys

Animal	Parkinsonian score	Dopamine (% decline)	Dopamine transporter (% decline)	Evoked endogenous dopamine release (# responses/10 stimuli) ^a
1	10	88	71	2
2	12	94	73	1
3	24	99	86	0

^a In control and moderately lesioned monkeys, release was observed after all stimuli, that is, 10 responses/10 stimuli.