Sprouting of dopamine terminals and altered dopamine release and uptake in Parkinsonian dyskinaesia

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Failed storage capacity, leading to pulsatile delivery of dopamine (DA) in the striatum, is used to explain the emergence of ‘wearing off’ and dyskinaesia in Parkinson’s disease. In this study, we show that surviving DA neurons in 6-OHDA lesioned rats sprout to re-innervate the striatum, and maintain terminal density until ~60% of neurons are lost. We demonstrate that DA terminal density correlates with baseline striatal DA concentration ([DA]). Electrochemical and synaptosome studies in 6-OHDA lesioned rats and primates suggest that impaired striatal DA re-uptake and increased DA release from medial forebrain bundle fibres contribute to maintaining striatal DA levels. In lesioned rats where terminal density fell by 60% or more, L-DOPA administration increased striatal DA levels markedly. The striatal [DA] produced by L-DOPA directly correlated with the extent of dyskinaesia, suggesting that dyskinaesia was related to high striatal [DA]. While sprouting and decreased dopamine uptake transporter function would be expected to contribute to the marked increase in L-DOPA induced [DA], the increased [DA] was most marked when DAergic fibres were >60% denervated, suggesting that other release sites, such as serotonergic fibres might be contributing. In conclusion, the extent of dyskinaesia was directly proportional to the extent of DA terminal denervation and levels of extra-synaptic striatal DA. We propose that sprouting of DA terminals and decreased dopamine uptake transporter function prevent the appearance of Parkinsonian symptoms until about 60% loss of nigral neurons, but also contribute to dysregulated striatal DA release that is responsible for the emergence of dyskinaesia and ‘wearing off’.

Keywords: dopamine uptake transporter; levodopa; Parkinson’s disease; striatum; voltammetry

Abbreviations: AIM = abnormal involuntary movement; AUC = area under curve; DA = dopamine; DAT = dopamine reuptake transporter; HPLC = high-performance liquid chromatography; IAS = index of arbour size; MFB = medial forebrain bundle; SERT = serotonin re-uptake transporter; TH = tyrosine hydroxylase

Introduction

L-DOPA ameliorates symptoms of early Parkinson’s disease, implying that increasing trans-synaptic delivery of dopamine (DA) by surviving terminals compensates for loss of release sites. In the absence of L-DOPA, motor function in Parkinson’s disease is reduced, reflecting impaired endogenous DA neurotransmission, whereas L-DOPA therapy improves motor function (Nutt et al., 2002) and by implication, DA neurotransmission. L-DOPA treatment improves motor function at the onset of disease, but within 5 years of treatment, about 50% of patients find that this beneficial effect of L-DOPA is marred by the development of involuntary, often debilitating, movements known as dyskinesias (Bezard et al., 2001) as well as motor...
fluctuations, characterized by shortened duration of efficacy, manifest as ‘wearing off’ (Poewe et al., 1986; Hely et al., 1994; Nutt et al., 1995, 2002; Reardon et al., 1999; McColl et al., 2002). The efficacy of l-DOPA is shortened rather than reduced and duration roughly correlates with plasma levels of l-DOPA (Cotzias et al., 1967; Nutt et al., 1995), suggesting that the capacity to store endogenously synthesized l-DOPA is compromised.

Dyskinaesia almost invariably accompanies the shortening benefit from l-DOPA and the threshold and time course for dyskinesia and the anti-Parkinsonian effect of l-DOPA are similar (Mouradian et al., 1988; Nutt et al., 1992, 2002; Metman et al., 1997; Nutt, 2001). While dyskinesias, motor fluctuations and the short-duration response are not necessarily the same thing, it seems likely that they are interrelated inextricably through some common, causal mechanism. Any theory explaining these phenomena must explain how, in the face of failing storage and endogenous DA neurotransmission, l-DOPA can deliver adequate DA to produce these increased unwanted movements. The failing endogenous capacity presumably occurs because of diminishing synthetic capacity, as a consequence of terminal loss. Yet, when these terminals are provided with the DA precursor l-DOPA, they can provide sufficient DA to not only improve motor response but also produce increased and superfluous movements.

Failing storage of synthesized DA, or the ‘storage hypothesis’ is the most commonly proffered explanation for ‘wearing off’. The normal nigrostriatal neuron is tonically active (Grace and Bunney, 1984a, b), firing at about 6 Hz and continuously releasing low levels of DA, but DA release is increased with bursts of activity associated with salient events (Mirenowicz and Schultz, 1994). Synthesis (Onali et al., 1988; Lindgren et al., 2001), release (Ungerstedt et al., 1982; Bowyer and Weiner, 1987), and re-uptake of DA (Hersch et al., 1997; Robinson, 2002) are under neuronal control to maintain its continuous delivery. According to the storage hypothesis, as the number of striatal DA terminals fall, synthetic capacity is increased so that DA neurotransmission can be maintained while the substrate is provided by l-DOPA. Eventually there is no endogenous DA storage because all DA synthesized from l-DOPA is immediately released. Thus, as compensatory mechanisms are overwhelmed and storage capacity fails, delivery becomes pulsatile and dysregulated. However there is surprisingly little direct evidence to support this theory or to explain how it may work. It seems unsatisfactory that release is maintained yet storage falls as these mechanisms are closely coupled. Recently, it has been suggested that serotonin innervation of the striatal complex, which survives the effects of Parkinson’s disease, may be important in converting exogenous l-DOPA to DA (Carlsson et al., 2007; Carta et al., 2007). While serotonergic neurons can store and release DA in an activity-dependent manner, they have no functional DA re-uptake and storage and release would be poorly regulated. The implication is that the role of serotonergic neurons becomes increasingly important as ‘wearing off’ and dyskinesia become prominent (Carta et al., 2007).

In this study, we propose that impaired DA transporter (DAT) function and dysregulated DA release, appearing as a consequence of compensatory sprouting of surviving neurons, may also contribute to impaired storage and abnormal DA release. DA neurons that survive injury sprout to maintain DA terminal density as a compensatory response (Stanic et al., 2003a). Because of this regulatory process, in rodents, normal DA terminal density is maintained in the striatum until >70% of nigral cells are destroyed (Finkelstein et al., 2000; Stanic et al., 2003a). Although sprouting maintains terminal density, newly formed DA terminals have altered structure and function (Stanic et al., 2003b) and have a diminished capacity for DA re-uptake. The net effect is that re-innervating terminals would be capable of delivering a bolus of DA in response to l-DOPA but impaired re-uptake will result in increased DA diffusion, affecting more sites than would be anticipated from the density of innervation alone, an effect compounded by the increased terminal arbour. New terminals formed by sprouting have both a greater number of vesicles and larger-sized vesicles, which would intuitively suggest that these terminals are capable of delivering larger amounts of DA into the synaptic cleft (Stanic et al., 2003b). Although larger vesicle numbers and size suggest increased capacity for DA release, it may also reflect increased demand for synthesis in lieu of the impaired transport through the DAT. As a consequence of large terminal arbour, impaired synaptic clearance of DA, and altered release capacity, there will be abnormal DA delivery to unusually large regions of the striatum. This would provide a mechanism for impaired storage yet augmented release implied by the storage hypothesis.

This study measured striatal DA release and re-uptake in dyskinetic animals and correlated this with the extent of terminal loss and sprouting in rodents and primates. The findings show that despite falling terminal density, extrasynaptic DA release increases. These data provide direct support for the storage hypothesis as an explanation for dyskinesia and also provide support for the view that as striatal terminal density falls, DA is released almost as soon as it is synthesized, and that re-uptake is greatly reduced to augment extra-synaptic DA.

Materials and Methods
All methods conformed to the Australian National Health and Medical Research Council published code of practice for the use of animals in research and were approved by the Monash University and Howard Florey Institute Animal Ethics Committee. One hundred male Wistar rats weighing 250–350 g were used. Twenty-two male Common Marmosets (Callithrix jacchus), aged between 2 and 3 years at the commencement of the study and weighing ~350–450 g were used in this study.
Lesion of SNpc in rats and marmosets

In rats, varying lesions of the SNpc were made by injecting 1.5–3 μl of a 2.5 μg/μl solution of 6-OHDA (Sigma-RBI, St. Louis, MO, USA) into the right SNpc at 3.5 mm anterior, 1.7 mm lateral to lambda and 7.1 mm below dura and 3.5 mm anterior; 2.1 mm lateral and 6.8 mm below dura (Paxinos and Watson, 1998). Anaesthesia was induced with sodium pentobarbitone; 60 mg/kg i.p. with atropine (0.24 mg/kg i.p.) and maintained with a mixture of xylazine (2.4 mg/kg i.m) and ketamine (28 mg/kg i.m.).

In Marmosets, partial lesions of the SNpc were made by injecting 2.5 μg/μl of 6-OHDA into four sites in the SNpc (site 1: AP = 6.0 mm posterior to the inter-aural line, ML = 1.5 mm lateral to the midline, DV = 6.5 mm below the dura; site 2: AP = 6.5 mm, ML = 2.5 mm, DV = 7.0 mm, site 3: AP = 5.0 mm, ML = 1.5 mm, DV = 6.0 mm, site 4: AP = 5.0 mm, ML = 2.75 mm, DV = 6.5 mm). Anaesthesia was induced with saffan (0.5 ml of 12 mg/ml solution, i.m.) and maintained with injections of 0.1 ml of a 7:3 mixture of ketamine and xylazine (28 and 2.4 mg/kg, i.m.).

Behavioural studies

Assessment of abnormal involuntary movements (AIMs) in rats

Rats with varying lesion sizes received l-DOPA and benserazide (20 and 6.5 mg/kg i.p., twice daily) for 2 weeks. The development and manifestation of AIMs, a correlate of dyskinesia in rodents, were monitored on treatment Day 1, 5 and 10, as previously described (Cenci et al., 1998; Winkler et al., 2002). To briefly summarize, rats were observed for 1 min every 20 min after the injection of l-DOPA (20 mg/kg i.p.) or vehicle for 3 h. Four subtypes of AIMs were scored: (i) axial AIMs: dystonic posturing or choreiform twisting of the neck and upper body towards the side contralateral to the lesion; (ii) limb AIMs: abnormal, purposeless movements of the forelimb and digits contralateral to the lesion; (iii) orolingual AIMs: empty jaw movements and contralateral tongue protrusion and (iv) locomotive AIMs: increased locomotion with contralateral side bias. Each subtype was scored as 0: absent; 1: present during less than half of the observation time; 2: present during more than half of the observation time; 3: present all the time but suppressible by a startling stimulus; 4: present all the time and not suppressible. The total AIMs from one observation were 16 and as there were 10 observations (200 min), allowing a maximum score of 160.

Assessment of dyskinesia in primates

Dyskinesia in Marmoset monkeys was measured for 60 min, commencing 30 min after oral administration of a single dose of 20 mg/kg l-DOPA and 3.75 mg/kg Benserazide (Madopar® tablets in honey and administered orally, Roche, Australia). Dyskinetic movements were assessed every 10 min and were scored as: 0 = absent, 1 = mild: fleeting and rare (<5 in 10 min) dyskinetic postures and movements, 2 = moderate: more prominent and abnormal movements (5–20 in 10 min), but not interfering with normal behaviour, 3 = marked: frequent (21–50 in 10 min) and at times continuous dyskinaesias intruding on the normal repertoire of activity and 4 = severe: virtually continuous dyskinetic activity, disabling to the animal and replacing normal behaviour as previously described (Pearce et al., 1995).

Bradykinesia

This was the time taken by marmosets to reach through a slot in a Perspex barrier and grasp a small piece of sweetened bread (~0.5 cm cube). This test was performed five times on each test week (Henderson et al., 1998).

DA release and uptake in vivo and in vitro

Measurement of striatal DA release using in vivo voltammetry in rats

Thirty micrometer nafion-coated (5% solution, Sigma–Aldrich) carbon fibre microelectrodes (Textx System, Lowell, MA, USA) were glued to a fused glass capillary (i.d. = 40 μm, SGE, VIC, Australia) with separation between carbon-fibre electrode tips and capillary delivery tube of ~200 μm with an Ag/AgCl reference electrode. Voltammograms were recorded at 10 Hz (potential +550 mV, square-wave pulses), electrode linearity and sensitivity were determined against standard solutions of dopamine (Sigma–Aldrich) and average responses (nA/ms) were translated into DA concentration. DA concentrations in the dorsal striatum were recorded by inserting carbon fibre electrodes and 325 ±70 nl of 200 μM DA was injected through the fused silica capillary. KCl (70 mM, 200 ±50 nl) was applied through the capillary to measure DA release from the dorsal striatum. Clearance was expressed in terms of the time (in seconds) for the DA electrochemical signal to decrease to 50% of peak amplitude (T50, shown in Fig. 3).

Measurement of evoked DA release with medial forebrain bundle (MFB) stimulation in rats and marmosets

MFB stimulation was delivered through a twisted, bipolar stimulation electrode (Plastics One, Roanoke, VA, USA) positioned in the right MFB (coordinates for rat: AP −4.4 to −4.6 mm from bregma, ML 1.4 mm and DV 8.0 to 9.0 mm below dura; marmoset: AP +6.5 mm anterior to the inter-aural line; ML 1.4 mm lateral to the midline; DV 5.0 to 5.5 mm below dura). The position of the stimulating electrode was adjusted to record a maximal response in the recording electrode. A constant current stimulator delivered 50 Hz trains of ±500 μA square wave pulses for 2 ms repeated every 90 s. Each train (50 Hz at 2 ms) evoked DA release with a ‘bell-shaped’ curve appearance (Fig. 3E). Stimulus trains were delivered in a block of six and the DA curves resulting from each train were averaged together to make a single DA release curve for that time point. DA release and uptake over longer periods were recorded by repeating this protocol every 20 min for 140 min. The DA signal was conditioned (Clampfit, Version 8.2, Axon Instrument, USA) by stimulus-locked averaging for 10 s before and 60 s after the stimulus, artefact rejection, application of high (0.00125 Hz cut-off) and low (1 Hz) pass filter (Clampfit, Butterworth IR filter) and a Boxcar filter with 9 points running average. This smoothed DA waveform was used for subsequent measurement and statistical analysis. Electrodes were electrochemically treated by passing 20 Hz triangular pulses for 50 s followed by a 5-s 2.2 V square pulses and then coated with two layers of nafion. Electrodes were calibrated before and after implantation and were found to have selectivity for DA over ascorbic acid of >200:1. Chloride-plated silver wire was used as a reference electrode. Twenty minutes was allowed for equilibration after implantation and prior to recording.
Measurement of extra-synaptic DA release using striatal in vivo microdialysis in rats

Rats were anaesthetized with urethane (0.8 g/kg i.p.) and a microdialysis probe (CMA/12, membrane length 3 mm, OD 0.5 mm: Stockholm, Sweden) was placed in the right striatum at the coordinates with respect to bregma; AP +0.50 mm; ML +2.5 mm; DV 6.5 mm below dura. A modified Ringer solution (147 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, 1.1 mM MgCl₂) was perfused through the probes at 1 µl/min. Following 120 min of equilibration, samples were collected on ice every 20 min (20 µl) into a vial containing 10 µl of 0.1 M perchloric acid. After three basal samples collections, l-DOPA and benserazide (20 and 6.5 mg/kg i.p.) was administered and another seven samples were collected. The in vitro recovery of the probes for DA was 15%.

Detection of DA levels with high-performance liquid chromatography (HPLC)

The DA levels in microdialysate samples were measured by HPLC. Detection was via a BAS LC4B glass carbon-working electrode operating at +650 mV versus an Ag/AgCl reference. The mobile phase comprised the following (mM): KH₂PO₄, 70; EDTA di-sodium salt, 0.5; octane sulphonic acid, sodium salt, 8; pH 3.0 with 15% HPLC grade methanol (Lawrence et al., 2005). Flow rate was 1 ml/min. The detection limit of this system, defined as masses of standards producing peak heights of double the baseline noise, was 1.5 pg for DA (all standards from Sigma-Aldrich, USA).

DA uptake and clearance in striatal synaptosomes

The rate of DA clearance and release was measured electrochemically in striatal synaptosome preparations, as previously described (Stanic et al., 2003b). To briefly summarize, rats were decapitated, brains removed and cut in a coronal plane (4.3 mm posterior to bregma) to separate the striatum from the SNpc. The striatum was then hemisected and the dorsal striatum placed immediately in KRH buffer (mM: NaCl, 125; K₂HPO₄, 1.5; MgSO₄, 1.5; CaCl₂, 1.25; d-glucose, 10; HEPES, 25; ascorbic acid, 0.1; pargylene, 1; and EDTA, 0.1, pH 7.4) at 4°C and oxygenated. The brain areas were homogenized in 25 ml of cold sucrose, centrifuged at 2000 g for 10 min at 4°C. The supernatant was centrifuged at 16,000 g for 15 min at 4°C and the resulting pellet remained on ice until it was re-suspended in KRH buffer so as to obtain a concentration of 1000 w/v for the transport assay or in vitro electrochemistry. One millilitre of synaptosome suspension was added to each well and pre-incubated at 37°C for 3 min. Recordings were made as described above for in vivo measurements. In preparations from normal and lesioned animals, 12 µl of 0.25 mM DA were injected by micropipette into the synaptosome suspension. At this concentration, clearance of DA into synaptosomes was too rapid in samples from normal animals to allow a meaningful comparison with clearance from lesioned animals. Therefore, for measurement of DA transport into synaptosomes from normal animals, the concentration of DA added to the preparation was subsequently increased to 0.5 mM. DA release from synaptosomes was examined by adding 50 µl 1 M KCl to the preparation once DA concentration in the well returned to a baseline level (i.e. when no further DA was being taken up into synaptosomes). Clearance was expressed as time (in seconds) for the DA electrochemical signal to decrease to 50% of peak amplitude ($T_{50}$).

Histology and immunohistochemistry

DAT, serotonin re-uptake transporter (SERT), Fos B and tyrosine hydroxylase (TH) immunohistochemistry

Twenty micro meter-thick coronal sections were cut serially through the striatum and immunoreacted against dopamine reuptake transporter (DAT), serotonin reuptake transporter (SERT) and Fos B antibodies. DAT immunohistochemistry was performed (Stanic et al., 2003b) by incubating sections in rat anti-DAT primary antibody (Chemicon, Temecula, CA, 1:3000) followed by a biotinylated secondary antibody (rabbit anti-rat IgG, 1:500, Vector, Burlingame, CA, USA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich Pty Ltd). SERT immunohistochemistry was performed by incubating sections with mouse anti-SERT primary antibody (Chemicon, Temecula, CA, 1:1000) followed by a biotinylated secondary antibody (sheep anti-mouse IgG, 1:500; Chemicon, Temecula, CA) and reacted with cobalt and nickel-intensified DAB. Fos B immunohistochemistry was performed by incubating sections in rabbit anti-Fos B primary antibody (Santa Cruz, CA, 1:800) followed by a biotinylated secondary antibody (anti-rabbit IgG, 1:800, Vector, Burlingame, CA) and reacted with cobalt and nickel-intensified diaminobenzidine (DAB, Sigma-Aldrich). Fifty micro meter-thick coronal sections were cut serially through the SNpc and every fifth section was stained with neutral red and a parallel series reacted with antibodies against tyrosine hydroxylase (TH), as previously described (Stanic et al., 2003b). The following antibodies and dilutions were used: Anti-TH primary antibody (Boehringer Mannheim, Castle Hill, Australia, 1:3000) and a biotinylated secondary antibody (Sheep, anti-Mouse IgG, 1:500, Silenus, Australia).

Fractionator sampling scheme

The fractionator design for estimating the number of SNpc neurons and the number of DAT-ir axonal varicosities was published in detail previously with the following modifications (Stanic et al., 2003a). Counts of SNpc neurons, stained for neutral red or TH-ir, were made at regular predetermined intervals (x = 125 µm, y = 200 µm) derived by means of a grid program (Stereo Investigator, MicroBrightField, VT, USA) with an unbiased counting frame (40 µm x 27 µm = 1080 µm²). Therefore, the area sampling fraction is 1080/(125 x 200) = 0.043. In all animals, 50 µm thick sections through the SNpc, each 180 µm apart, were analysed, the fraction of sections sampled being 0.25. Lesion size was the number of SNpc neurons estimated in lesioned animals, expressed as a percent of the average number determined in the normal SNpc.

DAT-ir and TH-ir varicosities in the dorsal 1.5 mm of the striatum ipsilateral to the lesioned SNpc were counted from 20 µm thick serial sections, each 500 µm apart (the section sampling fraction being 1/25). Counts were made at regular intervals (x = 800 µm, y = 800 µm) with the area of unbiased counting frame being 40 µm² (0 µm x 5 µm). Thus, the area sampling fraction is 40/(800 x 800) = 0.0000625. The entire z-dimension of each section was sampled, the section thickness sampling fraction being 1. Varicosities were identified as a dilated element (usually round or oval shaped) of immunoreactive axons (Finkelstein et al., 2000). The total number of SNpc neurons or axonal varicosities was estimated by multiplying the number of neurons or varicosities counted within the sampled regions with the reciprocals of the fraction of the sections sampled, fraction of the sectional area sampled and the fraction of the section thickness.
sampled (West et al., 1991). The density of axonal varicosities was calculated by dividing the estimated number of varicosities by the volume of dorsal striatum estimated by the sampling scheme. Coefficients of error and coefficients of variance were calculated as estimates of precision.

**Statistics**

For comparisons of $T_{50}$, peak [DA] and [DA] from baseline, a Student unpaired $t$-test was applied to estimate overall significance between control and lesion groups followed by post hoc $t$-tests. In DA release or AIMS studies, the comparisons of DA levels or AIM scores that had been recorded repeatedly, were performed using a repeated-measures ANOVA. Bonferroni post hoc tests were used to estimate overall significances where appropriate. Relationships between variables were studied using either linear or sigmoidal regression where appropriate. A probability level of 5% ($P < 0.05$) was considered significant for all statistical tests. Data are expressed as means ± SEM.

**Results**

6-OHDA induced lesions of varying sizes were made in the rat SNpc. Sixteen weeks was allowed for stable sprouting of DA terminals and re-innervation of the striatum to occur (Stanic et al., 2003a). At the end of the 16 weeks, animals were treated with L-DOPA or vehicle for 2 weeks and assessed for dyskinaesia and extra-synaptic DA levels and uptake in the striatum. The animals were then killed and the density of DAT-ir terminals in the striatum and TH-ir cells in the SNpc were estimated using formal stereological techniques.

**The relationship between lesion size, striatal DAT terminal density and sprouting of DA terminals following SNpc lesions in rats**

The density of DAT-ir terminals in the striatum was plotted against the number of surviving TH-ir neurones in the SNpc (Fig. 1A). In keeping with previous studies (Finkelstein et al., 2000; Stanic et al., 2003a), the density of DAT-ir terminals 16 weeks after a lesion was normal in animals with lesion sizes less than 60% (Fig. 1A). As lesion size exceeds 60%, terminal density fell progressively to zero. This implies that when lesion size is <60%, new terminals form or sprout, from the axons of the surviving neurons as a compensatory response. As discussed in detail previously (Parish et al., 2001, 2002; Finkelstein et al., 2004), an index of this sprouting can be obtained by dividing the density of DAT-ir terminals by the number of TH-ir neurons in the SNpc, to give an ‘index of arbour size’ (IAS). Expressing the IAS of the lesioned striatum as a percentage of the IAS of the intact striatum provides percentage of sprouting (Fig. 1B). Figure 1B demonstrates that the percentage of DA sprouting is proportional to lesion size, confirming that pre-synaptic DA terminals compensate for neuronal loss until the lesion size become >60%.

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**Fig. 1** SNpc lesions of varying sizes were made in 28 rats (with eight controls). (A) Estimates of DA terminal density (DAT-ir terminals) for each lesioned animal were plotted against lesion size (number of TH-ir neurons in the SNpc as a percentage of normal) and the mean (±SEM) terminal density of control animals is shown as a circle. (B) Extent of sprouting was estimated by expressing ‘index of arbour size’ (see Results section) of each animal as a percentage of the normal ‘index of arbour size’ and plotting this against lesion size (number of TH-neurones in the SNpc as a percentage of normal). (C–F) Photomicrographs of DAT-ir axonal varicosities and fibres in the dorsal striatum of (C) Normal, (D) normal lesioned (E) partial lesioned and (F) extensive lesioned animals, 16 weeks following SNpc lesion. (Scale bar = 20 μm.)
An important point is that the extent of denervation of the striatum is likely to be more relevant to DA delivery into the striatum than the extent of neuronal loss. To reflect this we will express terminal loss as a percentage of denervation compared with the normal striatum: % denervation = (1 − normal density) × 100. Normal, normal lesioned, partial and extensive denervations are defined as 0, 1–30, 30–60 and 60–100% denervation, respectively (Fig. 1C–F).

The relationship between striatal denervation, striatal DA concentration and dyskinesia in rats

To determine whether there was a relationship between the extent of denervation and the development of dyskinesias, we assessed AIMS in rats following a single dose of L-DOPA on days 1, 5 and 10. On Day 10 of testing, dyskinetic movements, measured by the total AIM score, appeared within 20 min of L-DOPA administration and peaked between 80 and 120 min (Fig. 2A). The severity of the dyskinesia was the greatest in the extensively denervated animals, whilst the normal animals did not exhibit any dyskinesia following L-DOPA administration (Fig. 2A). The total AIM scores (the sum of the AIM scores from each time point from 0 to 180 min) between Day 1, 5 and 10 were similar (P > 0.05, one-way ANOVA, Fig. 2B), indicating that severity of dyskinesia did not depend on previous exposure to L-DOPA. Dyskinesia only became apparent when there was loss >60% of DAT-ir terminals in the striatum and increased markedly in severity with further loss of terminal density (Fig. 2C).

The concentration of extra-synaptic DA in the striatum that followed the administration of a single dose of L-DOPA (20 mg/kg, i.p.) was measured using in vivo microdialysis and HPLC (Fig. 2D and E). L-DOPA treatment did not affect the baseline DA concentration in unlesioned animals, as there was no significant difference between 14 days of L-DOPA treatment (95.0 ± 5.2 fmol/sample) and 14 days of vehicle treatment (102.3 ± 3.4 fmol/sample). However, basal striatal DA levels fell progressively and linearly (r = 0.8) from ~100 fmol/20 min of dialysates to about ~50–70 fmol/sample of dialysates as the denervation fell from normal to extensive loss of terminals (Fig. 2F). A single dose of L-DOPA (20 mg/kg, i.p.) produced an insignificant rise in DA concentration in unlesioned animals and animals with lesions but normal density of DAT-ir terminals. However, when DAT-ir terminals were reduced, there was a robust rise in DA concentration: 210% (partial) and 510% (extensive) increase, respectively (Fig. 2D and E). The DA concentrations peaked (156 ± 17 and 354 ± 32 fmol/sample, respectively) between 40 and 60 min after L-DOPA administration (Fig. 2D and E). In rats with markedly reduced DAT-ir terminal density, striatal DA levels remained significantly elevated until recording ceased, 140 min after L-DOPA injection (two-way ANOVA, Fig. 2D and E). The rise in striatal [DA] was similar in vehicle and L-DOPA-treated rats.

Total striatal DA concentration was plotted against the density of DAT terminals in the striatum (Fig. 2G), showing that, following a dose of L-DOPA, striatal [DA] did not rise above baseline until there was about 60% loss of terminals. The relationship between density of DAT terminals and total [DA] (Fig. 2G) was similar to that between density of DAT terminals and total AIM score (Fig. 2C). This impression was confirmed by plotting AIMS score against DA concentration (Fig. 2H) and showed that severity of dyskinesia was linearly related to striatal DA concentration (r = 0.9).

Striatal DA re-uptake is impaired in newly sprouted DA axon terminals in rats

We examined DAT function in lesioned animals to establish whether altered re-uptake may contribute to the very high levels of striatal [DA] following a dose of L-DOPA. This was done using striatal synaptosome preparations and measuring clearance of DA from the striatum with carbon fibre electrodes located in the dorsal striatum of lesioned and normal rats. Rats with SNpc lesions >65% were used because it was not possible to estimate percentage of denervation, especially with the synaptosomal preparation.

Figure 3A provides an example of the measurement of DA concentration in the dorsal striatum in vivo, made before and after local application of DA (200 µM). In normal animals, DA concentration rose rapidly to a peak and was also cleared promptly. In a lesioned animal, the time to peak DA concentration was significantly longer and clearance was greatly prolonged. Clearance of DA, measured as the time taken for DA concentration to fall from peak level to 50% of peak level (T50), was approximately twice as long in lesioned animals (P < 0.001, in vivo, Fig. 3B). DA clearance was consistently prolonged at each of three sites in the striatum (data not shown). Peak amplitudes of DA concentration were similar in lesioned and control animals (Fig. 3C, in vivo DA) as was peak DA concentration evoked by KCl (P = 0.074, in vivo KCl, Fig. 3C). Rate of DA clearance (T50) was also measured from in vitro synaptosome preparations extracted from the dorsal striatum of normal (n = 6) and 6-OHDA lesioned rats (n = 12). Average lesion size was 59 ± 29%. Clearance of DA into synaptosomes was measured electrochemically by directly injecting known amounts of DA into the synaptosome preparation resulting in the same concentration of DA. Clearance of DA by synaptosomes from lesioned animals was markedly prolonged (Fig. 3B, in vitro). Clearance of 0.25 nM DA/mg by synaptosomes from lesioned animals was measured, but because the clearance by normal synaptosomes was so much more rapid, a greater concentration of DA was used for normal animals (0.5 nM DA). Peak amplitudes of DA concentration evoked by KCl were the same in both normal and lesioned rats (Fig. 3C, in vitro).
The binding properties of DAT in the rat dorsal striatum were measured using [3H]Mazindol and the data are represented in Table 1. As previously shown (Stanic et al., 2003b), the $K_d$ value of the high-affinity site ($K_{d1}$) in lesioned animals was similar to that observed in normal animals, although the density ($B_{max1}$) was reduced by almost 40%, $P<0.05$). The affinity of the second binding site ($K_{d2}$) was reduced by lesioning, concurrent with a 5-fold increase in the

Fig. 2 (A) Time course of total AIM scores/observation in rats following a single dose of l-DOPA (20 mg/kg i.p.) on Day 10 of behavioural testing. There was little evidence of dyskinaesia in animals without lesions (open triangle) or with lesions but normal density (filled square). Dyskinaesia was marked in animals with extensive (filled triangle) and to a lesser extent, partial denervation (filled circle). (B) The total AIM scores on Days 1, 5 and 10 of daily l-DOPA administration to rats with extensive striatal denervation. The total AIM score is the sum of scores from administration of a single dose of l-DOPA (20 mg/kg i.p.) to 180 min later. This plot shows that the dyskinaesia is present on the first dose of l-DOPA and duration of l-DOPA administration has no effect on severity of dyskinaesia. (C) Total AIM score plotted against % denervation [(l-density/normal density) x 100] and fitted with a sigmoidal curve. ($r=0.9$). (D and E) Time course of extra-synaptic DA release in the striatum, before (-40 to 0 min) and after (20 to 140 min) administration of l-DOPA (20 mg/kg) in animals with extensive (filled triangle), partial (filled circle), normal density lesioned (filled square) and normal density unlesioned (unfilled triangle). (D) Animals which had received 14 days of l-DOPA, and (E) animals which had received l-DOPA for the first time. (F) Baseline concentration of extra-synaptic striatal DA plotted against percentage of denervation ($r=0.8$). (G) Total [DA], following administration of a single dose of l-DOPA, plotted against percentage of denervation with a sigmoidal curve. ($r=0.9$). (H) Total [DA], following administration of a single dose of l-DOPA, plotted against total AIMs. This relationship was confirmed by plotting total AIMs against total [DA] to yield a linear line with an $r$-value of 0.9.
density (\(B_{\text{max}}\)) of the lower-affinity site (\(P<0.05\)). [\(^3\)H]DA transport measured in synaptosomes (Stanic et al., 2003b) from the dorsal striatum of rats with large lesions (\(>60\%\)) and small lesions (\(<60\%\)) was markedly reduced (Fig. 3D). In large lesions, DA transport falls to levels found with mazindol treatment. Taken together, these studies suggest that striatal DA re-uptake is impaired in the newly sprouted terminals following SNpc lesions.
The capacity for remaining nigral fibres to release DA was assessed by measuring the DA released into the striatum of the rat following MFB stimulation (Fig. 3E and F). Striatal DA release was evoked by a train of electrical stimuli delivered to the MFB and DA was detected in the dorsal striatum (Fig. 3E). There were 17 animals with lesions ranging from 59 to 82% and percentage of denervation ranging from 5 to 82%, as well as 10 normal animals. T50 rose from a mean of 13.6 ± 3.4 s in normal animals to as long as 29.5 s in rats with large lesions. These data were fitted with a logarithmic regression (r = 0.6, Fig. 3F), showing that T50 rose abruptly initially, and then plateaued. Examination of Fig. 3B shows T50 measured in vivo, is approximately twice as long as in lesioned animals as in normal animals, whereas T50 measured in vitro is ~30-fold greater in normal animals. In the case of in vitro preparations, re-uptake is the primary mechanism operating whereas in vivo, factors such as diffusion and metabolism operate and we speculate that these dominate when re-uptake becomes very slow and may explain why the curve plateaus.

**Relationship between DA denervation, striatal DA release and dyskinaesia in marmosets**

Partial lesions ranging from 20 to 90%, were made in 14 marmosets and compared with eight unlesioned animals. Histological estimates of lesion size and density of DAT-ir terminals in the striatum were made 6 months after the lesion. Presumably reflecting the greater heterogeneity of genetic background of marmosets compared with in-bred rats, the number of TH-ir cells in the SNpc and of striatal DAT-ir terminal density varies considerably. To overcome this problem, the lesioned side was expressed as a percentage of the unlesioned side (percentage of lesion size and percentage of DAT density) to allow reference parameters of DA release and re-uptake to be compared with striatal denervation in individual animals. Accordingly, lesions ranged from 20% to 90% (Fig. 4A). Unlike rats, percentage of DAT density did not return to normal but plateaued near 60–80% of normal, even with lesions <60% (Fig. 4A). With larger lesions, terminals were few and sparse and approached 100% loss. Six of these lesioned and six unlesioned marmosets were used in the studies described below.

Immediately after lesioning, the right arm of all lesioned monkeys was bradykinetic as measured by their performance of the staircase task (40.9 ± 24.7 s; mean ± SE compared with normal, 17.5 ± 4.6 s) but had returned to normal by 20 weeks after lesioning (20.9 ± 6.7 s, P = 0.13, t-test), although individual animals with large lesions remained significantly impaired. Prior to lesioning none of the marmosets rotated spontaneously. One week post-lesion, most animals spontaneously turned towards the lesioned side but 20 weeks post-lesion this plateaued at 1.8 ± 2.7 turns per hour even in animals with greatly diminished percentage of DAT density. This suggests that remaining terminals were capable of maintaining similar levels of DA to the contralateral side. Rotation in response to acute administration of L-DOPA was assessed at 1, 2, 6 and 10 months post-lesion and produced marked rotation in the opposite direction to the lesion (data not shown): this was greatest in animals with the fewest terminals (largest lesions). This implies that DA terminals in the partially innervated striatum can, in the presence of L-DOPA, produce nigrostriatal dopaminergic signalling that exceeds the normal side.

### Table 1: Scatchard analysis of [3H]Mazindol binding to DA transporter in the dorsal striatum of normal and SNpc lesioned animals 16 weeks after injury (mean ± SD)

<table>
<thead>
<tr>
<th>Dorsal striatum</th>
<th>Kd1 (nM)</th>
<th>Bmax1 (fmol/mg protein)</th>
<th>Kd2 (nM)</th>
<th>Bmax2 (fmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>768 ± 2.2</td>
<td>1441 ± 483</td>
<td>306 ± 182</td>
<td>4284 ± 1631</td>
</tr>
<tr>
<td>(n = 5)</td>
<td></td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>Contralateral</td>
<td>987 ± 2.7</td>
<td>1517 ± 439</td>
<td>126 ± 62</td>
<td>4688 ± 2308</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>(n = 8)</td>
<td>(n = 7)</td>
<td>(n = 7)</td>
</tr>
<tr>
<td>Lesioned</td>
<td>96 ± 3.1</td>
<td>954 ± 281</td>
<td>8349 ± 6938</td>
<td>22739 ± 11465</td>
</tr>
<tr>
<td>(n = 7)</td>
<td></td>
<td>(n = 7)</td>
<td>(n = 7)</td>
<td>(n = 6)</td>
</tr>
</tbody>
</table>

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**T**50 was unaltered by L-DOPA administration in either lesioned or normal animals (data not shown).

### Relationship between DA denervation and dyskinesia in marmosets

Dyskinetic movements were scored 30 min after administration of 20 mg/kg of L-DOPA and 3.75 mg/kg benzerazide p.o. and were assessed after 1, 2, 6, 10, 11 and 12 months of long-term L-DOPA treatment. Choreiform and dystonic movements, affecting upper limbs, face, trunk, neck and orolingual regions were observed in the four lesioned marmosets treated with L-DOPA (Fig. 4J) and these were prominent in animals with large lesions. Although there were only four animals, the extent of dyskinesia appeared related to the extent of striatal denervation. The percentage of denervation of the two markedly dyskinetic animals were 75 and 83% of normal (SNpc lesions of 77 and 80%, respectively), whereas percentage of denervation in the two animals with the less severe dyskinesia were 20 and 24% (SNpc lesion size of 41 and 48%). As with rats, rotation rates induced by L-DOPA were increased in animals with the greatest denervation and in the opposite direction to the lesioned side.

### Modelling of DA release in the striatum evoked by MFB stimulation

The methods and results illustrated in Figs 2–4 provide information about T_{50} and AUC but only indirect information about DA release. We therefore used the information from Figs 2F and 3F and a simple linear differential equation \( \frac{dC}{dt} = \frac{-C}{kC_1} \) to model the release of DA evoked by MFB stimulation. We have assumed that DA is released at a constant rate, represented by the constant \( C_1 \), over the first 10 s after stimulus. The re-uptake of DA was assumed to be directly proportional to the concentration and that re-uptake, diffusion and metabolic breakdown can be represented by a constant \( k \). T_{50} values from the logarithmic regression and the polynomial used to fit the data in Figs 2F and 3F were used to produce a family of 20 curves (Fig. 5A shows an example of one of the curves) corresponding to percentage of denervation from 1% to 100%. These were used to obtain the corresponding DA_{T_{50}}.
shown in Fig. 5B. The conclusion from this modelling is that there must be a sharp and dramatic increase in DA released from the remaining release sites to account for the increased DA release. While changes in $T_{50}$ may be important they are not sufficient alone to produce the marked increase when denervation is significant. We estimate that the amount of DA release by each remaining terminal when denervation is 95% is about 40-fold greater than normal.

**Fos B expression and SERT immunoreactive terminals in the striatum following l-DOPA treatment**

When lesions of the SNpc are made, sprouting of DA terminals is confined to the dorsal striatum. If sprouted terminals are responsible for abnormal DA release and activation of post-synaptic medium spiny neurons (MSNs) to produce dyskinaesia, then MSN activation should be constrained to the dorsal striatum. To address this, animals were killed 60 min after the administration of l-DOPA and processed for Fos B immunohistochemistry. As previously reported (Cenci et al., 1999), there was a marked upregulation of Fos B immunoreactive neurons in the dorsolateral striatum of lesioned animals treated with l-DOPA (Fig. 6B), that was not apparent in sham animals treated with l-DOPA (Fig. 6A).

One explanation for the marked increase in DA with large lesions is unregulated activity of serotoninergic axons that have sprouted new terminals in the MFB. However there was no change in the density of SERT-immunoreactive terminals in animals with large lesions compared to sham animals (Fig. 6C and D), making this an unlikely explanation.

**Discussion**

Dyskinaesias are produced by both pre and post-synaptic disturbances (Cenci and Lundblad, 2006). Excess striatal DA following a dose of l-DOPA appears to be an important
component of the pre-synaptic mechanism responsible for genesis of dyskinaesia (Meissner et al., 2006). The aims of this study were to examine in detail the relationship between striatal DA denervation, striatal DA concentrations and dyskinaesia.

An important consideration in this study is the examination of DAT-ir terminal density and the use of 6-OHDA lesioned animals that have been allowed to establish stable re-innervation of the striatum. In keeping with earlier reports (Finkelson et al., 2000; Stanic et al., 2003a), sprouting of surviving neurons prevents striatal denervation occurring until about 60% of neurons are lost. The process of compensation through sprouting is likely to be present in a slowly progressive condition such as Parkinson’s disease. When sprouting can no longer maintain terminal density, striatal [DA] begins to fall and is likely to be analogous to when humans with Parkinson’s disease become symptomatic.

While 1-DOPA replacement does not significantly raise striatal [DA] levels in rats with striatal denervation of <60%, striatal [DA] levels increased by 3-fold when the denervation was extensive (i.e. >60%). The point on the graph (Fig. 2G) where striatal [DA] begins to markedly increase corresponds to the point where dyskinaesia scores increase (Fig. 2C). Indeed, the extent of dyskinesia was linearly related to the striatal [DA] following 1-DOPA administration, suggesting that expression of dyskinesia was associated with high striatal [DA]. This reflects the findings of PET studies, demonstrating that pre-synaptic DA release in Parkinson’s disease patients correlates with higher dyskinaesia scores (Pavese et al., 2006).

We previously showed that DA terminals newly formed by sprouting have altered structure and function, including impaired DAT function, increased terminal bouton size, increased number of vesicles, mitochondria and contacts onto more proximal targets (Stanic et al., 2003b). The net effect is that re-innervating terminals could deliver a larger activity-dependent bolus of DA, which in the presence of impaired re-uptake could diffuse further, affecting DA receptor sites over a wider area of the striatum. As a consequence of large terminal arbours, impaired synaptic clearance of DA and altered release capacity, there will be abnormal DA delivery to unusually large regions of the striatum. The increased capacity for DA release and impaired DAT function will seriously compromise the capacity to store DA and contribute to the shortening duration of response to 1-DOPA observed in Parkinson’s disease. The benefit of 1-DOPA would progressively shorten as DAT function fails, reflected as ‘wearing off’ due to impaired storage and dyskinesia by higher DA levels. This is keeping with observations that pre-synaptic mechanisms are important in both short-duration and long-duration responses encountered in Parkinson’s disease and dyskinaesias (de la Fuente-Fernandez et al., 2004). These findings also support the clinical observations that greater DAT levels are directly associated with lower DA turnover and lower changes in synaptic DA concentration in Parkinson’s disease patients (Sossi et al., 2007). Thus, decreased levels and/or impairment of DAT, although potentially serving as a compensatory mechanism in early disease, may ultimately result in increased DA turnover and higher oscillations in synaptic DA concentration.

Stimulation of the MFB pathway could be considered to reflect activity-dependent release of DA. In the monkey, and most likely in the rat, AUC and DA_P were already increased in lesioned animals and 1-DOPA treatment did not increase it further. In unlesioned animals, both parameters approximately doubled and approached the (presumably maximum) output of lesioned animals. This implies that unlesioned animals were capable of storing the DA synthesized from 1-DOPA treatment whereas lesioned animals delivered it directly into the striatum.

This study has not fully resolved the question of the origin of the very high levels of DA in animals with few remaining terminals. While we have suggested that impaired transporter function and altered storage may be important factors, other contributions may also be important. Indeed the presence of DA in animals with effectively no DA terminals, and unstimulated with 1-DOPA, argues for a persisting site of DA synthesis and release. Convincing evidence of the role of serotonergic fibres capacity to provide DA as a false transmitter has been provided (Carta et al., 2005, 2007). While this mechanism would convert 1-DOPA to DA, the persisting levels of DA suggest that another source is active. The lack of increase in the density of SERT-positive fibres in lesioned animals suggest that any increased capacity of serotonergic fibres to convert 1-DOPA to DA is by upregulating DA synthesizing enzymes, rather than increasing release sites.

Another explanation for increased striatal [DA] and dyskinesia is the possible synthesis of DA from exogenous 1-DOPA in non-neuronal aromatic l-amino acid decarboxylase (AADC) containing cells (Melamed et al., 1980; Betarbet et al., 1997; Brown et al., 1999; Porritt et al., 2000a) found in rats (Betarbet et al., 1997), monkeys (Ikemoto et al., 1997) and humans (Porritt et al., 2000a). It is argued that striatal AADC immunoreactive neurons are induced by DA denervation (Meredith et al., 1999), but AADC positive neurons are present in the normal brain and denervation does not change their numbers (Lopez-Real et al., 2003). Their number is increased in Parkinson’s disease (Porritt et al., 2000b), but curiously their distribution is in areas where there is continuing nigral innervation (Porritt et al., 2000a; Lopez-Real et al., 2003). While Porritt and colleagues (2000a) argue that their numbers are sufficient to contribute to 1-DOPA conversion in the DA denervated striatum, the TH and AADC-positive cells are probably different populations (Ikemoto et al., 1997; Meredith et al., 1999) and the level of enzyme activity does not seem to be sufficient to convert 1-DOPA into DA (Nakamura et al., 2000). Perhaps most importantly, the evidence from this study shows that release of DA was from
Sprouting of dopamine terminals in dyskinesia


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