Survival of midbrain dopaminergic cells after lesion or deep brain stimulation of the subthalamic nucleus in MPTP-treated monkeys

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We have examined dopaminergic cell survival after alteration of the subthalamic nucleus (STN) in methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated monkeys. The STN was lesioned with kainic acid (B series) or underwent deep brain stimulation (DBS) at high frequency (C series). In another series, MPTP-treated and non-MPTP-treated monkeys had no STN alteration (intact animals; A series). Animals were treated with MPTP either after (B1, C1) or before (B2, C2) STN alteration. We also explored the long-term (~7 months) effect of DBS in non-MPTP-treated monkeys (D series). Brains were aldehyde-fixed and processed for routine Nissl staining and tyrosine hydroxylase immunocytochemistry. Our results showed that there were significantly more (20–24%) dopaminergic cells in the substantia nigra pars compacta (SNc) of the MPTP-treated monkeys that had STN alteration, either with kainic acid lesion or DBS, compared to the non-MPTP-treated monkeys (intact animals). We suggest that this saving or neuroprotection was due to a reduction in glutamate excitotoxicity, as a result of the loss or reduction of the STN input to the SNc. Our results also showed that SNc cell number in the B1 and C1 series were very similar to those in the B2 and C2 series. In the cases that had long-term DBS of the STN (D series), there was no adverse impact on SNc cell number. In summary, these results indicated that STN alteration offered neuroprotection to dopaminergic cells that would normally die as part of the disease process.

Keywords: Parkinson disease; kainic acid; neuroprotection; glutamate toxicity; substantia nigra; primate

Abbreviations: 6OHDA = 6 hydroxydopamine; AC–PC = anterior commissure-posterior commissure; b = B series; c = C series; d = D series; DBS = deep brain stimulation; MG = medial geniculate nucleus; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MRI = magnetic resonance imaging; PaG = periaqueductal grey matter; PBS = phosphate buffer saline; PD = Parkinson disease; PpT = pedunculopontine tegmental nucleus; SNc = substantia nigra pars compacta; SNr = substantia nigra pars reticulata; STN = subthalamic nucleus; TH = tyrosine hydroxylase; VTA = ventral tegmental area; ZI = zona incerta

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Introduction

Recent studies have suggested that glutamate excitotoxicity contributes to dopaminergic cell death in the SNc in Parkinson disease (PD; Lavoie and Parent, 1994; Blandini et al., 2004). This suggestion is based on the increased vulnerability of these cells to extracellular glutamate, due to a mitochondrial complex I defect (Schapira, 2001). In addition, there is evidence showing that after experimental reductions of glutamatergic inputs or receptor activities in the SNc of PD models, there is a saving of dopaminergic cells. For example, when NMDA receptor antagonists are infused into the SNc, the toxicity of MPTP on the dopaminergic cells is blocked (Brouillett and Beal, 1993). The same effect is achieved also by removing—whether by lesion or DBS—the glutamatergic inputs to SNc, namely those from STN (Hammond et al., 1983; Parent and Smith, 1987) and pedunculopontine tegmental nucleus (PpT; Saper and Loewy, 1982; Charara et al., 1996).
Both STN (Mitchell et al., 1989; Bergman et al., 1994; Bezard et al., 1999) and PpT (Breit et al., 2001) inputs are overactive in PD and hence presumed to impart glutamate excitotoxicity in the SNc. Indeed, many studies have shown that both lesion (Piallat et al., 1996; Nakao et al., 1999; Chen et al., 2000; Carvalho and Nikhah, 2001; Paul et al., 2004) or DBS (Maesawa et al., 2004) of the STN, prior to parkinsonian insult, saves dopaminergic cells. Similarly, in MPTP-treated monkeys with a prior PpT lesion, there are many more dopaminergic cells evident compared to controls (Takada et al., 2000). In addition, Temel and colleagues (2006) have shown recently that in cases of DBS of the STN in rats after parkinsonian insult [6 hydroxydopamine (6OHDA)], there is a saving of dopaminergic cells in the SNc also.

In this study, we explored the survival of dopaminergic cells with STN alteration in MPTP-treated monkeys. The STN was altered in two ways (i) the traditional method of kainic acid ablation (B series) and (ii) DBS at high frequency (C series). We used a subacute model of MPTP treatment, one designed specifically for examining dopaminergic cell survival (Ashkan et al., 2006). There were two approaches. In one set of each series (B1, C1), there was STN alteration before MPTP treatment. Removing glutamatergic pathways prior to the parkinsonian insult is a widely used experimental paradigm; it ensures that any dopaminergic cell loss occurs entirely in the absence of the excitotoxic pathway (e.g. Piallat et al., 1996; Maesawa et al., 2004). In a second set of each series (B2, C2), there was MPTP treatment followed by STN alteration. This is more like the clinical reality, where cell loss due to insult occurs prior to surgical intervention. In a final series (D series), we examined the survival of dopaminergic cells under chronic DBS of the STN in non-MPTP-treated animals. Our results on this primate model of PD—the most readily applicable to humans—will hopefully provide insights into the neuroprotective effects of STN alteration in PD cases.

Materials and methods

Subjects

Adult male macaque monkeys (Macaca fascicularis; Mauritius; n = 28) weighing 3.4–8 kg were used. Animals were maintained in individual cages under controlled conditions of temperature (∼25°C) and light (12 h light/dark cycle), were fed regularly on a diet of fruit and biscuits and had free access to water. The laboratory is authorized by the French Ministry of Environment (Agreement No B3851610003) and all experiments were performed in accordance with the European Communities Council Directive of 1986 (86/609/EC) for care of laboratory animals. All participants of the team had received proper training and certification for animal experimentation. Every effort was made to minimise animal suffering, while maximizing data output.

Experimental design

There were four experimental series in this study:

A series (n = 3)

This series formed the intact group. One monkey had no MPTP treatment (A1-1), while the other two did (A1-2, A1-3). None of these monkeys had STN alteration.

B series (n = 10)

This series had kainic acid injections into the STN area and were divided into two main groups:

B1 (n = 5): STN lesions followed by MPTP treatment (11.6 ± 3.3 days later).

B2 (n = 5): MPTP treatment followed by STN lesions (6 ± 0.7 days later).

C series (n = 10)

This series had continuous DBS into the STN area and were divided into two main groups:

C1 (n = 5): DBS of STN followed by MPTP treatment (7 ± 0.5 days later). Two of these monkeys (C1-1, C1-4) died during recovery after surgery and it was not possible to recover any data from them.

C2 (n = 5): MPTP treatment followed by DBS of STN (6 ± 0.5 days later).

The rationale behind the time periods for STN alteration after MPTP treatment (B2–C2 series) was to ensure adequate time for the dopaminergic cells to be exposed to the toxicity of MPTP and hence for the degenerative process to begin. In similar fashion, the rationale behind the time periods of MPTP treatment after STN alteration (B1–C1 series) was to ensure adequate time for the STN axons to degenerate or become under the influence of DBS; hence we could be confident of maximum effect of our lesions or DBS (Ashkan et al., 2004, 2006).

D series (n = 5)

This series of monkeys did not have MPTP treatment. All underwent continuous DBS of the STN for nearly 7 months (201 ± 4.5 days).

For the B, C and D series, STN alterations (lesion or DBS) were always on the right-hand side (RHS) of the brain; the left-hand side (LHS) was left untouched and served as a control (see later).

Surgical technique

Anaesthesia was a combination of ketamine (20 mg/kg loading dose, 5 mg/kg maintenance im) and diazepam (0.2 mg/kg iv). In addition, 1% lidocaine with adrenaline was used for local anaesthesia of the scalp. Saline was infused intravenously during the operation for drug access and hydration. Anteroposterior and lateral ventriculography X-rays were performed through a stereotactically placed right lateral ventricular puncture through which 2 ml of ventricular contrast (Iopamiron® 200, iodine 200 mg/ml, Italy) was injected after 0.5 ml of confirmatory air (Fig. 1A and B). Similar to the protocol used in surgery for PD patients, the coordinates for stereotactic targeting were based on III ventricular landmarks [Anterior (AC) and posterior (PC) commissures] obtained from the ventriculograms.

Electrophysiological localization

Electrophysiology was performed—to refine the radiographic target—simultaneously through four microelectrodes held by a custom designed electrode holder (Fig. 1C and D). The microelectrodes (FHC, USA, Tungsten Microelectrode w/Cannula
2–4 Meg) were preloaded in guide tubes which were then placed into the four channels of the holder (Fig. 1D). The trajectory was parallel to the sagittal plane and 20° anterior to the coronal plane. Electrophysiology was performed simultaneously through four microelectrodes and from the AC–PC plane, through the STN, to the SNr. (E) Indicates the characteristic firing pattern seen in the STN by the microelectrodes; high background noise and cells that show bursting activity. (F) The quadripolar Medtronic electrode used for DBS. Arrow indicates one of the four contacts; the most appropriate contact (one located within the STN, determined electrophysiologically) was activated. (G) Shows a lateral X-ray of monkey with DBS electrode within STN (arrow). This electrode was connected to an implantable pulse generator (arrowhead). (H) Shows an MRI scan, along transverse plane. In each case, the electrode tract location within STN checked with MRI (arrow).

**Fig. 1** Lateral (A) and antero-posterior (B) ventriculography X-rays, performed through a stereotactically placed lateral ventricular puncture (arrows) through which ventricular contrast was injected; * indicates location of III ventricle. (C) Shows the electrode holder and (D) shows the holder with the microelectrodes in place. Electrophysiology was performed simultaneously through four microelectrodes and from the AC–PC plane, through the STN, to the SNr. (E) Indicates the characteristic firing pattern seen in the STN by the microelectrodes; high background noise and cells that show bursting activity. (F) The quadripolar Medtronic electrode used for DBS. Arrow indicates one of the four contacts; the most appropriate contact (one located within the STN, determined electrophysiologically) was activated. (G) Shows a lateral X-ray of monkey with DBS electrode within STN (arrow). This electrode was connected to an implantable pulse generator (arrowhead). (H) Shows an MRI scan, along transverse plane. In each case, the electrode tract location within STN checked with MRI (arrow).

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2–4 Meg) were preloaded in guide tubes which were then placed into the four channels of the holder (Fig. 1D). The trajectory was parallel to the sagittal plane and 20° anterior to the coronal plane. Electrophysiology was performed from the AC–PC plane, through the STN, to the substantia nigra pars reticulata (SNr) in 200 μm steps using the Leadpoint™ system (Medtronic, USA). During a typical track, the first structure encountered was the thalamus, recognized by a bursting pattern of discharge. There was then an area of decreased activity as the electrode tip passed through the region of the zona incerta. STN was usually encountered 3 mm below the AC–PC line and was characterized by a sudden increase in the background noise reflecting high cellular density and cells with bursting activity at frequencies of 40–50 Hz (Fig. 1E). The electrode location within the motor area of STN was verified by the response of cells (increased firing audible through signal transduction and amplification) to passive movement of the contralateral limbs. The entrance of the electrode into the more ventrally located SNr was identifiable by high, but regular discharge rate of about 70 Hz. Control X-rays were utilized throughout the procedure to confirm electrode and catheter location.

**STN alterations**

**Kainic acid lesion**

Kainic acid (Sigma, USA) was dissolved in 0.9% NaCl at a concentration of 1 mg/1 ml. After the optimal injection site was determined (by electrophysiology; see above), the microelectrode was removed and replaced with a cannula of identical length to the microelectrode. The cannula was then attached to a 25 μl
Hamilton syringe driven by a micropump (KD Scientific, USA). Approximately 5 μl of kainic acid solution was infused at a rate of 0.5 μl/min. After the injection was completed, the cannula was left in place for 10 min prior to withdrawal.

**DBS**

After electrophysiological determination of the STN, a chronic quadriporal DBS electrode (Fig. 1F; Medtronic 3389-28) was inserted and secured to the skull. The distal part of the electrode was connected through an extension (Medtronic 7482) to an implantable pulse generator (Soleta 7426) inserted in a subcutaneous pouch between the shoulders (arrowhead Fig. 1G). The implantable pulse generator was programmed transcutaneously in a non-invasive manner using programming consoles (Medtronic N’Vision). The animals were stimulated in a monopolar fashion with the electrode contact designated as the cathode (−), and the case of the pulse generator designated as the anode (+). The stimulating active contact (e.g. arrow Fig. 1F) was chosen in the awake monkey, 24–72 h after implantation, based on clinical observation of benefits, such as improvement of rigidity or development of dyskinesia and side effects, particularly muscular contractions. Frequency was set at 130 Hz, pulse width at 60 μs and the amplitude between 2 and 3 V, immediately below the threshold of side effects. After surgery, the location of the DBS electrode within the STN region was checked using MRI (arrow Fig. 1H).

**MPTP treatment**

A subacute MPTP model was used as it produces an effective primate model of PD and it is the most appropriate for dopaminergic cell survival studies (Ashkan et al., 2006). MPTP (Sigma, USA; 0.2 mg/kg) was administered intramuscularly for eight days consecutively. Twenty days after the last MPTP injection, animals were aldehyde perfused (see later). The non-MPTP-treated monkey of the A series (A1) was perfused after 21 days of clinical evaluation while the monkeys of the D series (D1-5) were perfused ~7 months (201 ± 4.5 d) of continuous DBS of the STN, ~8 months (241 ± 17.4 d) after implantation of the electrode.

**Clinical evaluation**

Animals were assessed in their home cages starting 4 weeks after their initial arrival. Assessments were performed on five occasions before the start of MPTP treatment to establish the baseline and thereafter occurred on a daily basis until sacrifice. On each occasion, animals were scored independently by two observers over a 30 min period and video taping was used to facilitate this. A modified Benazzouz scale (Benazzouz et al., 1995; Ashkan et al., 2006) was applied and the following parameters were rated: Tremor (0–3), bradykinesia (0–3), change in posture (0–3), vocalization (0–1), frequency of arm movements (0–3 for each arm) and the general level of activity (0–3). Minimum score was zero and maximum score was 19.

**Immunohistochemistry and histology**

Animals were anesthetized after intraperitoneal injection of sodium pentobarbital (50–60 mg/kg) and perfused transcardially with 0.9% saline followed by 4% buffered formaldehyde. Brains were removed, blocked, immersed in the same fixative for 24 h and then placed in saline with the addition of 30% sucrose until the block sank. They were then sectioned coronally on a cryostat at a thickness of 50 μm. Every section was collected in sequence; one series was collected onto gelatinized slides and processed for routine cresyl violet staining while five series were collected in phosphate-buffered saline (PBS; free floating) for immunohistochemistry. Sections were immersed in a solution of 10% normal goat serum and 1% bovine serum albumin (made up with PBS) for 1 h. In some cases, sections were placed in 10% H2O2 in 70% ethanol for 10 min in order to block endogenous peroxidase activity. Sections were incubated in anti-TH for 48 h at 4°C (1:500; Chemicon, France). The sections were then incubated with biotinylated anti-mouse (1:200; IgG, Sigma, USA) for 2 h at room temperature. Finally sections were incubated in the avidin–biotin–peroxidase complex (1:50; Vectastain, France) for 1 h at room temperature. The bound peroxidase molecule was visualized using 3,3-diaminobenzidine (Sigma, USA). In between each incubation, the sections were washed several times with PBS. Sections were mounted on gelatinized slides, dried overnight, dehydrated in ascending alcohols, cleared in Histoclear and coverslipped with DPX. For control experiments, the primary and/or secondary antibodies were replaced by PBS and then reacted as above. Control sections were immunonegative.

**Stereological analysis**

The total number of cells was estimated using stereological methods. Cell counts were made from the ventral sector of the SNc (Fig. 2). This region was the most clearly defined of the greater nigral complex and hence formed the focus of our analysis. Furthermore, this region has been shown to suffer the most death after parkinsonian insult (Rinne, 1993). TH immunoreactivity and Nissl staining was used to assess cell number, as to explore whether cell loss after MPTP insult was due to loss of antigen expression (TH) or to a loss of the cells themselves (Nissl). In other words, we explored whether there was functional and/or true cell saving. TH immunohistochemistry labels ‘healthy’ cells that express the dopamine phenotype, that is, this labelling provides an index of function of dopaminergic cells (Javoy-Agid et al., 1990; Paul et al., 2004). On the other hand, Nissl staining labels all cells regardless of phenotypic expression. Following procedures as outlined by previous studies, the outline of the SNc (from TH-immunostained sections that defines the nucleus most distinctly; Fig. 2A and B) was drawn and the volume of the region was calculated using Cavalieri’s formula (e.g. Coggeshall, 1992; Earle and Mitrofanis, 1996; Oorschot, 1996; Wadwa, 2003; Wright et al., 2004). The estimated total number of cells within the SNc was obtained using the optical fractionator method (West, 1999; Bonthius et al., 2004; Schmitz and Hof, 2005). Systematic random sampling of locations (with an unbiased counting frame) within the defined SNc boundaries of every fifth section was undertaken (by two observers). All cells (nucleolated only) that came into focus within an unbiased virtual counting space were counted. As a general rule, the number of cells and volumes (mean of two observer counts) were expressed as the mean ± standard error. The number of SNc cells on the lesion (RHS) side was expressed as a ratio of cell survival compared to the contralateral (LHS) side. The number of cells in the STN, from Nissl-stained sections, was estimated using the same procedures as described earlier. A percentage of remaining SNc cells in the RHS was generated by
direct comparison to the number of cells on the LHS in the same cases. For comparisons between groups, and because of the small size of the series, a one-way ANOVA test ($F$-test) was performed. Comparison of the number of SNc cells ($\text{TH}^+$ and Nissl-stained) from the two sides of a single animal (or same group) was undertaken using a paired sample Student $t$-test (for inequality). Finally, Pearson’s correlation coefficient ($r$) was used to assess the degree of correlation between STN alterations (by volume) on SNc cell counts.

**Results**

The results below will be presented in two major parts; (i) clinical evaluations (ii) anatomical analysis.
Clinical evaluations

Using a modified Benazzouz scale, each monkey was evaluated clinically (see ‘Materials and methods’ section). The higher the scores, the greater the degree of clinical impairment (Benazzouz et al., 1995). A score of \( \geq 8 \) indicated the presence of severe parkinsonian symptoms, including akinesia, bradykinesia, rigidity, postural instability, mask-like faces and reduced vocalization. Tremor was less common and when it occurred, it was of the intention rather than of the rest type. These clinical evaluations were mainly global (bilateral) measures. The only one that was lateralized (unilateral) was arm movements. There were only minimal differences in the movements of the two arms, with or without STN alteration; further there were no observable involuntary movements after STN lesion in any of our cases. Clinical scores during the period of time prior to any STN alteration or MPTP treatment remained at zero for all cases. The scores of the non-MPTP-treated monkeys, A1-1 and D series, remained at zero.

Figure 3 shows the clinical scores for each of our series (except non-MPTP-treated cases). Only the peak scores are shown; as a general rule, they occurred about 10 days after the last MPTP injection in each case. After reaching their peak score, all cases showed improved scores, stabilizing at about 15 days after the last injection. From our evaluations, it was clear that there were no major differences in the timetable of recovery between the groups, whether STN altered or not. Hence, we did not find any major changes in behaviour and in clinical scores with STN alteration nor did we detect any behavioural signs of neuroprotection. This was due, in part, to unilateral alterations (bilateral ones might have shown changes more clearly) and a limited survival period (a longer period of more sustained evaluation may have revealed clearer clinical changes).

Most of the cases in the different groups developed severe parkinsonian symptoms, although with some exceptions (Fig. 3). In the C series, two monkeys (C1-5, C2-5) showed no change from their baseline scores of zero, while in the B series, one monkey (B1-3) showed only mild symptoms. These cases nonetheless showed the expected decrease in SNc cell number with MPTP treatment (see later section); they are testimony to the variability in clinical symptoms—and their lack of clear correspondence to SNc cell loss—in different individuals after MPTP treatment.

Anatomical analysis

This section, that forms the main focus of our work, will consider results in each of the experimental series separately. First, a consideration of dopaminergic cell morphology and SNc volume in all our series will be given.

Morphology of dopaminergic cells

Figure 2A and B indicate the SNc region from where the quantitative analysis was undertaken. Figure 2C shows Nissl-stained cells, while Fig. 2D shows TH\(^+\) cells of the normal control (A1-1). They serve as typical examples of the labelling seen in all the experimental series. In general, there were no differences in the morphology of Nissl-stained or TH\(^+\) cells of the SNc after MPTP treatment and/or after STN alteration (not shown).

SNc volume

The SNc volume in the normal control (A1-1) was 17 mm\(^3\) on the LHS and 18 mm\(^3\) on the RHS. In the MPTP-treated intact animals (A1-2, A1-3), volumes were slightly smaller, averaging 14.1 \(\pm\) 1.1 mm\(^3\) on the LHS and 14.8 \(\pm\) 1 mm\(^3\) on
the RHS. In the B series, the SNc volume was very similar to the MPTP-treated intact animals (A1-2, A1-3), averaging 14.5 ± 0.7 mm$^3$ on the LHS and 13.6 ± 1 mm$^3$ on the RHS (lesion). These values were not significantly different ($P = 0.44$). In the C series, the SNc volume averaged 13.2 ± 0.3 mm$^3$ on the LHS and 12.8 ± 1 mm$^3$ on the RHS (DBS). These values were not significantly different ($P = 0.28$). Finally, for the D series, the SNc volume averaged 14.4 ± 0.4 mm$^3$ on the LHS and 14.1 ± 0.1 mm$^3$ on the RHS (DBS). Again, these values were not significantly different ($P = 0.40$).

Thus, although STN alteration (either lesion or DBS) generated a small decrease in the volume of the SNc, this decrease did not reach significance. When comparing the volumes of the SNc between series, no significant differences were found between the B and C series ($P = 0.14$; $F = 0.19$) and the C and D series ($P = 0.90$; $F = 1.14$).

**Intact animals: no STN alteration (A series)**

Figure 4 shows that the estimated total number of Nissl-stained (Fig. 4A) and TH$^+$ (Fig. 4B) cells of A1-1 (non-MPTP-treated intact animal) was ~50% higher than in A1-2 and A1-3 (MPTP-treated intact animals). Our estimates of the total SNc number in the SNc are in the range to those reported previously for normal (~200 000, Hardman et al., 2002; ~65 000, Poirier et al., 1983) and for MPTP-treated (~140 000; Luquin et al., 2006) monkeys (Fig. 4). It should be noted that the fewer overall number of TH$^+$ compared to the Nissl-stained cells was probably due to (i) antibody penetration (Mitrofanis, 1992) and (ii) not all SNc cells express TH (see McRitchie and Halliday, 1995; Hardman et al., 2002). This phenomenon was evident in all our series (Figs. 4, 6, 8 and 9).

In these intact animals, there was minimal difference in the number of Nissl-stained and TH$^+$ cells of the LHS and RHS. All three monkeys had a RHS : LHS ratio about 1; if the value was less than 1, for example, then that would represent a higher number of cells on the LHS. Overall, there was slightly more cells on the LHS than on the RHS in these cases (Fig. 4). The RHS : LHS ratio of cell counts for A1-1 was 0.98 for Nissl-stained and 0.93 for TH$^+$ cells. For A1-2 and A1-3, the RHS : LHS ratio of cell counts combined was 0.97 ± 0.07 for Nissl-stained and 0.91 ± 0.01 for TH$^+$ cells.

In summary, these results indicated that our MPTP regime was effective, mimicking the pathology of PD, that is, there was a substantial decline in the SNc cell population after MPTP treatment. Also, the results showed that there was no significant skew of SNc cell number to one side or the other in the intact animals.

**Kainic acid lesions in STN region (B series)**

On the basis of STN and nigrostriatal fibre involvement, three groups of monkeys were evident from the B1 (STN lesion followed by MPTP) and B2 (MPTP followed by STN lesion) series. These will be considered later.

**bSTN-lesioned**

The cases within this group ($n = 7$; B1-2, B1-3, B2-5, B1-5, B2-1, B1-1, B2-2) had lesions that included the majority of the STN. Figure 5A shows the percentage of SNc that was lesioned in each case; in the bSTN-lesioned group, the average loss of the SNc was ~80%. Figure 5B shows the topography of kainic acid lesion in one of our cases (B1-1); Fig. 5C and D show the lesion site (and non-lesioned side) of the same animal. In this case, as in all others, the lesion site not only included the STN, but surrounding structures as well. These structures included the zona incerta, SNc, SNr and thalamus. A small portion of the rostral SNc was lesioned in three of the seven monkeys (B1-2, B2-1, B2-2). In these cases, the lesions did not have a major effect on our overall counts of cells in the SNc (see later). Figure 6 shows the estimated number of Nissl-stained (Fig. 6A) and TH$^+$ (Fig. 6B) cells in this group. In each case, there were always more cells, both Nissl-stained (Fig. 6A) and TH$^+$ (Fig. 6B), on the RHS (lesioned) than on the LHS (non-lesioned). This clear skew of cell number towards the RHS was in contrast to the trend apparent in the control A series. All seven monkeys in the bSTN-lesioned group had a RHS : LHS ratio >1 (compare to A series where ratios were <1). The average RHS : LHS ratio...
Fig. 5  (A) Graph indicating the percentage of remaining cells in the STN of the RHS after kainic acid injections in the B series of experiments. The percentage of Nissl-stained cells in STN in RHS was generated compared to the LHS (total number determined using stereological methods—see text for details). The inset indicates the different experimental groups, determined after examination of lesion sites and degree of STN lesion. (B) Topography of kainic acid lesion in one case of the B series of experiments (B1-1). This case serves as an example of a successful lesion of much of the STN. Schematic diagrams were constructed with reference to monkey atlas of Paxinos et al. (1998). The numbers in italics refer to the plates of that atlas. (C) And (D) photomicrographs of Nissl-stained sections of the STN in B series in one case (B1-1). (D) Is of the kainic acid lesion site within the STN (indicated by *), while (C) is the non-lesioned side in same case. Figures are of coronal sections, dorsal to top, lateral to left (C) or right (D). Scale bar = 100 mm.
was 1.20 ± 0.08 for Nissl-stained and 1.24 ± 0.12 for TH⁺ cells. The differences between the RHS and LHS in both Nissl-stained ($P = 0.02$) and TH⁺ ($P = 0.0002$) cell number were significant.

**bSham**

Two of the ten monkeys (B2-3, B2-4) had very small, if any lesioning of the STN (average loss of ~7%; Fig. 5A). In one monkey (B2-3), no kainic acid was administered from the catheter, probably because of a mechanical failure of the delivery system. In this case, there was a clear gliosis surrounding the catheter track on the outskirts of the STN and within the zona incerta, but there was no kainic acid lesion evident. Although a kainic acid lesion was present in the second monkey (B2-4), it included little of the STN, sparing nearly the entire nucleus (Fig. 5A). This lesion was centred mainly on the overlying zona incerta. For both cases in this group, the number of both Nissl-stained (Fig. 6A) and TH⁺ (Fig. 6B) cells on the RHS and LHS were similar. In fact, there was a slight skew towards the LHS. The average RHS:LHS ratio in this group was 0.92 ± 0.01 for Nissl-stained and 0.96 ± 0.01 for TH⁺ cells.

**bFibres**

In one of the ten monkeys of this series (B1-4; Fig. 5A), there was a lesion of the passing dopaminergic nigrostriatal fibres; these fibres pass dorsal to the STN, within the region of the lesion site (Paxinos et al., 1998). This monkey had an almost complete lesion of the STN, but no lesion to the overlying zona incerta or underlying SNc. In the SNc, there were fewer Nissl-stained (Fig. 6A), and in particular, TH⁺ (Fig. 6B) cells of the RHS (lesioned) compared to the LHS (non-lesioned). The RHS:LHS ratio for this animal was 0.80 for Nissl-stained and 0.60 for TH⁺ cells. As noted earlier, this loss of SNc cells was not due to the lesion-site encroaching the SNc itself; rather, it was more likely that the lesion site ablated the nigrostriatal fibres, that then generated the loss of SNc cells.

Following this latter point, it should be noted that in some animals of the bSTN-lesioned group, the lesion site on the RHS did encroach on regions of the rostral SNc (B1-2, B2-1, B2-2). Despite this, their Nissl-stained and TH⁺ cell numbers were still higher on the RHS than on the LHS (Fig. 6). Thus, in these cases, we may have in fact underestimated the RHS:LHS ratios; there should have

**Fig. 6** Number of Nissl-stained (A) and TH⁺ (B) cells in the SNc (ventral sector) in the B series of experiments. The columns within the grey background shading involve cases that had a kainic acid lesion to the STN (bSTN-lesioned); those with a striped background were sham-injected (bSham), while those with no background involved a kainic acid lesion of the STN that included passing nigrostriatal fibres (bFibres). All cases were MPTP-treated.
Fig. 7  (A) Graph of the percentage of remaining cells in STN in the RHS after implantation of the DBS electrodes in the C and D series of experiments. The percentage of Nissl-stained cells in STN in RHS was generated compared to the LHS (total number determined using stereological methods—see text for details). (B) And (E) Topography of electrode implants in C (B) and D (E) series of experiments. Schematic diagrams were constructed with reference to monkey atlas of Paxinos et al. (1998). The numbers in italics refer to the plates of that atlas. Note that in these cases, the active electrode contact was within the STN itself. (C) And (D) photomicrographs of Nissl-stained sections of the STN in once case of the C (C) and D (D) series of experiments. The figures show the electrode implant sites within the STN (indicated by *). All figures are of coronal sections, dorsal to top, lateral to right. Scale bar = 100 mm.
been even more cells on the RHS, except for their ablation by kainic acid.

**DBS in STN region (C series)**

On the basis of electrode location, two groups of monkeys were evident from the C1 (DBS of STN followed by MPTP) and C2 (MPTP followed by DBS of STN) series.

**cSTN-DBS**

The cases in this group had electrode implants within the STN ($n=5$; C1-2, C1-3, C1-5, C2-4, C2-5). Figure 7A indicates that the physical location of the electrode within the STN did not cause a major loss of cells within the nucleus of this group. In each case, the side that had the STN electrode implant (RHS) had only $\sim$5% difference in total cell number to the side that did not have an implant (LHS). Figure 7B shows the electrode location in one of our cases (C2-4); Fig. 7C shows a Nissl-stained section of the STN with the electrode site in the same case. It should be noted that, in the cSTN-DBS group, although the electrode tract was within the STN, there was also parts of electrode outside of the STN. As mentioned earlier, only one of the contacts was activated—the one within the STN—while the others were not (those outside of the STN). In each case, there were always more cells, both Nissl-stained (Fig. 8A) and $\mathrm{TH^+}$ (Fig. 8B), on the RHS (DBS) than on the LHS (no DBS). This clear skew of cell number towards the RHS was in contrast to the trend apparent in the A series, but similar to the B series. The average RHS: LHS ratio was $1.19\pm0.10$ for Nissl-stained and $1.20\pm0.12$ for $\mathrm{TH^+}$ cells. The differences between the RHS and LHS in both Nissl-stained ($P=0.006$) and $\mathrm{TH^+}$ ($P=0.009$) cell number were significant.

**cSham**

The cases in this group ($n=3$) had electrode implants outside the STN, either just medial to it within the lateral hypothalamus (C2-1, C2-2) or the zona incerta (C2-3). In these cases, the number of both Nissl-stained (Fig. 8A) and $\mathrm{TH^+}$ (Fig. 8B) cells on the RHS and LHS were similar. In fact, there was a small skew towards the LHS. The average RHS:LHS cell count ratio was $0.97\pm0.05$ for Nissl-stained and $0.97\pm0.08$ for $\mathrm{TH^+}$ cells. The differences between the RHS and LHS in Nissl-stained ($P=0.33$) and $\mathrm{TH^+}$ ($P=0.67$) cell number were not significant.
Chronic DBS of STN (non-MPTP-treated; D series)

On the basis of electrode location, three groups of monkeys were evident from the D series. These are described later.

**dSTN-DBS**

Two monkeys had electrode implants within the STN (D4, D5). Figure 7A indicates that, as with the C series, the physical location of the electrode within the STN did not cause a major loss of cells within the nucleus. Figure 7E shows the topography of the electrode in one of the D series (D5); Fig. 7D shows a Nissl-stained section of the STN with the electrode site in the same case. The number of Nissl-stained (Fig. 9A) and TH$^+$ (Fig. 9B) cells on the RHS and LHS (no DBS) were approximately equal in both monkeys of this group (D4, D5). The RHS:LHS ratios averaged 1.04 ± 0.1 for Nissl-stained and 0.96 ± 0.5 for TH$^+$ cells. There was no clear skew of SNc cell numbers towards any particular side of the brain.

**dSham**

One monkey (D1) had the electrode located outside of the STN, within the zona incerta. The number of both Nissl-stained (Fig. 9A) and TH$^+$ (Fig. 9B) cells on the RHS and LHS were similar in this one case (D1), with the RHS:LHS ratio being 0.95 for Nissl-stained and 1.15 for TH$^+$ cells.

**dFibres**

The other two monkeys (D2, D3) had their electrodes within the STN but also included passing nigrostriatal fibres; the most distal part of their electrodes also encroached the main body of the SNc. The two monkeys of this group (D2, D3) had fewer Nissl-stained (Fig. 9A) and TH$^+$ (Fig. 9B) cells on the RHS compared to the LHS. The RHS:LHS ratio averaged 0.17 ± 0.02 for Nissl-stained and 0.12 ± 0.04 for TH$^+$ cells. The fewer SNc cells in the RHS of these cases was presumably as a result of physical damage of the nigrostriatal fibres (just dorsal to STN) and/or of the SNc by the electrode itself. Such instances may occur more readily in the monkey brain, mainly because we used the same DBS electrodes used for the larger human

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**Fig. 9** Number of Nissl-stained cells (A) and TH$^+$ cells (B) in the SNc (ventral sector) in the D series of experiments. The columns within the grey background shading involve cases that had an electrode implant within the STN (dSTN-DBS); those with a striped background had electrode implants outside of the STN (dSham) and those with no background had the electrodes within the STN but included many nigrostriatal fibres of passage (dFibres). All cases were not MPTP-treated.
Comparisons between series and groups

In the section that follows, the number of Nissl-stained and TH\textsuperscript{+} cells in the SNC of the different series and groups will be compared.

**STN alterations**

There were no significant differences in the number of both Nissl-stained ($P = 0.54$; $F = 1.19$) and TH\textsuperscript{+} ($P = 1.14$; $F = 1.01$) cells on the RHS (altered) of the bSTN-lesioned and cSTN-DBS groups.

**Controls (MPTP-treated intact, b,cSham, LHS of STN alteration cases)**

When comparing all our MPTP-treated ‘control’ cases, we found no significant differences in the number of cells on the RHS of the bSham, cSham and A2–A3 groups. This was the case for both Nissl-stained ($P = 0.96$; $F = 0.032$) and TH\textsuperscript{+} ($P = 0.49$; $F = 0.83$) cells. In addition, we found no significant differences between the number of cells on the RHS of these controls and the LHS of the STN alteration (bSTN-lesioned and cSTN-DBS) groups. This was the case for both Nissl-stained ($P = 0.08$; $F = 2.68$) and TH\textsuperscript{+} ($P = 0.12$; $F = 2.12$) cells. Thus, the LHS of the STN alteration groups may be considered satisfactory controls.

**STN alterations versus controls**

There was a significant difference in the number of Nissl-stained ($P = 0.002$; $F = 15.56$) and TH\textsuperscript{+} ($P = 0.002$; $F = 10.0$) cells on the RHS (altered) of bSTN-lesioned and cSTN-DBS groups, compared to the MPTP-treated controls (MPTP-treated intact, b,cSham and LHS of STN alteration cases; see above).

**STN lesion versus SNC number**

There was a significant correlation between the percentage of cells lesioned in the STN (Fig. 5A) to the number of both Nissl-stained ($P = 0.0001$; $r = 0.89$) and TH\textsuperscript{+} ($P = 0.01$; $r = 0.81$) cells in the SNC in each case (Fig. 6). Thus, the larger the STN lesion, the higher number of cells in the SNC were evident.

**MPTP insult versus SNC cell saving**

We also found that the severity of MPTP insult on Nissl-stained cell number was related inversely to the magnitude of asymmetry in the SNC on the RHS and LHS ($P = 0.0001$; $r = -0.81$). Hence, the more severe the MPTP insult, than the smaller the dopaminergic cell saving with STN lesion. A similar relationship was apparent for the TH\textsuperscript{+} cells, but this did not reach statistical significance ($P = 0.29$; $r = -0.43$).

Discussion

Our most striking finding was that there were significantly more (20–24%) dopaminergic cells in the SNC of the MPTP-treated monkeys that had STN alteration, either with kainic acid lesion or DBS, compared to ‘controls’ (MPTP-treated intact animals, b,cSham and LHS of STN alteration cases). This indicated that removal of the glutamatergic STN in MPTP-treated monkeys saved dopaminergic cells. Our results also showed that after long-term DBS of the STN, there was no change in SNC cell number.

Comparison with previous studies

Our study is the first to report on the patterns of dopaminergic cell survival in SNC after both lesion and DBS of the STN in MPTP-treated monkeys. There have been several other studies that have explored cell survival using different approaches and/or animal models of PD. Such studies have involved STN lesions (6OHDA-lesioned rats: Piallat et al., 1996; Chen et al., 2000; Carvalho and Nikhah, 2001; Paul et al., 2004; 3 nitropropionic acid-lesioned rats: Nakao et al., 1999) or DBS (6OHDA-lesioned rats: Maesawa et al., 2004; Temel et al., 2006), as well as PpT lesions (MPTP-treated monkeys: Takada et al., 2000). These studies have reported between 15 and 50% brain. Hence, some adverse damage to surrounding structures was perhaps not surprising.

B1 and C1 versus B2 and C2 series

Finally, we compared the differences in estimated number of Nissl-stained and TH\textsuperscript{+} cells on the RHS (altered) between the B1 and B2 and C1 and C2 series. Only monkeys within the bSTN-lesioned or cSTN-DBS group were considered. There were no significant differences in the estimated number of Nissl-stained ($P = 0.16$; $F = 2.23$) and TH\textsuperscript{+} ($P = 0.75$; $F = 5.97$) cells between the B1 and B2 series and between the C1 and C2 series. Thus, although there might have been differences in the time-periods between lesion and perfusion in the B series (B1 = ~40 d; B2 = ~14 d) and between DBS and perfusion in the C series (C1 = ~35 d; C2 = ~15 d), these differences did not manifest in significantly different numbers of cells in the SNC.

Summary

A clear pattern was evident from our results. After kainic acid lesion or DBS of the STN, there were always more Nissl-stained and TH\textsuperscript{+} cells in the SNC on the side that had STN alteration. This finding is illustrated further in Fig. 10. Results from two cases are shown, one from a kainic acid lesion of the STN (Fig. 10A–D) and the other from a DBS of the STN (Fig. 10E–H). These cases serve as examples of the patterns seen in all other corresponding cases. In both cases, there were more Nissl-stained cells in the SNC of the side that had the STN either lesioned with kainic acid (Fig. 10D) or underwent DBS (Fig. 10H), compared to the non-lesioned (Fig. 10C) or non-DBS (Fig. 10G) sides.
Fig. 10  Schematic diagrams (A, E) and photomicrographs (C, D, G, H) of Nissl-stained cells in the SNc after kainic acid lesion (A–D) or DBS (E–H) of the STN. Two cases are shown; B1-1 (A–D) and C1-2 (E–H). The lesion (B) and electrode (F) sites are shown also. In the schematic diagrams, one black circle represents one cell (A, E). All figures of coronal sections. The grey box in the schematic diagrams (A, E) indicate general region where photomicrographs were taken from. Note that there were more cells in the SNc on the side that had the STN alteration (RHS) than on the non-alteration LHS) side. Scale bar = 100 mm.
saving of SNc cells after STN or PpT alteration in parkinsonian cases. Despite differences in design, the overall magnitude of dopaminergic cell survival found in our study is comparable to that found in these previous studies.

It should be noted that Luquin et al. (2006) have reported recently no dopaminergic cell saving in MPTP-treated monkeys with prior STN lesion. This result is against the trend of previous (and our) findings, and considering that they followed an approach similar to the previous (and our) studies, it is difficult to interpret. However, their MPTP dosing was severe and this resulted in a massive loss of dopaminergic cells (~85%). This was perhaps too acute for any saving to occur; our study, for instance, generated a subacute dopaminergic cell loss (~50%) and hence was more likely to reveal any saving (see later). Further to this point, our results indicated that the greater the MPTP insult, then the less cell saving occurs in the SNc with STN alteration.

Although most studies on animal models of PD provide evidence for dopaminergic cell saving (see earlier), the clinical evidence for such a phenomenon is largely lacking. On one hand, there are observations of PD patients with slower rates of disease progression after DBS (Ostegaard and Sunde, 2006; Benabid, personal observations). On other hand, a recent PET study reports that DBS does not halt disease progression (Hilker et al., 2005). It is clear that more clinical studies are needed to clarify the issue of dopaminergic cell saving, particularly some that address the issue directly.

Finally, the fact that both TH\(^+\) and Nissl-stained cell number was higher in the MPTP-treated monkeys that had STN alteration, indicated that there was both a functional and a true cell saving. That there was an actual increase in cell survival in the SNc, not just a rescue of the dopaminergic phenotype. This result confirms the findings of Takada et al. (2000), but not of Paul et al. (2004). One may argue that it is difficult to compare the findings of these previous studies and ours, mainly because of the different experimental designs. Each study had a different means of cell labelling (tracer-labelled versus Nissl-stained), animal models of the disease (6OHDA-lesioned rats versus MPTP-treated monkeys), method of elimination (kainic acid lesion versus DBS) and neural centre eliminated (STN versus PpT). Perhaps a more insightful comparison on the issue of functional versus true saving may come from a study that incorporates a common design.

**Evidence for neuroprotection of dopaminergic cells with STN alteration**

We were confident that the skew in SNc cell number towards the STN alteration sides (RHS) of the bSTN-lesioned and cSTN-DBS groups was not due to a natural asymmetry in the MPTP treatment. This was because we found symmetry in SNc cell number in all the MPTP-treated controls (MPTP-treated intact animals, b Sham and LHS of STN alteration cases), indicating that MPTP affected the SNc alteration cases). Further, our finding of a consistently higher number of dopaminergic cells of the RHS in every single case of the bSTN-lesioned and cSTN-DBS groups supported the presence of dopaminergic cell saving after STN alteration. Further to this point, we found that in the bSTN-lesioned group, the magnitude of the SNc cell saving was correlated to the size of the STN lesion; the greater the STN lesion the larger the SNc cell saving.

The mechanism that generated dopaminergic cell neuroprotection in the bSTN-lesioned and cSTN-DBS groups is not known. However, one may make the following speculation. The STN has been reported to have a small but distinct input to the SNc (Hammond et al., 1983; Parent and Smith, 1987). In parkinsonian cases, the STN (and consequently the SNc input) is overactive and hence may generate glutamate excitotoxicity (e.g. Piallat et al., 1996; Maesawa et al., 2004; Temel et al., 2006). This phenomenon could exacerbate the degeneration of dopaminergic cells generated initially by the parkinsonian insult. Thus, our STN alterations may have removed or inhibited a source of glutamatergic input to the SNc and lead to the neuroprotection or saving of dopaminergic cells. Indeed, the STN—largely because of its hyperactivity in PD and its amenability to surgical manipulation (see 'Introduction' section)—has been a primary target for reducing the glutamatergic input to SNc.

It is important to note that we did not find any neuroprotection in the SNc of our sham cases, those that had lesions or electrode locations outside of the STN. In particular, we would like to consider the sham cases that included the overlying zona incerta (B2-4; C2-3). The zona incerta has also been shown to be overactive in parkinsonian cases (Perier et al., 2000), has a small glutamatergic projection to the SNc (Heise and Mitrofanis, 2004), and when lesioned or under DBS, has been reported to alleviate parkinsonian symptoms (e.g. Nandi et al., 2002). Our results show that, unlike the STN, alteration of the zona incerta did not result in dopaminergic cell saving. Perhaps the efficacy of the glutamatergic input from the STN is greater than the one from the ZI, and this may result in greater glutamate toxicity by the STN in parkinsonian cases.

**Impact on SNc from long-term cases and mechanism of DBS**

Although our study did not address the issue of the mechanism of DBS directly, our outcome(s) prompt the following comments. There were no differences in SNc cell number between the bSTN-lesioned and cSTN-DBS groups indicating that DBS of STN generated the same effect as STN lesion. Furthermore, in the long-term DBS cases (dSTN-DBS), there were no differences in SNc number between the DBS and non-DBS sides. Hence, our results support the idea that DBS inhibits activity in the STN.
rather than perhaps activate it (see Benabid et al., 2002; Ashkan et al., 2004). If DBS activated the STN, then one would have expected more glutamatergic activity in the SNc and perhaps more cell death (particularly in dSTN-DBS). This result is prevalent because there are studies that have reported that DBS of STN actually increases, not decreases, the glutamate release in target structures (Windels et al., 2000; Bruet et al., 2001). These previous reports do not find strong support in our findings.

Limitations of study

A major limitation of this study was the small number of animals used. Ideally, we would have examined more cases for each series and developed another series that involved longer periods of DBS. Furthermore, it would have been advantageous to explore patterns of neuroprotection in cases with different MPTP dose regimes. Our regime, although ideal in the sense that it did not generate an acute death of dopaminergic cells, thereby allowing time for therapeutic intervention (Ashkan et al., 2006), was limited in the following way. It did not mimic PD in humans in the fact that cell death was limited to 50%, and not as extensive as would be seen normally in PD when first diagnosed (70–80%; Ashkan et al., 2004); furthermore, we found that the greater the MPTP insult then there is a smaller degree of dopaminergic cell saving. The ethics and restricted availability of using primates for research were the overall limiting factors. Nonetheless, we feel that with the animals available to us, we have generated important information on issues relating to PD, information that is applicable to humans.

Another limitation of this study—and this is related to limited animal numbers also—was that we could not undertake an extensive clinical evaluation of the MPTP-treated monkeys that had STN alteration. This was partly because the MPTP model we used was designed to explore dopaminergic cell survival as measured from anatomical analysis (histological staining) and not from clinical evaluations and also because of our particular behavioural scoring assessments (Benazzouz et al., 1995). We made unilateral STN alterations, and in theory, such alterations would have affected the contralateral limbs. Of our behavioural assessments (e.g. tremor, bradykinesia, posture, vocalization, arm movements and general activity level), only one of the six was a unilateral measure (arm movements), while all others were global bilateral assessments. Hence, the impact of the unilateral STN alteration in these assessments would have been minimal (there was, at best, only mild asymmetry in arm movements; see ‘Results’ section). Furthermore, if our survival period was longer, we would have undertaken a more sustained evaluation and this might have revealed clearer clinical changes.

Finally, although our results are highly suggestive, we do not show conclusively that a reduction in glutamate excitotoxicity after STN alteration in parkinsonian cases was the cause of the dopaminergic cell saving. Future studies may explore this issue further with, for example, pharmacological methods.

Conclusions

Our results indicated that after either STN lesion or DBS in MPTP-treated monkeys, there was a 20–24% saving of dopaminergic cells. Thus, STN alteration may have offered neuroprotection to dopaminergic cells, those that would normally die as part of the disease process. Despite the limitations of our use of a primate model of PD, our results are highly applicable to humans; they offer hope that here we may have a therapeutic tool that not only treats the symptoms of the disease (e.g. see Benabid et al., 2002; Ashkan et al., 2004), but slows down the pathology also. This is a feature currently available drug therapy does not do.

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Survival of dopaminergic cells

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