A new model to study compensatory mechanisms in MPTP-treated monkeys exhibiting recovery

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The cardinal symptoms in Parkinson's disease (PD), akinesia, rigidity and tremor, are only observed when the striatal level of dopamine is decreased by 60–80%. During the preclinical phase of PD, compensatory mechanisms are probably involved in delaying the appearance of motor symptoms. In a MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) monkey model of PD, a spontaneous recovery has been reported after initial intoxication suggesting that compensatory mechanisms are activated in this model as well. Assuming that mechanisms are similar in these phenomena, the study of recovery in monkeys following MPTP intoxication may enable identification of compensatory mechanisms involved in the preclinical phase of PD. In order to maximize the temporal similarity between PD and the MPTP model, we assessed a new progressive monkey model in which spontaneous recovery is expressed systematically and to characterize it based on (1) its behavioural features, and (2) the presence of compensatory mechanisms revealed by an immunohistological approach comparing dopaminergic and serotoninergic innervation between monkeys either exhibiting behavioural recovery or stable motor symptoms. This immunohistological study focused on the substantia nigra, striatum and pallidum, and their anatomical and functional subdivisions: sensorimotor, associative and limbic. The behavioural analysis revealed that with progressive MPTP intoxication motor symptoms were initially expressed in all monkeys. Observable recovery from these symptoms occurred in all monkeys (7/7) within 3–5 weeks after the last MPTP injection, and most exhibited a full recovery. In contrast, acute intoxication induced stable motor symptoms. Despite this obvious behavioural difference, immunohistological methods revealed that the loss of dopaminergic cell bodies in substantia nigra was substantial and similar in both MPTP-treated groups. However, quantification of fibres revealed that recovered monkeys displayed more dopaminergic and serotoninergic fibres than those with stable motor symptoms in sensorimotor and associative territories of striatum and more dopaminergic fibres in internal pallidum. This study provides a new model of PD where all monkeys expressed functional recovery from motor symptoms despite a large dopaminergic neuronal loss. The immunohistological results suggest that both dopamine and serotonin could be implicated in the compensatory mechanisms.

Keywords: Parkinson's disease; monkey; MPTP; behaviour; immunohistology; compensation

Abbreviations: TH = tyrosine hydroxylase; DAT = dopamine transporter


Introduction

Parkinson’s disease (PD) is characterized by a progressive, irreversible and ultimately disabling motor deficit, with a triad of major symptoms: akinesia, rigidity and tremor. This pathology is related to dopamine (DA) depletion in the basal ganglia, a consequence of degeneration of dopaminergic neurons localized in the substantia nigra pars compacta (SNC).

In order to study the pathophysiology of this disease, many animal models were developed (Jenner et al., 1984;
Beal, 2001). Among them MPTP (1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine) intoxication in monkeys is probably the most relevant model available. This model can reproduce not only destruction of DA systems but also the entire triad of symptoms, for example in African Green Monkeys, where even resting tremor, the most difficult symptom to produce in an animal model, can be observed (Redmond et al., 1985; Bergman et al., 1998). These studies have greatly increased the understanding of the neural basis of the various Parkinsonian symptoms (DeLong, 1990; Bergman et al., 1998; Boraud et al., 2002; Pessiglione et al., 2005).

Nevertheless, while this model closely parallels many aspects of PD, it has several limitations. First, the severity of symptoms is highly variable between monkeys even when using the same protocol and similar doses of MPTP. For example, in a study by Elsworth (Elsworth et al., 2000) involving 32 monkeys treated with the same protocol, symptoms ranged from severe (9 monkeys), to moderate (8), mild (6) or even absent (9). A second limitation of the MPTP model to date is the difficulty in producing a gradual destruction of dopaminergic neurons and a corresponding progressive appearance of symptoms as is observed in PD. In response to this limitation, researchers developed long-term progressive protocols, with repetitive small doses injections spread over extended periods (Schneider and Kovelowski, 1990; Hantraye et al., 1993). Although this did result in a progressive appearance of symptoms, it required extremely long treatment periods [e.g. 17 to 21 months (Hantraye et al., 1993)]. A final aspect of the MPTP model that has been considered as a limitation is that spontaneous recovery from motor symptoms has often been reported after MPTP intoxication (Eidelberg et al., 1986; Taylor et al., 1997). This phenomenon is not observed in humans suffering from idiopathic PD, and can interfere with both the study of stable motor symptoms in the model and evaluation of therapeutic interventions. While some investigations have attempted to eliminate this problem by establishing a stable state of symptom expression for studying ongoing dysfunctions (Taylor et al., 1997), the phenomenon of recovery itself seems to be worthy of further study, providing insight into the role of behavioural adaptation versus neural compensation.

For example, we have previously shown, in MPTP-treated monkeys, that visual tracking during execution of movements (Pessiglione et al., 2003) combined with an increase in motivational processes (Pessiglione et al., 2004b) could help to overcome executive dysfunction observed in the early stages of parkinsonism in the monkey model. Such behavioural adaptations were not sufficient to explain all the functional recovery, particularly reductions in some of the motor symptoms, such as tremor or rigidity, indicating that other compensatory mechanisms must be involved. This could involve dopaminergic compensation in the striatum: e.g. an increase of synthesis and release from remaining terminals, sprouting of axonal collaterals from remaining fibres in, as well as denervation supersensitivity in the dopaminoceptive striatal neurons. Moreover it is important to note that, according to the distribution of cortico-striatal inputs, the striatum can be subdivided into three anatomo-functional territories (Alexander et al., 1986): sensorimotor territory receiving cortical inputs primarily from sensory and motor cortex, associative territory with cortical inputs primarily from prefrontal cortex and limbic territory with major inputs from cingulate cortex as well as the amygdala (Kunishio and Haber, 1994; Parent and Hazrati, 1995). Corresponding functional attributes for these three territories (sensorimotor integration, cognitive processes and reward and motivational processing) have been identified through behavioural studies. In both PD (Kish et al., 1988; Brooks et al., 1990; Frost et al., 1993) and the MPTP model (Jan et al., 2003) consideration of these territories had revealed that striatal dopaminergic degeneration is heterogeneous: sensorimotor and associative territories of striatum are severely affected, whereas limbic territory is relatively preserved, providing a relatively large source of potential collaterals. It is possible that sprouting of axonal collaterals from limbic territory into the adjacent associative and sensorimotor territories is involved in a recovery of parkinsonian symptoms in the MPTP model. Alternatively, dopaminergic compensation could be related to the DA innervation present in another structure of basal ganglia (Bezard et al., 2001), such as the pallidum which is less affected than the striatum in both patients and MPTP monkeys (Jan et al., 2000), also consists of anatomo-functional territories (Haber et al., 1990; Flaherty and Graybiel, 1994) and also expresses an heterogeneous denervation. Finally compensation could be mediated by non-DA systems in the striatum such as GABAergic (Schroeder and Schneider, 2002), glutamatergic (Bezard et al., 1998) and/or serotoninergic systems (Gaspar et al., 1993). Reviews are available summarizing these and other possible compensatory mechanisms (Zigmond, 1997; Bezard and Gross, 1998).

In humans with PD it is commonly assumed that the cardinal symptoms are observed only once the striatal DA level is decreased by 60–80% (Bernheimer et al., 1973; Hornykiewicz and Kish, 1987). Presumably, compensatory mechanisms during the preclinical phase of PD delay the appearance of motor symptoms. Because these mechanisms may well parallel those involved in recovery of function in MPTP-treated monkeys, a greater understanding of these mechanisms might lead to the development of new therapeutic approaches, especially for early stages of PD.

With this in mind, the first aim of this study was to develop a progressive protocol for MPTP intoxication in order to consistently induce a full expression of motor symptoms followed by substantial functional recovery. A second aim was to use this model to observe the temporal evolution of symptom appearance and subsequent...
recovery. A final aim was to investigate potential compensatory mechanisms by comparing DA and serotoninergic (5HT) innervation in different striatal and pallidal territories (sensorimotor, associative and limbic) in MPTP-treated monkeys that exhibited behavioural recovery versus those expressing stable motor symptoms. Portions of the data in the current article were previously presented in abstract form (Mounayar et al., 2005).

Material and methods
Animals and MPTP treatment

Fifteen male vervet monkeys (Cercopithecus aethiops) between 4 and 6 years old (young adults) and weighing 4–7 kg, provided by the Barbados Primate Research Centre (Farley Hill, Barbados, West Indies) were used in this study. Care and treatment of these monkeys were in strict accordance with NIH guidelines (1996) as well as with the European Community Council Directive of 1986 (86/609/EEC) and the recommendations of the French National Committee (87/848).

Five monkeys served as controls and the other 10 were treated with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, Sigma, St Louis, MO, USA). All MPTP injections were intramuscular, 0.4 to 0.6 mg/kg and performed under light anaesthesia (ketamine 0.5 mg/kg, atropine 0.05 ml/kg). Two different protocols of administration were used: progressive intoxication (injections spaced by 4 to 5 days) or acute intoxication (daily injections).

The protocol for progressive intoxication (Fig. 1, part 1, A) was used for seven monkeys. Injections were stopped after appearance of the triad of symptoms (see ‘Behavioural analysis’ section later). After behavioural recovery, two of these seven monkeys also received a short acute protocol (two or three injections at 0.6 mg/kg) in order to produce strong and stable symptomatic state (Fig. 1, part 1, C). Acute intoxication alone was used for three monkeys and consisted of two series of daily injections (Fig. 1, part 1, B). For one of these monkeys, supplementary injections were required to maintain the level of the clinical signs. Monkeys were sacrificed either after recovery (progressive intoxication only) or after at least 1 month (Taylor et al., 1997) of stable Parkinsonian symptoms (progressive + acute or acute only) with the exception of one monkey (CA 9) who died of an infectious disease 3 weeks after the end of MPTP injections.

Experimental schedule

Before MPTP treatment, monkeys were trained to sit in a primate chair for 1 h daily, 5 days a week. This period allowed observation of spontaneous behaviour (2 periods of 20 min), behavioural responses during joint manipulations, and performance on a simple reaction task (repeated 3 times, before and after spontaneous behaviour). Following this, monkeys were observed in their home cages for half an hour. They were also observed 2 times between MPTP injections for progressive protocol and every 3 or 4 days after treatment in both protocols. These evaluations allowed rating of their Parkinsonian symptoms in order to follow the evolution of symptom onset and recovery.

Behavioural analysis

The severity of parkinsonism was evaluated by using the rating scale proposed by Schneider and Kovelowski (1990). This scale includes 12 items rated between 0 and 2 or 3, with a total score of 29. It takes in account classical motor symptoms (bradykinesia, rigidity, tremor, freezing, posture and arm posture) but also spontaneous activities (arm movements, spontaneous eyes movements and homecage activity) and other activities (vocalization, triggered eyes movements and feeding).

The presence of many symptoms was checked for both in the task and during spontaneous behaviour, both in the primate chair and the home cage (bradykinesia, tremor, freezing, feeding, arm movements and arm posture). Posture was rated only in the home cage because the monkeys had their heads fixed in the chairs. Home cage activity was additionally evaluated using an activity digitizing system (Vigie Primates, Viewpoint, Lyon; see for details Pessiglione et al., 2003). Muscular rigidity and eyes movements were assessed only in the primate chair. Rigidity was assessed by joint manipulation and spontaneous eyes movements were rated by counting saccades during 3 min after each period of task execution. Triggered eyes movements were tested in chair especially during the task. Finally, vocalization was not rated because it was rarely observed in normal contexts with our Vervet monkeys.
Histological analysis
At the end of the experiments, the monkeys received a lethal overdose of anaesthesia and then were transcardially perfused with saline followed by fixative solution. Brains were removed from the skull, cut into 50 μm thick transverse sections and stored for immunohistochemical procedures (see details in Jan et al., 2000). Dopaminergic and serotonergic innervations were studied by immunocytochemical localization of tyrosine hydroxylase (TH), dopamine transporter (DAT) and serotonin (5HT). The aim was to compare the denervations between intoxicated monkeys, in substantia nigra (SN), striatum, external and internal globus pallidus (GP). Subregions were identified for SN [the peri- and retrorubral cell group (A8), the Substantia Nigra Pars Compacta (SNc) cell group (A9) and the ventral tegmental area cell group (A10)] as well as for striatum and GP (sensorimotor, associative and limbic territories). Anatomo-functional territories were delineated (Fig. 1, part 2) according to the literature on the topography of cortico-striatal projections for striatal territories (Parent and Hazrati, 1995) and striato-pallidal projections (Haber et al., 1990; Flaherty and Graybiel, 1994). TH positive cells were counted in SN. Dopaminergic and serotonergic innervations were evaluated in striatum by optical density and in striatum and GP by quantification of TH, DAT and 5HT positive fibres.

Immunocytochemical techniques
Immunoreactivity for TH was localized using the protocol in Francois et al. (1999). Briefly, sections were incubated in a mouse anti-TH antibody (1/500 dilution: Incstar, Stillwater, MO, USA), followed by a secondary biotinylated antibody (1/250 dilution: goat anti-mouse IgG, Vector Laboratories). Visualization was achieved using avidin–biotin–peroxidase complex (ABC standard kit; Vector, 1:125 in Phosphate-Buffered Saline) and diaminobenzidine (DAB: Sigma).

For DAT, sections were incubated in a rat anti-DAT antibody (1/4000 dilution: Chemicon) followed by a secondary biotinylated antibody (1/200 dilution: goat anti-rat, IgG, Vector Laboratories). Visualization was achieved using avidin–biotin–peroxidase complex (ABC standard kit; Vector, 1:125 in Phosphate-Buffered Saline) and DAB (Sigma) and Glucose oxidase.

Finally, for 5HT, sections were incubated in rabbit anti-5HT antibody (1/10 000 dilution: Diasorin) followed by a secondary biotinylated antibody (1/200 dilution: goat anti-rabbit, IgG, Sigma). Visualization was achieved using Streptavidine peroxidase (1/400) and DAB (Sigma).

Quantification methods
In the SNc, TH-positive cells were counted in nine regularly spaced sections covering the antero-posterior extent of the structure with an image analysis system (Mercator, ExploraNova, La Rochelle, France). The sections were matched anatomically in each of the animals, verifying that the cross-sections of the midbrain were similar in controls and MPTP-treated monkeys. The total number was estimated after correction by the Abercrombie method as previously described (Herrero et al., 1993). The percentage of neuronal loss in the SNc was evaluated by comparison with control values of intact vervet monkeys (for details see Francois et al., 1999).

In order to quantify TH, DAT and 5HT-labelled fibres, functional territories of the striatum and pallidum were first delineated in one monkey at four levels. Analogous sections were then selected for analysis in the others. One rostral section level [anterior commissure (AC) + 4.3 mm by comparison to a standard atlas] permitted measurement on limbic and associative territories of striatum. At a caudal level (AC-3.7), measurements were performed for sensorimotor territory of striatum, GPe and GPi. Associative and limbic territory of GPe were assessed at an intermediate level (AC + 0.5), as was the associative territory of GPe (AC-1.5). In the striatum, due to the high density of TH, DAT and 5HT fibres in control monkeys, dopaminergic and serotonergic innervations were evaluated by measuring optical density of immunostaining using an image analysis system (Mercator). Optical density is a measure based on the difference of luminosity between a defined area and reference taken in a band of fibres. In order to complement these optical density measurements, individual fibres were quantified visually in sensorimotor and associative territories only for MPTP-treated monkeys. This was done by counting the number of fibres crossing the perimeter of 25 circles (diameter = 10 μm) pseudo randomly distributed by the computer (Mercator) within the drawn limits of the sensorimotor striatum as previously described (Jan et al., 2000). The same procedure was used to quantify fibres in different territories of pallidal segments (for details of quantification methods see Jan et al., 2000).

Statistical analysis
Optical density and quantification of cells or fibres in different territories were analysed using Mann–Whitney U-test to compare control, recovered and stable motor symptoms monkeys. We considered three levels of significance: $P<0.05$, $P<0.01$ and $P<0.001$. Unless otherwise specified, values are mean and ($\pm$) standard error of mean.

Correlation coefficients (Pearson’s $r$) were calculated in order to investigate the relation between behavioural recovery (motor score total, motor scores for individual symptoms and recovery time) and immunohistological indices of DA and 5HT innervations of the different territories of the basal ganglia (cell loss in dopaminergic areas of mesencephalon, TH, DAT and 5HT positive fibres in striatum and TH positive fibres in pallidum). This test indicates a correlation factor $r$ which is close to $r=1$ when the correlation is significant. Three levels of significance were considered: $P<0.05$, $P<0.01$ and $P<0.001$.

Results
Behavioural aspects
Monkeys undergoing progressive intoxication received 3 to 7 (mean 4.7) injections of MPTP for a cumulative dose of 1.2 to 2.5 mg/kg (mean 1.8 mg/kg). The dose was higher for monkeys with acute intoxication [6 to 12 injections (mean 7.8)]; total dose of 2.8 to 5.2 mg/kg (mean 3.2 mg/kg: see Table 1 and Fig. 1]. All monkeys under progressive protocol (7/7) developed motor symptoms and then exhibited recovery after stopping treatment (Fig. 2). Recovery was complete for most of the monkeys (6/7), while one monkey (CA37) presented only a partial recovery with mild motor symptoms, bradykinesia, postural trouble and tremor persisting until sacrifice. Monkeys treated with the acute intoxication protocol (5/5) developed all the motor
symptoms but exhibited a weak recovery at best, remaining severely symptomatic until sacrifice (Table 1, Figs. 1 and 2). One of these monkeys (CA2) required supplementary MPTP injections in order to maintain a high motor score. With a single exception (the monkey with the lowest overall symptom score, CA21, who did not exhibit resting tremor), all these animals developed the complete triad of motor symptoms.

Evolution of total score over time

With the progressive protocol, symptoms appeared abruptly after the third MPTP injection, with a maximal effect 4 or 5 days after the last injection (see time = 0 on Fig. 2). Even though most of monkeys developed the triad of motor symptoms, akinesia, rigidity and tremor, the maximal score obtained for recovered monkeys (16.6 ± 1.7) was inferior to the maximal score observed in monkeys with stable motor symptoms (23.2 ± 0.6). Recovery after the maximal effect and cessation of MPTP injections was gradual over 3 to 5 weeks (mean 28.6 ± 3.9 days to maximal recovery see Fig. 2). In general, the greater the maximal effect, the longer the time to maximal recovery. An exception to this occurred in monkey CA33 which presented a high score of 19 and had the fastest recovery, returning to a normal state in 17 days.

Evolution by individual symptoms

When averaged across monkeys treated with the progressive protocol, bradykinesia was the first symptom to appear whereas tremor was the last (Figs 3 and 4). In fact,
bradykinesia was one of the first symptoms to appear in all monkeys whereas tremor appeared later, not developing at all in one case (CA21). Other early appearing and long-lasting symptoms included a decrease in spontaneous activities such as homecage activity and spontaneous eyes movements (Figs 3 and 4).

Symptoms in some cases appeared locally and/or asymmetrically (Table 2). For example, symptom expression

Fig. 3 Appearance and disappearance of individual symptoms. This graph represents the average day of appearance and disappearance for each symptom, centred on the day of maximal score. All averages were computed from the seven animals treated with the progressive protocol (recovered monkeys). Error bars indicate the SD for appearance and disappearance of symptoms.

Fig. 4 Example of the evolution of Parkinsonian symptoms in one monkey treated with the progressive protocol (CA23, maximal score = 19). Each symptom was rated between 0 and 2 or 3 during the intoxication and the recovery.
was stronger or earlier for the arms than at the legs in five monkeys out of six. Symptoms appeared first on the left side for four monkeys, on the right side for one monkey and symmetrically for one. The laterality of dysfunction did not appear to be dependent on the left versus right-side dominance of the monkeys.

**Immunohistochemical aspect**

**Dopaminergic cells in the mesencephalon**
An extensive loss of TH positive neurons was observed in both groups of MPTP-treated monkeys compared to control monkeys (Fig. 5, P<0.01). In contrast this cell loss was not statistically different between recovered (62%) and stable motor symptoms monkeys (73%). This pattern of loss is found in the three areas: A8, A9 and A10. However somewhat greater variability was noted in the group of recovered monkeys, in which CA21, the monkey with the lowest score has the smallest loss of DA cells (32%; see Table 3).

**Table 2 Asymmetry of symptoms**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Dominant side</th>
<th>Earlier appearance or stronger expression</th>
<th>Side</th>
<th>Limb</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA11</td>
<td>Right</td>
<td>Right</td>
<td>Arm</td>
<td>Arm</td>
</tr>
<tr>
<td>CA13</td>
<td>Right</td>
<td>Left</td>
<td>Arm</td>
<td>Arm</td>
</tr>
<tr>
<td>CA23</td>
<td>Left</td>
<td>Bilateral</td>
<td>Leg</td>
<td></td>
</tr>
<tr>
<td>CA33</td>
<td>Left</td>
<td>Left</td>
<td>Arm</td>
<td>Arm</td>
</tr>
<tr>
<td>CA34</td>
<td>Left</td>
<td>Left</td>
<td>Arm</td>
<td>Arm</td>
</tr>
<tr>
<td>CA37</td>
<td>Right</td>
<td>Left</td>
<td>Arm</td>
<td>Arm</td>
</tr>
</tbody>
</table>

CA21 did not develop enough symptoms for inclusion in this analysis.

**TH, DAT and 5HT innervation of the striatum**
In the striatum, TH labelling measured using optical density was greatly decreased in all three territories for monkeys expressing a recovery from Parkinsonian symptoms (P<0.01) and in sensorimotor and limbic territories for those with stable motor symptoms (P<0.01) compared to controls (Fig. 6A and B). The decrease of 62% in the associative territory was not statistically significant (Fig. 6B). The sensorimotor territory is the most depleted region for both groups of MPTP-treated monkeys with a loss of about 80% (83% for stable motor symptoms monkeys and 78% for recovered monkeys), whereas the denervation is about 65% for associative and 57% for limbic territory. In contrast, when comparing the TH immunostaining between the two MPTP intoxicated

**Table 3 Dopaminergic cell loss**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Dopaminergic cell loss (%)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A8</td>
<td>A9</td>
</tr>
<tr>
<td>CA2</td>
<td>33</td>
<td>80.7</td>
</tr>
<tr>
<td>CA9</td>
<td>24</td>
<td>79</td>
</tr>
<tr>
<td>CA11</td>
<td>60</td>
<td>81</td>
</tr>
<tr>
<td>CA13</td>
<td>64</td>
<td>88</td>
</tr>
<tr>
<td>CA35</td>
<td>64.7</td>
<td>90.9</td>
</tr>
<tr>
<td>Mean</td>
<td>49.1</td>
<td>83.9</td>
</tr>
<tr>
<td>SD</td>
<td>8.6</td>
<td>2.3</td>
</tr>
<tr>
<td>CA21</td>
<td>7.7</td>
<td>40.5</td>
</tr>
<tr>
<td>CA23</td>
<td>45</td>
<td>82.3</td>
</tr>
<tr>
<td>CA33</td>
<td>49.5</td>
<td>64.1</td>
</tr>
<tr>
<td>CA34</td>
<td>17.3</td>
<td>78.7</td>
</tr>
<tr>
<td>CA37</td>
<td>55.2</td>
<td>87.1</td>
</tr>
<tr>
<td>Mean</td>
<td>34.9</td>
<td>70.6</td>
</tr>
<tr>
<td>SD</td>
<td>9.4</td>
<td>8.4</td>
</tr>
</tbody>
</table>

A8 = peri- and retrorubral cell group; A9 = substantia nigra pars compacta cell group; A10 = ventral tegmental area cell group.

**Fig. 5 TH labelling in the mesencephalon.** (A) Depiction of areas used to define the A8, A9 and A10 cells groups and examples of the distribution of TH immunostaining in the mesencephalon from control, stable motor symptoms and recovered monkeys. Scale bar, 2 mm. (B) Percentage of TH-positive neurons remaining in the mesencephalon of stable motor symptoms and recovered monkeys relative to control. Results from total counts as well as counts in three subareas, A8, A9 and A10, are presented. Mann and Whitney U: *P<0.05; **P<0.01.
groups, and despite the obvious behavioural difference between these two groups, there was no significant difference for any of the three territories (Fig. 6B). In order to assess the DA innervation at a finer level of resolution, fibre quantifications were performed in the sensorimotor and associative territories of striatum. In contrast to the optical density measures, these measures revealed that recovered monkeys displayed more TH-labelled fibres than stable motor symptoms monkeys (Fig. 6C and D). Thus recovered monkeys had more TH fibres than stable motor symptoms monkeys above all in sensorimotor (23.8 times more for recovered monkeys, \(P<0.01\)) but also in associative territory (5.8 times more). Limbic territory was relatively preserved and fibre density prohibited quantification using this method.

Assessment of DA innervation using immunohistological visualization of the dopamine transporter was also performed initially using measures of optical density. This revealed a tendency for a decrease in sensorimotor and associative territories in both groups of MPTP-treated monkeys, although only the decrease in the sensorimotor territory in the stable motor symptom monkeys was significant (decrease of 45%, \(P<0.01\); see Fig. 7A and B). The labelling was relatively unaffected in limbic territory. Once more no significant difference was observed between recovered and stable motor symptoms monkeys. In contrast, quantification DAT fibres revealed a significant difference between stable motor symptoms and recovered monkeys in both sensorimotor and associative territories (Fig. 7C and D). Recovered monkeys had more DAT fibres than stable motor symptoms monkeys (25 times more in sensorimotor territory, 3.8 times more in associative territory, \(P<0.01\)).

Finally, measures of optical density for 5HT-immunoreactivity in the striatum showed a strong tendency for an increase in recovered monkeys in all three territories (Fig. 8A and B). However this increase was only significant for the sensorimotor territory (\(P<0.01\) control versus recovered), and for the associative territory (\(P<0.05\) recovered versus stable motor symptoms). There was no significant difference in limbic territory between the three groups of monkeys.
Quantification of 5HT fibres helped to confirm this tendency and allow further analysis (Fig. 8C and D). First, a significant increase of fibre number was evident in recovered compared to stable motor symptoms monkeys in all three territories ($P < 0.01$). Moreover, a decrease of the number of 5HT fibres was observed in stable motor symptoms monkeys in comparison to control monkeys in associative and limbic territories ($P < 0.01$).

**Globus pallidus**

A similar pattern of loss in TH positive fibres was found in the GPe as in the striatum using the fibre quantification procedure. Thus a large decrease in the quantity of fibres was observed in sensorimotor and associative territories of recovered and stable motor symptoms monkeys compared to controls. However no significant difference was found between the MPTP-treated groups. No significant reduction was noted in the limbic territory (Fig. 9). In the GPi, only the sensorimotor territory showed a decrease in TH positive fibres with MPTP treatment ($P < 0.01$; both groups). In addition a significant difference was detected in this territory between the two MPTP groups, with fibre number greater in the recovered monkeys ($P < 0.05$). The associative territory did not show any modification in MPTP-treated monkeys compared to control when only means were considered. However counts were highly variable in the group of recovered monkeys and for those that exhibited the greatest recovery (CA23, 33, 34), counts were superior to those of control and stable motor symptoms monkeys. Moreover, fibre counts for the two monkeys which expressed recovery before receiving an acute intoxication were greater than those for the three monkeys that received only the acute protocol.

**Correlation between behavioural and immunohistological approaches**

The strongest correlation observed was between motor score and A8 cell death ($r = 0.99$, $P < 0.01$; Fig. 10A). Cell death in no other area of mesencephalon was significantly correlated with motor scores or recovery time. In contrast, in the striatum correlations were detected with recovery time but not with motor score. There was a correlation on
one hand between recovery time and the number of TH fibres in associative territory \((r = 0.94; P < 0.05; \text{Fig. 10B})\) and on the other hand between recovery time and the number of DAT fibres in sensorimotor territory \((r = 0.94; P < 0.05; \text{Fig. 10C})\). Finally in pallidum there was a correlation between the number of TH + fibres in limbic GPe and the recovery time. There were no significant correlations at all for GPi. Data concerning stable motor symptoms monkeys are indicated on the graph with their real motor score but at an arbitrary recovery time after all recorded recovery times.

A few correlations between individual symptom scores and immunohistological variables were significant. These include those between tremor and number of TH fibres in sensorimotor territory of GPe \((r = 0.91; P < 0.05)\) and between homecage activity and number of 5HT fibres in sensorimotor and associative territories of striatum \((r = 0.91; P < 0.05)\).

### Discussion

This report presents evidence that a progressive protocol of MPTP administration (injections every 4–5 days) allows the study of the progressive appearance of parkinsonian symptoms. Moreover, a recovery from parkinsonian symptoms was observed in all monkeys treated with this progressive protocol. In contrast, monkeys treated with an acute protocol (two series of repeated daily injections of MPTP) failed to show such recovery, exhibiting a stable level of parkinsonian symptoms. Despite this difference, immunohistochemical analysis revealed that DA cell death in the substantia nigra is as extensive for monkeys showing a recovery as for monkeys with a stable expression of symptoms. In contrast, indices of striatal DA (TH-IR, DAT-IR) and 5-HT (5-HT-IR) innervation were greater in several striatal and pallidal territories of recovered monkeys than those with stable motor symptoms.

### Special features of this present progressive protocol

The first aim of our study was to create a new model of MPTP administration in order to follow the evolution of symptoms, to observe behavioural recovery and to investigate potential compensatory mechanisms involved in the phenomenon of recovery. Recovery has been reported in
monkeys after acute MPTP intoxication but with a large amount of variability in its occurrence (Eidelberg et al., 1986; Taylor et al., 1997; Elsworth et al., 2000). In standard acute intoxication procedures, monkeys received four or five intramuscular injections of MPTP (0.4 mg/kg) over 5 days leading to a total dose between 1.6 and 2.5 mg/kg (Elsworth et al., 2000). As in previous studies (Eidelberg et al., 1986; Elsworth et al., 1989; Taylor et al., 1997), such an acute protocol produced a large range in symptom expression, from the stability of the parkinsonian symptoms to the expression of recovery. Taylor et al. (1997) reported that the expression of recovery, when it occurred, was dependent on the initial severity of symptoms. In the present progressive protocol, the chosen interval of 4 to 5 days between each injections leaves time for elimination of MPTP (Przedborski et al., 2001), as well as expression and evaluation of symptoms resulting from the last injection. The latter was evident in a previous study (Pessiglione et al., 2003), using the same interval between MPTP injections showing that the behavioural effects of a particular injection, measured by the number of errors in a motor task, were detectable by the fourth day following an injection. The spacing of injections in the current progressive protocol takes into account this time lag between injection and maximal effect. This allows much greater control of the maximal symptom severity in an individual monkey so that MPTP administration can be stopped once all core symptoms are evident, but while recovery is still possible. Additional MPTP administration could surpass a threshold beyond which full recovery is not possible, potentially close to the maximal score of CA37, the only monkey treated with the progressive protocol that did not exhibit complete recovery. The ability to closely monitor the effects of individual MPTP injection and cease administration in an individual-specific fashion thus appear to be central to the use of this progressive protocol for consistently producing monkeys exhibiting recovery.

Another progressive protocol (Bezard et al., 1997) involving daily injections of MPTP (0.2 mg/kg, i.v.) has been used to produce a stable expression of parkinsonian symptoms without recovery. This yields a larger cumulative dose of MPTP (3 ± 0.2 mg/kg) than the present progressive protocol (1.2–2.5 mg/kg) or standard acute protocols (1.6–2.5 mg/kg) (Eidelberg et al., 1986; Elsworth et al., 1989; Taylor et al., 1997). It is noteworthy that a similar stable expression of parkinsonian symptoms was obtained with our acute daily injection protocol with a similar cumulative dose of MPTP (3.2 mg/kg). This suggests that with daily injections, 3 mg/kg may be required to obtain a stable expression of parkinsonian symptoms without recovery.

Two additional factors could explain why the progressive protocol with spaced injections results in expression of recovery more consistently than daily injections. First, MPTP and its metabolites are fully excreted within a period of 3 days after injection. With injection of MPTP spaced by 4 to 5 days, accumulation of the toxic form of MPTP (the MPP⁺) would be avoided (Przedborski et al., 2001). With daily injections, accumulation of MPP⁺ could lead to an underestimation of the impact of daily dose of MPTP. This could explain why in a daily progressive protocol (Bezard et al., 1997) the expression of symptoms continued to increase to a full and stable state even after the injections of MPTP had been stopped. A second, and perhaps more important, reason why the spaced progressive protocol may promote recovery is the fact that the interval between injections may make it possible for compensatory mechanisms to be initiated gradually, before the threshold level, around a loss of 60–70% of dopaminergic neurons, resulting in the expression of the motor symptoms. We hypothesize that the compensatory mechanisms in the progressive protocol are initiated earlier, at the beginning of dopaminergic lesion (with the first injection), and progress during the interval between each step of intoxication. They are pushed over their limit if an excess of MPTP was administered, but permit a progressive recovery from the symptoms when MPTP intoxication is stopped before such excesses are achieved.
A new monkey model to study early stages of Parkinson’s disease

Another advantage of this protocol lies in the possibility to observe a progressive appearance of Parkinsonian symptoms. Obviously symptoms emerge faster in this model than in PD in human. The present protocol is a compromise between acute protocols and other progressive protocols leading to a slower progression of symptoms but requiring an intoxication schedule extended over several months (Hantraye et al., 1993; Schneider and Pope-Coleman, 1995). Thus Hantraye et al. (1993) treated five monkeys over a period of 21 months and observed that bradykinesia and hypokinesia were often the first symptoms to appear, whereas resting tremor appeared last. Moreover, several studies by Schneider’s team used a chronic intoxication protocol that, at least in some cases (Schneider and Kovelowski, 1990; Schneider and Pope-Coleman, 1995) resulted in cognitive deficits appearing prior to motor deficits. Although the progressive protocol used in the present study is much shorter, similar characteristics can be observed. Bradykinesia is the first motor symptom to appear, whereas tremor is the last one (Figs. 3 and 4). Using the same progressive protocol in a previous study (Pessiglione et al., 2004a), we reported that before emergence of overt motor symptoms, cognitive difficulties may produce hypokinesia (lack of initiation), bradykinesia (abnormal slowness) and executive disorders (hesitations and freezing). Thus the evolution of symptoms over time appears to be similar, although condensed in time, in the present protocol, compared to longer, chronic intoxication protocols. The ability to study the evolution of

Fig. 10 Correlation between immunohistology and behaviour. Correlations were observed between dopaminergic cell loss in A8 and motor score (A), and also between recovery time and three different histological variables, TH fibres in associative striatum (B), DAT fibres in sensorimotor striatum (C) and TH fibres in limbic GPe (D). Full shapes correspond to recovered monkeys and empty shapes correspond to stable motor symptom monkeys. Continuous lines represent regression lines and dotted lines represent the extrapolation of regression lines.
symptoms without having to use such extended protocols should be of great advantage.

This relatively slow emergence of the entire triad of symptoms may also allow more appropriate comparisons with the clinical disorder. Tremor is the most difficult symptom to produce in monkeys (Bergman et al., 1998; Guehl et al., 2003), so its presence with this new protocol supports the similarity of the model to PD at the level of symptoms. The variability described in PD in the progressive appearance and expression of symptoms could be revisited in conjunction with results from this model. One relatively consistent observation is that bradykinesia and tremor are generally the most prominent symptoms (Kang et al., 2005; Uitti et al., 2005). This has led to a classification of two clinical subtypes in Parkinson’s disease: akinetic-rigid or tremor-dominant. According to post-mortem studies (Jellinger, 1999), the akinetic-rigid subtype is attributed to loss of dopaminergic neurons in the ventrolateral SNc (A9), whereas cell loss in the medial SNc and retrorubral field (A8) is associated with the tremor-dominant subtype. Correlational analysis in the present study did not show such a clear division. Although a significant correlation was observed between the loss of dopaminergic neurons in A8 and the overall severity of motor impairment ($r = 0.99, P < 0.01$), the correlation between tremor and the dopaminergic cell loss in A8, although strong ($r = 0.87$), was not statistically significant. Nevertheless, one of the few significant correlations detected between individual symptoms and histological markers was the correlation between tremor and the number of dopaminergic fibres in the sensorimotor GPe, one of the target structures of A8 (Jan et al., 2000). Taken together, these results from human and monkey provide convergent evidence for the possible role of this extra-striatal projection from A8 to the pallidum in the expression of this particular symptom, as has been previously suggested by some authors (Bernheimer et al., 1973; Bergman et al., 1994).

According to clinical studies, Parkinsonian symptoms may appear first in the upper limb (Schlosky and Poewe, 1990), in the lower limb (Vidailhet et al., 1994) or equally in the upper and lower limbs (Dickson and Grunewald, 2004). In our study symptoms appeared mostly in the upper limb initially (5/6). However it is not clear whether this result is dependent on evaluation strategies used. The tasks used involve particularly arm movements and when the monkey is in the primate chair, observation of legs is limited. This could explain the earlier detection of symptoms in the upper limbs. Comparison of localization of initial symptoms in the progressive protocol to the evaluations made in humans could help to understand the variability observed in these studies. It is also clear that PD is commonly an asymmetric disease, as evidenced by the fact that major ratings scales of Parkinsonian symptoms take this asymmetry into account (see e.g. Hoehn and Yahr, 1967; Martinez-Martin et al., 1994; Goetz et al., 1994). This phenomenon had not been described previously in animal models, but we were able to observe an asymmetry in the emergence of symptoms, with one side affected first or more strongly in five monkeys out of six. No systematic relation between the affected side and a monkey’s dominant side was apparent.

### Compensatory mechanisms behind recovery

In considering the compensatory mechanisms involved in delaying the onset of symptoms during preclinical stages of PD and in animal models, the residual dopaminergic system has received the most attention. One focus has been the role of increased DA release (Zigmond et al., 1984; Snyder et al., 1990; Schneider et al., 1994) or turnover (Agid et al., 1973; Bernheimer et al., 1973; Zigmond et al., 1984; Altar et al., 1987) from remaining axons. This biochemical aspect will be addressed in another study, already presented in abstract form (Boulet et al., 2005), but one aspect that can be addressed here is whether any increase in DA release would come exclusively from fibres spared by the degenerative process or where such compensation could also be due to a sprouting of dopaminergic fibres, as has been suggested from results using both a rat model (Finkelstein et al., 2000) and the MPTP-treated monkey (Song and Haber, 2000).

The present study indicates that despite a similar loss of dopaminergic cells, and a substantial loss of TH and DAT labelling in striatum of both monkeys that recovered and those with stable motor symptoms, differences could be detected between the two groups. These differences were not, however apparent using gross measures of optical density: it was necessary to utilize a finer level of analysis involving numerical quantification of fibres. These results based on fibre quantification revealed that recovered monkeys displayed more TH and DAT-labelled fibres than stable motor symptoms monkeys. The possibility that this reflects a greater sparing of fibers in the recovered group cannot be entirely ruled out. However, the similarity in nigral cell loss in our two groups of monkeys, as well as the studies cited earlier (Finkelstein et al., 2000; Song and Haber, 2000), suggest that collateral sprouting from remaining DA fibres makes a contribution to the observed difference.

Thus sprouting of dopaminergic fibres could be implicated in the phenomenon of recovery and, furthermore, in the compensatory mechanisms in early phases of PD. Indeed correlations were observed between dopaminergic fibres quantification and recovery time but not motor score.

Consideration of the different striatal territories individually reveals other details regarding potential compensatory mechanisms. Fibre quantifications and correlations with recovery time in recovered monkeys suggest that collateral sprouting in both sensorimotor and associative territories could be involved. As it is generally assumed that parkinsonian motor symptoms come from dysfunction of
neuronal activity in the sensorimotor territory of striatum, it is not surprising that the few remaining fibres in this territory could participate to the recovery. More surprising is the correlation with the fibres present inside the associative territory. This suggests that dopaminergic fibres remaining or sprouting inside this non-motor territory of the striatum could be also involved in compensatory mechanisms for delaying or reducing symptoms. This raises another question: what is the contribution of any dysfunction inside the associative territory to the parkinsonian symptoms? Specific investigations of this new question could shed light on both symptoms and treatments.

Non-striatal DA systems could also be involved in compensation. For example, in the pallidum a decrease of TH labelling has been seen in different territories of external and internal subdivision in MPTP-treated monkeys (Jan et al., 2000). However this decrease is less in pallidum than in striatum, suggesting that the pallidum could contribute to compensation. The DA could act at different levels in basal ganglia (striatum and other nuclei) to increase the selectivity of information that passes through the entirety of basal ganglia (see Tremblay et al., 1989 and Pessiglione et al., 2005). The relative preservation of the DA projection to the GP could help to reduce this loss of selectivity observed in primate model of Parkinson’s disease. In humans it has been suggested that GPi in particular could be implicated in compensatory mechanisms (Whone et al., 2003). This study indicated that an increase of 18F-dopa uptake was seen in early phase of PD in the GPi but not in the GPe. In the current study, no statistical difference in TH labelling was detected in the GPe between recovered monkeys and those with stable motor symptoms, regardless of the territory considered. There was, nevertheless, a correlation between recovery time and the number of TH fibres in the limbic GPe, indicating that GPe could be implicated in compensatory mechanisms. Additionally an increase of TH labelling has been noticed in sensorimotor internal pallidum of recovered monkeys. Moreover, a strong tendency to an increase of TH labelling has also been reported in the associative GPi among recovered monkeys, notably for those with the most complete recovery. Indeed the monkey with incomplete recovery had a very low number of TH fibres in comparison to the others. This data suggests that both these GPi territories could be implicated in compensatory mechanisms, as has been shown in early phases of PD (Whone et al., 2003). This further validates the hypothesis that compensatory mechanisms in recovery in monkeys and in early phases of PD could be the same.

Systems other than the dopaminergic one could be involved in compensatory mechanisms and in particular the serotoninergic system. As was the case for dopaminergic fibres, we focused on immunohistological than biochemical data (for this aspect see Boulet et al., 2005). Indeed serotoninergic sprouting has been reported in MPTP-treated monkey (Gaspar et al., 1993). This sprouting could simply be a reflection of a phenomenon of competition due to the decrease of the number of dopaminergic fibres. However, it could be also involved in the functional recovery as those authors suggest. Studies in adult primates have produced inconsistent results: some reveal a serotoninergic hyperinnervation in striatum after a 6-hydroxy-dopamine lesion (Zhou et al., 1991), whereas others report a decreased serotonin innervation (Takeuchi et al., 1991). These conflicting results make it difficult to determine the role of serotonin in compensatory mechanisms, but using the progressive protocol, in which the behavioural recovery can be used as evidence that true compensation has occurred at some level, the results are consistent with a positive role of serotonin in compensation. Thus we have observed an increase in 5HT labelling in the three functional territories of the striatum of recovered monkeys, contrasted with a decrease in such labelling in those monkeys with stable motor symptoms in comparison to controls. This would appear to rule out the serotoninergic hyperinnervation as a simple artifact of decreased competition, further implicating it in recovery and compensation. The increase in serotoninergic innervation raises the question of whether treatment of parkinsonian symptoms with serotoninergic agents might be helpful, potentially reinforcing endogenous compensatory mechanisms and delaying the increased severity of motor symptoms. Such a treatment with serotoninergic agents has been suggested previously by Iravani and collaborators (Iravani et al., 2003) who reported that the indirect 5HT agonist MDMA could transiently decrease motor symptoms in MPTP-treated monkeys and decrease dyskinesia when coadministered with L-Dopa. However, it would be necessary before testing such a treatment to have a better understanding of the role of serotonin in this system. A study in adult mice (Rozas et al., 1998) produced apparently contradictory results with respect to behavioural recovery and striatal 5HT innervation. They reported that the lower the degree of recovery is, the higher 5HT innervation is. It is not clear whether differences between species or MPTP treatment protocols or other variables account for this discrepancy. Even in the current study, no correlation was found between serotoninergic fibre quantification and recovery time, whereas a correlation between 5HT fibres and homecage activity was observed. An additional limitation in interpretation of these results is the fact that few things are known on the functional role of 5HT in the striatum, making it difficult even to speculate as to the mechanism by which 5-HT could compensate for the loss of dopamine in the striatum. Complementary approaches using the progressive protocol can be used to elucidate the role of 5HT in compensatory mechanisms, and potentially in the striatum in general.
Conclusion
Our study describes a relatively short-term, progressive protocol for MPTP administration and demonstrates its use as a model for studying compensatory mechanisms in PD. This protocol allows production of extensive degeneration of the central dopaminergic systems, resulting in expression of three of the major motor symptoms in PD, while also consistently yielding monkeys that show complete, or nearly complete, behavioural recovery.

Using this protocol confirmed the importance of the residual dopaminergic system in compensation in the face of degeneration, not only in the three territories of striatum but also in extra-striatal structures and in particular in Gpi. This model also provides preliminary support for the role of serotonin in compensation. Future studies are needed to determine which of these changes is causative and which is simply correlated with the behavioural improvements. This could then inform us on possible therapeutic interventions to promote the biochemical changes responsible for compensation and thus potentially increase compensation in order to delay the emergence and/or worsening of symptoms in early phases of PD. Indeed, as we have indicated, compensatory mechanisms in our model could be similar to those in early stage of PD. It is important to note that the absence of behavioural recovery in patients with PD does not exclude the possibility of compensatory mechanisms: because PD is a progressive condition, compensation may manifest itself solely as a slowing of progression of clinical symptoms. However even if these specific compensation do not occur naturally in PD, understanding the mechanisms of recovery in MPTP-treated monkeys could be nevertheless prove useful in opening the way to new treatments by artificially activating compensation in the early phases of PD.

This model could provide a useful experimental tool for studying other aspects of compensation as well: receptor up- or down-regulation, role of other neurotransmitters and neuromodulators, electrophysiological aspects, etc.

Finally such a model could be used to study the role of dopamine and basal ganglia in other brain functions, such as learning (Matsumoto et al., 1999) and reward-guided behaviour (Schultz et al., 2003; Nakamura and Hikosaka, 2006).

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References


