Reversal of akinesia in experimental parkinsonism by GABA antagonist microinjections in the pedunculopontine nucleus

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Summary
Recent studies, mainly in animals, have shown that the pedunculopontine nucleus (PPN) in the upper brainstem has extensive connections with several motor centres in the CNS. This structure has also been implicated in the akinesia seen in patients with Parkinson’s disease. Here we demonstrate that microinjection of γ-aminobutyric acid (GABA) receptor A antagonist substance, bicuculline, into the PPN of non-human primates (n = 2) rendered parkinsonian with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) results in significant improvement of akinesia. The effect of bicuculline microinjection in the PPN matches that of oral administration of L-dopa. This finding opens up new possibilities in the management of akinesia, the most intractable symptom of advanced Parkinson’s disease.

Keywords: pedunculopontine nucleus; akinesia; gamma-aminobutyric acid; bicuculline

Abbreviations: GABA = γ-aminobutyric acid; GIC = Global Impression of Change; GPi = medial globus pallidus; MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PPMRS = Primate Parkinsonism Motor Rating Scale; PPN = pedunculopontine nucleus; STN = subthalamic nucleus; VPPMRS = Video PPMRS

Introduction
Parkinson’s disease is characterized by rigidity, resting tremor, bradykinesia (slowness of movement), akinesia (inability to initiate movement), postural instability and gait disturbance. The last three are by far the most disabling and difficult to treat (Imai, 1996). Current models of the pathophysiology of motor symptoms in Parkinson’s disease emphasize the abnormal increase in the activity of the subthalamic nucleus (STN) (Albin et al., 1989; DeLong, 1990; Wichmann and DeLong, 1996). This drives the medial globus pallidus (GPi) to inhibit the thalamus and thalamocortical pathway. This in turn results in reduced cortical activity and accounts for the motor disturbances associated with Parkinson’s disease.

In recent years, a number of reports have suggested a significant role for the upper brainstem, and in particular the pedunculopontine nucleus (PPN), in the genesis of some of the motor symptoms in Parkinson’s disease, like akinesia, gait dysfunction and postural abnormalities (Kojima et al., 1997; Aziz et al., 1998; Munro-Davies et al., 1999; Pahapill and Lozano, 2000; Keating and Rye, 2001; Nandi et al., 2001; Nandi et al., 2002). The PPN is a nuclear structure in the rostral brainstem tegmentum with a pars compacta (cholinergic neurones) and a pars dissipata (glutamatergic, dopaminergic, cholinergic and adrenergic neurones) (Mesulam et al., 1989; Geula et al., 1993; Lavoie and Parent, 1994a). The PPN receives dense afferents from the GPi and the substantia nigra pars reticulata (SNr) (Spann and Grofova, 1991; Shink et al., 1997). It sends ascending efferents to the substantia nigra pars compacta (SNc), the STN and the thalamic nuclei (Spann and Grofovna, 1989; Lavoie and Parent, 1994b). Descending projections from the PPN target lower brainstem, cerebellar and spinal motor centres (Rye et al., 1988). In advanced Parkinson’s disease and other clinical entities associated with akinesia, such as multisystem atrophy and progressive supranuclear palsy, there is degeneration of the PPN (Jellinger, 1988; Zweig et al., 1993).
The degree of akinesia in Parkinson’s disease has been linked to the extent of loss of the large cholinergic PPN neurones (Zweig et al., 1989). Stimulating the PPN electrically in decerebrate cats and monkeys elicited stepping movements and increasing the intensity of the current drove the stepping progressively from a walk to a trot to a gallop (Eidelberg et al., 1981; Garcia-Rill et al., 1987). Several studies have demonstrated that unilateral lesions in the PPN in a normal monkey cause temporary hemi-akinesia while bilateral lesions result in a profound long-lasting akinetic state (Kojima et al., 1997; Aziz et al., 1998; Munro-Davies et al., 1999). In previous studies (Nandi et al., 2001, 2002), we have shown that unilateral electrical stimulation of the PPN in a normal monkey results in frequency dependent motor effects: low frequencies (<30 Hz) elicit contralateral proximal limb tremor while high frequencies (>60 Hz) cause loss of postural control and severe akinesia.

\[ \gamma \text{-Aminobutyric acid (GABA) is the predominant afferent neurotransmitter in this region (Noda and Oka, 1986; Granata and Kitai, 1991). We have suggested that akinesia in Parkinson’s disease may be caused by excessive GABAergic inhibition of the PPN by descending projections from the GPi and substantia nigra pars reticulata. In this study, therefore, we have investigated the effects on motor function of pharmacologically manipulating the GABA input to the PPN in two normal monkeys and subsequently, after treating them with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), to make them parkinsonian. Two separate sets of experiments were performed in one of these monkeys after inducing moderate and severe parkinsonism, respectively.}\]

## Material and methods

### Outline of the study design

Two macaque monkeys were chair-trained and normal parameters of motor activity and behaviour were recorded for one week. A cannula was then implanted, which was stereotactically targeted on the PPN on one side. Observations of motor activity and behaviour were then repeated over the next two weeks beginning 48 h after surgery to allow recovery. Following this, intra-cerebral microinjections of 2 \( \mu \)g of muscimol hydrobromide (Sigma, St. Louis, MO, USA) or bicuculline methiodide (Sigma), each in 2 \( \mu \)l of saline, or 2 \( \mu \)l of saline alone, were delivered to the unilateral PPN in each monkey. This was repeated over five sessions for each substance. Motor activity and behaviour were recorded following each microinjection.

In the next stage of the experiment, the animals were rendered parkinsonian with intravenous MPTP (MPTP HCl; Sigma). Once a stable parkinsonian state was reached and motor parameters recorded, the intra-cerebral microinjections were repeated. In one of the monkeys, two sequential parkinsonian states were induced, i.e. moderate and severe. A complete set of intra-cerebral microinjections were given

### Table 1  PPMRS features

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Score</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous activity</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Walks, but reduced</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Does not walk; actively moves limbs and trunk</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moves eyes and head only</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>No movement</td>
</tr>
<tr>
<td>Speed of movement</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Moderately reduced</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Severely reduced</td>
</tr>
<tr>
<td>Facial expression</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Moderately reduced</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Severely reduced</td>
</tr>
<tr>
<td>Provoked response</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slow; reduced activity and expression</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Does not walk; moves limbs and trunk</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Moves eyes and head only</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>No response</td>
</tr>
<tr>
<td>Posture</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Stands/sits up with support</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderately stooped</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severely stooped</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Flat</td>
</tr>
<tr>
<td>Stability</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Leans; unsteady</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Topples over</td>
</tr>
<tr>
<td>Tone</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Moderately increased</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Severely increased</td>
</tr>
</tbody>
</table>

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during each stage. Finally, the animals were sacrificed and the brains processed histologically.

**Monkeys**

An elderly (18 years old) ex-breeding female rhesus macaque weighing 11 kg (monkey A) and a male cynomolgus macaque (7 years old and weighing 7.5 kg) (monkey B) were used for these experiments. The monkeys were housed singly in a primate cage under standard conditions of controlled humidity (50 ± 5%), light (12 h light/dark cycles) and temperature (24 ± 1°C) in accordance with the UK Home Office guidelines. The animals were trained to sit in a primate chair and to allow handling. This was necessary to permit estimation of tone and to deliver intra-cerebral microinjections during the study. There was free access to food and water at all times. Care was shared by trained animal technicians and veterinary staff. All procedures were licensed by the UK Home Office and the Local Animal Ethics Committee, University of Oxford.

**Activity counts**

As a baseline, the daily activity counts of the macaques were recorded over 7 days in the home cage. In addition, half-hourly activity counts were recorded in a glass-fronted observation cage. The half-hourly activity counts were then recorded in the observation cage at every stage of the experiment following each intra-cerebral microinjection. The home cage has an infrared motion detector mounted in front of it. The sensitivity was adjusted to detect whole body movements. The observation cage has three infrared beam based motion detectors criss-crossing the cage. Each detector has an independent counter. Hence, the counts per unit time in the observation cage, taken as the sum of the three counters,
were higher than those in the home cage. These latter counts were the ones used for all statistical analyses.

**Behavioural assessment**

We used a Primate Parkinsonism Motor Rating Scale (PPMRS) as described in Table 1 for clinical rating of the motor features of parkinsonism. This scale is a modification of several existing scales used by other groups (Clarke et al., 1987; Kurlan et al., 1991; Smith et al., 1993; Schneider et al., 1995; Imbert et al., 1997). We found that, for our experimental model of severe Parkinsonism, none of these scales highlighted adequately the motor changes induced by chemical manipulation, even though these changes were apparent on observation, automated activity counts and global impression of change (GIC) scoring (see below). Our scale, therefore, differs in several key aspects from other rating scales in the literature. It does not include features like grooming, tremor, vocalization, freezing and gait. The primary reason for this lies in the severity of the akinesia of our monkeys in the second stage. The animals were severely akinetic and rigid; they had minimal tremor and did not walk at all. This made reliable assessment of the parameters mentioned above impossible. Instead, we have focused on two primary aspects of motor performance—activity (both spontaneous and provoked) and posture (including stability). We found that our scale consistently detected changes at different stages of the experiment and correlated well with the changes in the automated activity counts and the GIC scores. The PPMRS was used both clinically and to assess the video recordings made during each step of the experiment using a Pro 8 220 Compact Camcorder, Ferguson Thomson Technology, Enfield, Middlesex, UK. For video assessment, the PPMRS was slightly modified in that tone was not assessed; this scale was called video PPMRS (VPPMRS). The maximum score in the VPPMRS was thus reduced to 18 compared with 20 in the PPMRS. Video clips were scored in a blinded fashion by one observer (N.G.), who is an experienced neurologist specializing in movement disorders using a Goldstar Intelliset VCR, DV131 viewer by Goldstar, Korea. Two independent observers performed clinical scoring of the same sessions on the PPMRS scale. One of these observers...
was blinded to the nature of the intervention conducted. The mean of their scores over each of the sessions was used for statistical examination.

GIC scores in motor behaviour were assessed in comparison with the baseline, which was set after stabilization of motor function following MPTP administration. GIC was rated on a scale of -3 to +3. A score of 0 was given when there was no change in the motor behaviour. A score of -1, -2 and -3 was indicative of mild, moderate and severe worsening in the state of parkinsonism, respectively. In the opposite range, +1, +2 and +3 indicated mild, moderate and marked improvement in parkinsonian motor parameters, respectively.

In the case of monkey A, the GIC was rated from independent baselines following induction of moderate and severe parkinsonism with low and high doses of MPTP, respectively. The scoring was performed independently by two observers, both of whom were in daily contact with each animal during the entire period of the experiment and were well versed with their behaviour. One of these observers was blinded to the nature of the intervention conducted. The mean of the scores by the two observers over each of five sessions (except in monkey A after induction of severe parkinsonism, when three sessions in each intra-cerebral microinjection category were performed) of microinjections, control, L-dopa and end control, was used to calculate the change in motor behaviour.

**Stereotactic implantation of indwelling cannula**

Under general anaesthesia with intravenous alphaxalone/alphadalone (Saffan®, Schering-Plough Animal Health, Welwyn Garden City, UK) at 2 ml/h, the monkey was positioned in a stereotactic frame (Kopf®, David Kopf Instruments, Tujunga, CA, USA) such that the orbito-meatal line was parallel to the surface of the table. A ventriculogram was performed using Omnipaque® contrast medium (300 mg/ml iodine, Nycomed, Amersham, UK) (Fig. 1A). The anterior commissure–posterior commissure distance was measured from the ventriculogram. After proportional correction for both size and radiological magnification, the coordinates for the PPN (right side in one animal and left side in the other) were derived using a standard primate brain atlas (Martin and Bowden, 1996). These were 2 mm lateral to midline and 6 mm inferior to the posterior commissure. Using a microdrive, a stainless steel cannula (internal diameter 0.75 mm) was then inserted in the monkey’s skull targeted on the unilateral PPN. The cannula was positioned 5 mm above the centre of the PPN (Fig. 1B). It was closed off with an obturator, which could be unscrewed to permit microinjections. The cannula was fixed to the skull with dental acrylic anchored around three T-bolts. The animal was given antibiotic cover [Synulox (Pfizer Ltd, Sandwich, Kent, UK) 0.06 ml/kg, intramuscularly].

**Intra-cerebral microinjections**

The injections were given through a 5 μl Hamilton syringe with an attached stainless steel needle through the implanted cannula after unscrewing the obturator. Each injection was aimed at the centre of the PPN, 5 mm deep to the distal end of the cannula. Two micrograms of muscimol hydrobromide (Sigma) or bicuculline methiodide (Sigma), each in 2 μl of saline, or 2 μl of saline alone, were delivered. The injections were given over 5 min and the syringe was kept in place for 5 min after the end of the injection to prevent backflow along the injection track. Each substance was injected on five occasions and every injection was separated by 24 h. After the first monkey was rendered severely parkinsonian, only three microinjections each of muscimol, bicuculline and saline were given. The sessions were distributed randomly at various times of the day. The entire assembly was cleaned regularly with alcoholic chlorhexidine (Zenecahdol,
Macclesfield, Cheshire, UK) and aqueous solution of Betadine® (Seton Healthcare Group, Oldham, UK).

Onset of clinical effects was within 5 min after completion of an injection and the peak effect lasted from 0.5 to 2 h following each injection. No effects could be observed after 4 h. Activity counts were recorded for a period of 30 min, starting half an hour after completion of each injection. Video clips, activity counts and motor rating scores during this period of peak effect from all the sessions were used for all analytical and statistical studies.

**Oral L-dopa (Madopar®)**

After the monkeys had been rendered parkinsonian with MPTP, Madopar® tablets (Roche, Welwyn Garden City, UK) containing 50 mg of L-dopa and 12.5 mg of benserazide were given as a suspension in fruit juice to enable them to feed and groom. In the moderate parkinsonian stage, half a tablet (and in the severe stage one tablet) was given twice a day. Onset of action was within 30 min and the effects lasted for ~4–6 h. Counts, motor scores and video clips were recorded for analysis between 1 and 2 h after the drug was given. The morning dose was omitted on experimental days. The animals were weighed three times a week to monitor adequacy of food intake.

**Intravenous MPTP**

Monkey A was sedated with ketamine hydrochloride (Fort Dodge Animal Health, Ltd, Southampton, UK) (10 mg/kg, intramuscularly.). For the first stage, 0.5 mg/kg of MPTP dissolved in 10 ml of sterile saline was infused through an indwelling 20 G cannula (Venflon® from Ohmeda, Hatfield, Herts, UK) in the popliteal vein at the rate of 1 ml/min. This was repeated three times at weekly intervals. For the second stage, performed six weeks after the last of the MPTP injections of the first stage, a bolus of 2 mg/kg of MPTP dissolved in 20 ml of sterile saline was infused at 1 ml/mine. In the second animal, MPTP was given only once as a bolus.

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**Fig. 4** (A and B) These figures plot the mean (±SD) half-hourly activity counts against different interventions in the severely parkinsonian state in monkeys A (n = 3) and B (n = 5), respectively. The post-MPTP counts serve as a control with which post-microinjection and Madopar (L-dopa) counts are compared. Bicuculline and Madopar® significantly improve activity counts in both monkey A (*P < 0.005, two-sample t-test, n=3) and monkey B (**P < 0.001, paired t-test, n = 5).
of 1 mg/kg of MPTP dissolved in 20 ml of sterile saline infused at 1 ml/min.

Radio frequency lesion and histopathological processing

After completion of the pharmacological experiments in monkey A, two weeks after the induction of severe parkinsonism, a small radio frequency lesion was made at the site of the injection. A lesioning electrode (0.5 mm diameter, Radionics Inc., Burlington, MA, USA) was inserted through the cannula and advanced to the centre of the PPN, 5 mm deep to the distal end of the cannula. Current was passed to reach a temperature of 60°C and was kept in place for 30 s. The animal was sacrificed immediately with pentobarbital (Rhone Merieux Ltd, Harlow, Essex, UK) (50 mg/kg, intravenous bolus). Transcardiac perfusion was performed with heparinized saline (CP Pharmaceuticals, Wrexham, UK) and 10% formalin. The brain was then blocked in the stereotactic frame and fixed in formalin for another week. Coronal sections from the area of the brainstem were cut manually in a microtome (Shandon AF 325, Shandon Scientific Ltd, Runcorn, Cheshire, UK) and selected sections stained with haematoxylin and eosin, cresyl violet and luxol fast blue. In monkey B, instead of making a radio frequency lesion, 0.5 mg/kg of MPTP dissolved in 20 ml of sterile saline was infused into the lateral ventricle, 24 h after surgery. After completion of the pharmacological experiments in monkey B, two weeks after the induction of severe parkinsonism, a small radio frequency lesion was made at the site of the injection. The lesioning electrode was inserted through the cannula and advanced to the centre of the PPN, 5 mm deep to the distal end of the cannula. Current was passed to reach a temperature of 60°C and was kept in place for 30 s. The animal was sacrificed immediately with pentobarbital (Rhone Merieux Ltd, Harlow, Essex, UK) (50 mg/kg, intravenous bolus). Transcardiac perfusion was performed with heparinized saline (CP Pharmaceuticals, Wrexham, UK) and 10% formalin. The brain was then blocked in the stereotactic frame and fixed in formalin for another week. Coronal sections from the area of the brainstem were cut manually in a microtome (Shandon AF 325, Shandon Scientific Ltd, Runcorn, Cheshire, UK) and selected sections stained with haematoxylin and eosin, cresyl violet and luxol fast blue.

Fig. 5 (A and B) The motor scores on the PPMRS (mean and SD, n = 5; except as indicated in A stage 3 where n = 3) recorded during the different sessions in the pre-MPTP, post-MPTP (moderate parkinsonism, monkey A) and post-MPTP (severe parkinsonism) stages of the experiment. Each session was 15 min long. Each score for each session represents the mean of the scores of two independent observers (one blinded). The controls in the three stages (two shown in B) are the post-operative scores, the post-MPTP (first set) and post-MPTP (second set), respectively. *P < 0.02, **P < 0.002, ***P < 0.005, ****P < 0.001. Abbreviations: B = bicuculline; C = control; C (end) = control at the end of that stage of the experiment; L = l-dopa (Madopar®); M = muscimol; S = saline.
frequency lesion, India ink dye (3 µl) was injected at the centre of the PPN through the cannula. The brain was then blocked, fixed and processed histopathologically in the same manner.

**Results**

The daily and half-hourly activity counts, motor assessments and video clips were repeated over the two weeks following the operations to implant indwelling cannulae in the monkeys’ skull stereotactically targeted on the unilateral PPN. This monitoring revealed that there was no effect of surgery on the mean half-hourly activity counts (Fig. 2A and B) and on the motor scores (Figs 5A, 5B, 6A and 6B). Intracerebral microinjections of GABA agonist (muscimol), GABA antagonist (bicuculline) and saline were then delivered sequentially into PPN through the cannula. Videotape, PPMRS and activity count records were maintained. Muscimol significantly reduced the mean half-hourly activity counts of both monkeys A and B. There was no effect following saline or bicuculline microinjection (Fig. 2A and B). However, bicuculline caused some axial motor effects such as sustained titubation and vertical nystagmus—more prominently in monkey A. The PPMRS and VPPMRS scores showed a consistent significant decrease in motor function following injection of muscimol (Figs 5A, 5B, 6A and 6B).

![Fig. 6](http://brain.oxfordjournals.org/)

**Fig. 6** (A and B) The motor scores on the VPPMRS (mean and SD, n = 5; except as indicated in A stage 3 where n = 3) from the video clips recorded during the different sessions in the pre-MPTP, post-MPTP (moderate parkinsonism, monkey A) and post-MPTP (severe parkinsonism) stages of the experiment. Each clip, corresponding to a separate session, was 5 min long. The scorer was blinded. The controls (for statistical calculations) in the three stages (two are shown in B) are the post-operative scores, the post-MPTP (first set) and post-MPTP (second set), respectively. *P < 0.001.
**Moderate parkinsonism**

In the next stage of the experiment (conducted only in monkey A), the animal was rendered parkinsonian by weekly intravenous injections of MPTP. Parkinsonism was rated on the clinical rating scale. After three injections, the monkey slowed down markedly and developed limb and head tremor as well as a stooped posture. It scored 10.6 ± 1.35 (n = 5) on the PPMRS (baseline score 1.3 ± 0.67, n = 5) (Fig. 5A) and 11 ± 1 (n = 5) on the VPPMRS (baseline score 1.6 ± 0.58, n = 5) (Fig. 6A), which suggests moderate parkinsonism. The mean half-hourly activity counts were significantly reduced (Fig. 3). The reduction in activity was reversed by oral suspension of L-dopa (Figs 3, 5A and 6A).

The series of intra-cerebral microinjections was then repeated. There were significant increases in the activity counts following the bicuculline injections. The tremors were unaffected by bicuculline. Saline and muscimol did not have any effect (Fig. 3). Motor scores also demonstrated significant improvement in activity following intra-cerebral bicuculline and oral L-dopa (Figs 5A and 6A). This was consistent with the results for the GIC scores (Fig. 7A).

**Severe parkinsonism**

In the final stage of the experiment, both the monkeys were given a bolus dose of intravenous MPTP (2 mg/kg in monkey A and 1 mg/kg in monkey B). This made the animals severely akinetic, stooped and rigid. The decline in motor function was first noted 1 day after the injection in both monkeys and stabilized after 10 days in monkey A and after 12 days in monkey B. Severe parkinsonism was confirmed by the clinical scores (18.1 ± 1.6, n = 5, monkey A; 17 ± 0.35, n = 5, monkey B) on the PPMRS (Fig. 5A and B) and those

![Fig. 7 (A and B) The GIC scores (mean and SD, n = 5; except as indicated in A stage 3 where n = 3) recorded during the different sessions in the post-MPTP (moderate parkinsonism, monkey A) and post-MPTP (severe parkinsonism) stages of the experiment. Each session was 15 min long. Each score for each session represents the mean of the scores of two independent observers (one blinded). The controls in the two stages (one shown in B) are the post-MPTP (first set) and post-MPTP (second set) scores, respectively. *P < 0.002, **P < 0.001.](http://brain.oxfordjournals.org/doi/fig/10.1093/brain/awv191)
(16.3 ± 1.3, n = 5, monkey A; 16.6 ± 0.54, n = 5, monkey B) on the VPPMRS (Fig. 6A and B). The monkeys required intensive nursing and had to be hand-fed regular doses of L-dopa in order to feed and groom themselves. The mean half-hourly activity counts were markedly reduced (Fig. 4A and B). The series of intra-cerebral microinjections was repeated. Due to its severely affected condition, the number of injections was reduced in monkey A. There was a remarkable increase in the animals’ activity following the bicuculline injections, but there was no effect with saline or muscimol (Fig. 4A and B). Within 5 min after the bicuculline microinjection, the animal was able to sit up from its previous flat position; it reached out and grasped food and was able to feed, and it groomed itself. The peak effects lasted for ~45 min before gradual slowing of activity over the next 30 min. There was some evidence of contralateral turning behaviour in monkey B. Videotaping was performed through the glass front before and after every intra-cerebral micro-injection. The clinical scores of motor function (PPMRS and VPPMRS; Figs 5A, 5B, 6A and 6B) revealed a highly significant increase following injection of bicuculline, which matched the degree of increase seen with oral L-dopa. In monkey B, there was some decrease in response to both Madopar® and to bicuculline towards the end of the experiment. This was matched by an increase in the severity of the parkinsonian condition. However, as both the clinical and video clip motor scores show, there was no significant change to the initial baseline of motor function recorded at the start of this phase of the experiment (Figs 5A, 5B, 6A and 6B). The GIC scores recorded during this stage of the experiment supported the observation of improvement with intra-cerebral bicuculline and oral L-dopa (Fig. 7A and B).

**Histological examination**

Coronal sections through the area of the brainstem were examined after staining with haematoxylin and eosin, cresyl violet and luxol fast blue. The sections were referenced to the primate brain atlas (Martin and Bowden, 1996). This confirmed the site of the microinjections as the right PPN in monkey A and the left PPN in monkey B. In the magnified (×44) histological section from the brain of monkey A stained with cresyl violet, large basophilic neurones are seen in the left pedunculopontine area of the relevant coronal sections between the medial lemniscus and the decussation of the superior cerebellar peduncle. On the right side, the pre-terminal radio frequency thermal lesion has coagulated these neurones (Fig. 8A). The intensity of the lesion was kept deliberately low to avoid widespread destruction of tissue,

**Fig. 8** (A) Photomicrograph (×44) of the coronal section through the brainstem of monkey A stained with cresyl violet. It shows the presence of the large neurones of the PPN in the area between the medial lemniscus and the superior cerebellar peduncle on the left side (black arrow). There is a loss of these neurones in the corresponding area in the right side (black arrow and letter R), due to the pre-terminal radio frequency lesion. When compared with the primate brain atlas (Martin and Bowden, 1996), it confirms the site of the lesion to be the centre of the right PPN. (B) Coronal section of the brain of monkey B (×5.5) stained with cresyl violet. It shows the area of the left PPN with India ink dye. The dye was injected through the cannula just before blocking the brain in the stereotactic frame and marks the site of the intracerebral microinjections. Abbreviations: Aq = cerebral aqueduct; L = left side; ppn = pedunculopontine nucleus; xscp = decussation of superior cerebellar peduncle.
which would have made microscopic examination unreliable. In Fig. 8B, a coronal section from the brain of monkey B (×5.5) stained with cresyl violet and India ink is seen in the area of the left PPN. It marks the site of the intra-cerebral microinjections.

Discussion
Our results support the findings of earlier studies that suggest an important role for the inhibition of the PPN region in parkinsonian akinesia. The current surgical treatment of akinesia in Parkinson’s disease by lesioning or high frequency stimulation of the STN or the GPi has been shown to be effective (Benabid et al., 1998; Scott et al., 1998). However, axial symptoms like gait, akinesia and postural disturbance do not respond as well as tremor, rigidity and bradykinesia (Lozano and Lang, 1998). It has been suggested that part of this beneficial effect of STN and GPi lesions is achieved by reducing the descending GABA inhibitory influence from these structures to the PPN region (Aziz et al., 1998). In support of this hypothesis, this pathway has been shown to be overactive in primates of parkinsonism (Mitchell et al., 1989). Here, we have managed to achieve significant reversal of akinesia in severely parkinsonian primates by blocking the overactive GABAergic inhibitory input to the PPN region. Our finding that disinhibition of the PPN improves akinetic signs in the parkinsonian primate—while these particular signs of Parkinson’s disease are relatively less responsive to dopaminergic drugs and/or to surgical interventions in the basal ganglia—may point to an interesting area in the pathophysiology of akinesia, not just in Parkinson’s disease but also in related disorders like multi-system atrophy and progressive nuclear palsy, i.e. some of these clinical signs are mediated by non-dopaminergic pathways.

Based on previous studies of the spread of lidocaine and muscimol in the cortex and brainstem in rats following microinjections, the effects of our microinjections should have been restricted to a spherical diameter of <2 mm (Martin, 1991). There is an exponential decrease in concentration of the injected drug from the centre of the injection site to the periphery (Martin, 1991). Moreover, in a subsequent study by the same group in behaving cats, the maximal spread of both drugs was achieved within 10–20 min of the injection (Martin and Ghez, 1999). Muscimol was found to persist for ~2 h in the tissue in the site of injection, though there was no significant change in its extent of spread (Martin and Ghez, 1999). Our studies showed a similar time course and thus support our conclusion that the reversal of akinesia was due to suppressing ongoing GABAergic inhibition in the PPN. This is also similar to that reported recently in a study involving injections of muscimol into the GPi and STN in non-human primates (Baron et al., 2002). Saline injections in the same site and of the same volume had no effect indicating that neuronal inactivation, and not the mechanical or osmotic effects of the injection, resulted in the observed changes in motor activity. However, it is important to note that even with the infusion of such small volumes, it is difficult to be absolutely certain about the specific cell groups being affected by the pharmacological agent. This is especially true when one is injecting in an area such as the rostral brainstem tegmentum with its closely packed groups of cells and fibre tracts.

We would like to emphasize the importance of identifying the anterior and posterior commissures (and hence the inter-commissural line) in accurate localization in stereotactic operations in the non-human primate. This is especially true for targeting deep structures like the basal ganglia and brainstem nuclei. The ventriculogram also enabled us to ensure that the inter-commissural line was in a plane parallel to the horizontal stereotaxic plane, thus approximating the orientation of the brain as represented in the atlas (Martin and Bowden, 1996). It has been shown that this can reduce stereotaxic error by ≈40–80% compared with conventional bony landmarks (Dubach et al., 1985).

It is interesting that both muscimol in the normal state and bicuculline in the parkinsonian post-MPTP state given unilaterally in the PPN had such a marked global effect on motor activity in both monkeys. This may be explained by the bilateral distribution of efferents from the PPN established in the rat (Woolf and Butcher, 1986), cat (Edley and Graybiel, 1983) and non-human primates (Lavoie and Parent, 1994b). This holds true for both ascending projections to the thalamic nuclei and the basal ganglia as well as descending projections to several midbrain, pontine and medullary areas (for review, see Pahapill and Lozano, 2000). There were some unilateral driven effects, like the contralateral turning, seen more in monkey B following bicuculline microinjection in the parkinsonian state.

We also found that the GABA antagonist did not suppress tremor (more obvious in the stage of moderate parkinsonism in monkey A when there was obvious baseline tremor) while L-dopa did. This supports the theory that separate pathways may control tremor and akinesia in Parkinson’s disease. Thus, clinical reports show that thalamic or pallidothalamic intervention successfully suppresses tremor in Parkinson’s disease (Benabid et al., 1998), but does not improve akinesia, when the pallidotegmental pathway is left intact (Laitinen and Vikki, 1973; Iacono et al., 1994).

We expected increased activity to follow bicuculline injection in the normal monkey, because GPi is thought to exert tonic inhibitory effect on its output targets including the PPN; but it did not. Similarly, in both the moderate and severe parkinsonian states, muscimol did not further reduce activity as might have been expected. Perhaps the explanation lies in the difference of baseline motor activity in the normal and parkinsonian states that might alter the sensitivity to neurotransmitter influence. This is seen in a similar situation wherein chemical or electrical inhibition of the STN in a normal monkey causes hemiballismus (Crossman et al., 1984; Beurrier et al., 1997) while STN lesions in a parkinsonian monkey improve akinesia while causing no...

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hemiballismus and only transient dyskinesias (Bergman et al., 1990; Aziz et al., 1991).

It has been reported that the effects of intravenous MPTP may wear off with the passage of time (Eidelberg et al., 1986). To ensure that our controls were reliable, we scored the motor performance at the end of the entire series of intracerebral microinjections after each stage of the experiment, i.e. after both the moderate and the severe parkinsonian states. These assessments confirmed that there had been no significant change in the motor scores during the course of each stage of the experiment (Figs 5–7).

We used an elderly rhesus macaque as one of the subjects in our study. Based on our prior experience with primate studies (Aziz et al., 1998; Munro-Davies et al., 1999; Nandi et al., 2002) and compared with the other subject, a relatively younger monkey, this animal had less baseline motor activity as recorded both on automated activity counts and on clinical rating scores. Previous studies have found correlation between age-related nigral dopaminergic neuronal loss and impairment in home cage activity as well as decline in motor rating scores (Emborg et al., 1998). The fact that this study was performed in an elderly hypoactive monkey strengthens the results of the pharmacological intervention observed. It supports the validity of our model as it conforms to the usual age of onset of symptoms in humans with idiopathic Parkinson’s disease.

Conclusions
Our results are potentially of great clinical significance. They support the case for the involvement of the PPN in akinesia and suggest a possible clinical application for surgical or pharmacological excitation in the PPN region for the treatment of advanced Parkinson’s disease. It may even be possible to use this target for alleviation of akinetic symptoms in the hitherto untreatable conditions of L-dopa unresponsive Parkinson’s disease, multi-system atrophy and progressive supranuclear palsy. However, further experimental work is necessary before these findings can be translated into human clinical settings.

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