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CHRONIC ADMINISTRATION OF MPTP TO MONKEYS: BEHAVIOURAL MORPHOLOGICAL AND BIOCHEMICAL CORRELATES

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It is well established that the administration of 1-methyl–4-phenyl–1,2,3,6-tetrahydropyridine (MPTP) to human beings or to non-human primates brings about a parkinsonian syndrome closely resembling idiopathic Parkinson's disease (Snyder and D'Amato, 1986). With the exception of a single report (Langston and Ballard, 1983), all human cases of accidental exposure to MPTP were caused by the acute administration of this toxin (i.e., by one or by few repeated injections; Langston, 1987). Similarly, in order to obtain experimental animals which are clinically affected, monkeys are usually injected with repeated doses of MPTP over 2 to 5 days. The regimen varies according to the species. In marmosets, 4–5 daily injections of 1–4 mg/kg/day i.p. are effective so as to obtain animals which are obviously parkinsonian, but who can maintain themselves (Jenner *et al.*, 1984).

The resemblance of MPTP-induced parkinsonism to Parkinson's disease is still an unsolved issue (Albanese, 1990.) Recently, Kish *et al.* (1988) found that in patients affected by Parkinson's disease dopamine depletion is more severe in the putamen than it is in the caudate nucleus; to the contrary, in monkeys poisoned acutely with MPTP dopamine loss affects both nuclei equally.

Based on the consideration that Parkinson's disease is a chronic and slowly progressive clinical condition associated with the degeneration of dopaminergic neurons, the present study was undertaken in order to evaluate the effects of a chronic administration of low doses of MPTP.

The question as to whether low doses of MPTP administration are capable to induce the behavioural and pathological signs of parkinsonism is relevant for a better understanding of the putative pathogenetic role played by environmental toxins in human pathology (Barbeau *et al.*, 1986; Rajput *et al.*, 1986). If environmental pyridines do really play a role in the natural human disease, they must be able to produce pathological and biochemical alterations by the additive effect of low doses over time. Recently Tetrud *et al.* (1989) reported several cases of patients affected by a mild parkinsonian symptomatology resulting from a discrete exposure to MPTP. In addition, the same authors were able to detect cognitive changes in individuals exposed to MPTP, who were not symptomatic and did not meet diagnostic criteria for Parkinson's disease (Stern *et al.*, 1990). Furthermore, studies based on PET scans have shown that, compared to healthy individuals, subjects exposed to MPTP have a reduced uptake of 6-fluorodopa in the striatum (Martin *et al.*, 1986). Therefore, MPTP can induce not only the symptoms and signs of Parkinson's disease, but also a subclinical derangement of brain dopamine metabolism.

EXPERIMENTAL PROCEDURES

Thirty common marmosets (*Callithrix jaccus*) of either sex were used in the study. The animals were housed in couples; the cages were kept at 25 °C with a natural daylight cycle.

Dose-response study

Twenty animals were divided in five experimental groups, each one consisting of 4 sex- and age matched individuals. Sixteen monkeys were dosed i.p. twice a week with MPTP: group A received 0.25 mg/kg of the toxin in each injection; group B received 0.50 mg/kg; group C received 0.75 mg/kg; group D received 1.25 mg/kg. Four monkeys, belonging to group E, were injected twice a week with vehicle only (saline solution, 0.5 ml, i.p.). Assessment of parkinsonian disability was performed before starting the experiment, and at weekly intervals thereafter, by means of a disability scale for MPTP-treated marmosets (Jenner and Marsden, 1986; Ueki *et al.*, 1989; Table 1). In addition, the animal behaviour was observed for at least one hour every day and after each injection.

Three months after treatment, the animals were killed by decapitation, under deep general anaesthesia with a mixture of alphaxalone and alphadolone acetate (Saffan, 15 mg/kg). After extraction, the brain stem was fixed by immersion in 4% phosphate buffered paraformaldehyde for approximately 2 weeks, then transferred to 30% phosphate buffered sucrose for 3 days, before being processed for morphological analysis. Coronal sections were cut at 30 μm intervals, by

Table 1. Global disability scale for marmosets

Clinical condition	Score
Normal	0
Slowing of movements, with normal balance and posture	1
Abnormal posture of the limbs, trunk or tail; abnormal balance when pushed or pulled	2
Very slow, unable to keep balance on a perch	3
Immobile, bound to the floor	4

means of a freezing microtome. Alternate adjacent sections were stained by means of cresyl violet or processed for tyrosine hydroxylase (TH) immunohistochemical staining, according to the following procedure. All antisera were diluted in phosphate buffered saline (PBS) containing 0.1% Triton X–100, 3% goat serum, and 1% (w/v) bovine serum albumin. Sections were treated for 30 min. in PBS containing 0.2% (v/v) hydrogen peroxide followed by repeated wash in PBS. Incubation in TH antisera was overnight at room temperature. Sections were then washed in PBS, incubated with goat anti-rabbit IgG serum (1:20), washed again and then incubated for 1 h at room temperature with rabbit peroxidase-antiperoxidase antiserum (Miles) diluted 1:100. After washing, the tissue sections were then exposed to the chromogen solution (1,3-diaminobenzidine, 0.05% w/v) in PBS containing 0.02% (v/v) hydrogen peroxide as substrate for approximately 10 min. Sections through the midbrain were studied by means of a microcomputer based image analyser, blindly, by a single observer. Cell counts were performed in the pars compacta: the relative density of TH-positive cell bodies was computed in medial, intermediate and lateral segments of this nucleus. In addition, the cross-sectional area of all neurons displaying a well-defined non stained nucleus was measured in the substantia nigra and in the ventral tegmental area. Cell counts of glial cells were performed on Nissl-stained sections. Data obtained from the five experimental groups were compared individually, by means of pooled t two-sample analysis.

Chronic administration and follow-up

Ten marmosets of either sex, aged between 2.5 and 7 years (average 6 years), were used in this experiment. Five animals were treated with 1 mg/kg MPTP, which was dissolved in 0.5 ml saline and was injected i.p., twice a week, consecutively for four months. Five monkeys, used as controls, were injected with vehicle only using the same schedule of administration. The monkeys were observed before each dose of MPTP was administered and for half an hour afterwards. Parkinsonian disability was assessed by means of a rating scale specifically devised for assessing parkinsonism in this animal species (Table 2).

In each monkey, ratings were performed before starting the experiment and periodically for twelve months (i.e., for eight months after MPTP administration was discontinued). Each evaluation was performed by two observers, whose scores were averaged. The animals were killed eight months after MPTP was discontinued. Dopamine, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured in the caudate nucleus and in the putamen by means of high pressure liquid cromatography with electrochemical detection.

Table 2. Analytical rating scale for marmosets with parkinsonian symptoms

Item	Score
Alertness	0–1
Reaction to stimuli	0–3
Head motility	0–2
Eye motility	0–1
Posture alterations	0–4
Balance impairment	0–2
Motility (akinesia)	0–3
Vocalisation	0–2
Tremor	0–1
Fur condition	0–1
Total disability score	0–20

RESULTS

Dose-response analysis

After chronic administration of different doses of MPTP it was observed that parkinsonian signs occurred in all the experimental groups. In groups C and D all monkeys were obviously affected, whilst some monkeys belonging to groups A and B were rated normal. As shown in figure 1, average disability scores increased with time in all groups; in addition, average scores were always higher in the experimental groups treated with higher doses of MPTP.

Acute motor abnormalities, which commonly occur after acute administration of MPTP (Jenner *et al.*, 1986) were not seen during chronic treatment. The earliest behavioural changes consisted in a progressive bradykinesia, which affected monkeys of groups C and D, starting less than one month after the beginning of treatment. Rigidity of the limbs, abnormalities of posture and balance and blepharospasm appeared later in the same experimental groups. Monkeys belonging to group A and B became bradykinetic only by the third month of treatment. Motor symptoms were never as severe as those observed following an acute sub-lethal treatment. All the animals were able to feed themselves and to take care of themselves. After three months of poisoning, group C and D animals were grossly parkinsonian: they were all bradykinetic; most of them suffered from dystonia. Tremor only occurred in animals belonging to group D. Most monkeys of group B only showed a mild bradykinesia and some additional abnormalities, while most monkeys of group A were rated normal.

In all monkeys, histological analysis of the brain stem allowed to observe TH-containing neurons located in the substantia nigra and in the ventral tegmental area. In MPTP-treated monkeys, both

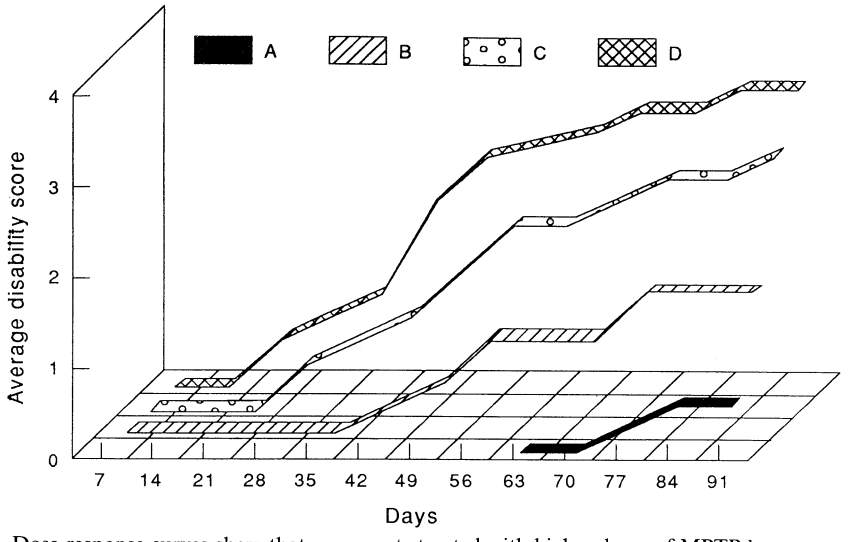

Fig. 1. Dose-response curves show that marmosets treated with higher doses of MPTP have more severe behavioural alterations. This experiment shows that a clear parkinsonian picture is observed in groups C and D after three months of MPTP administration.

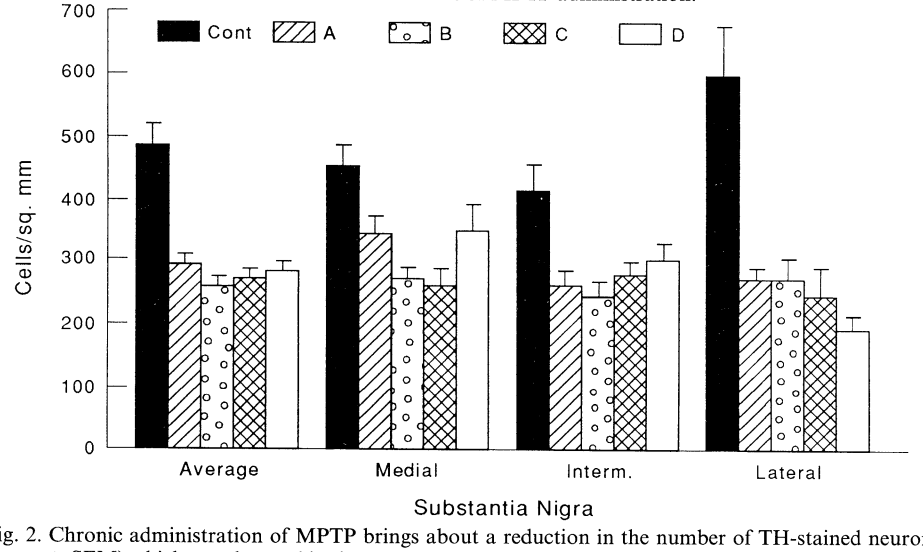

Fig. 2. Chronic administration of MPTP brings about a reduction in the number of TH-stained neurons (mean ± SEM) which are observed in the pars compacta of the substantia nigra. In monkeys belonging to group D, cell depletion is particularly severe in the lateral part of the nucleus.

regions were partially depleted of TH-stained perikarya. Morphometric analysis of the substantia nigra showed that, as compared to controls, the number of TH-stained neurons located in the substantia nigra was reduced in MPTP treated monkeys; however, no linear correlation could be found between morphometric data and the regimen of MPTP administration (Fig. 2).

No differences were observed when the medial, intermediate and lateral segments of pars compacta were compared. Average cell density in control animals was 491.87 cells/μm^2; in group A it decreased by 40.16% to 294.35 cells/μm^2; in group B it was 263.18 cells/μm^2 (–46.49%); in group C it reached 256.29 cells/μm^2 (–47.90%); and in group D it was 283.88 cells/μm^2 (–42.29%). Morphometric analysis also showed that the size of TH-stained neurons located in the substantia nigra and in the ventral tegmental area was not affected by MPTP treatment (Fig. 3).

In fact, between-group comparison showed that the cross-sectional area did not differ significantly among

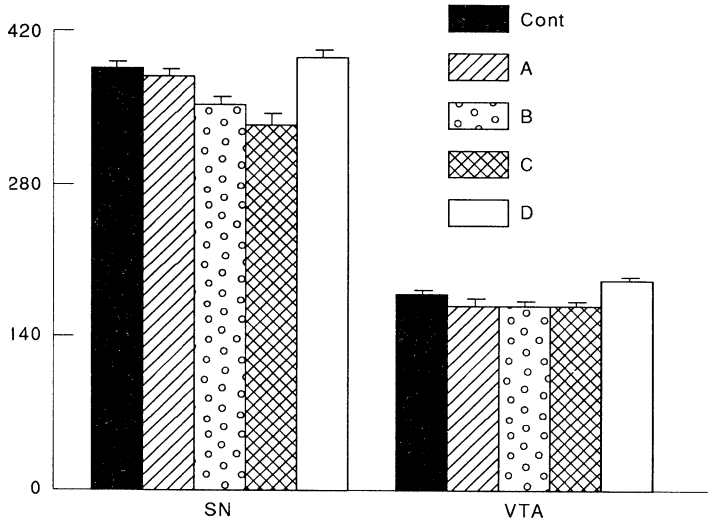

Fig. 3. Chronic MPTP treatment did not affect the size (mean ± SEM) of TH-stained neurons located in the pars compacta of substantia nigra (SN) or in the ventral tegmental area (VTA).

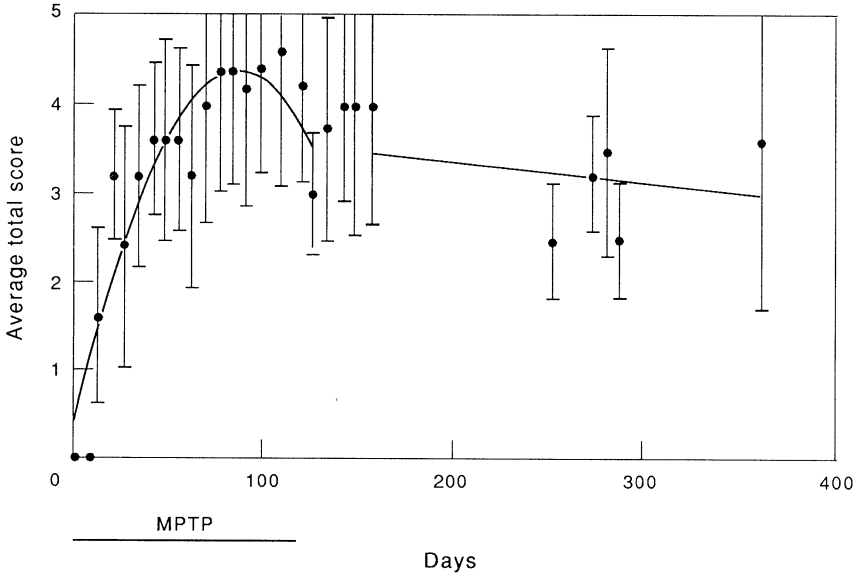

Fig. 4. Chronic administration of 1 mg/kg MPTP induced a progressive parkinsonian syndrome. Average total scores (± SEM) are plotted together with a second order polynomial curve fit. This indicates that a gradual recovery of clinical signs occurs shortly after discontinuance of the toxin; afterwards, the neurological impairment becomes stable.

groups A,B, C, D and controls. Finally, when the number of glial nuclei located in the pars compacta was compared, no significant differences were found between MPTP-treated and control monkeys.

Chronic administration and follow-up

The present study is in keeping with earlier observations reporting that chronic treatment with MPTP (1 mg/kg, twice a week) brings about a parkinsonism, that progressively worsens when subsequent doses of the toxin are administered (Albanese *et al.*, 1990). The average of total disability scores showed that a marked and progressive worsening of symptoms occurred during the first month of treat-

ment, and that clinical disability progressed at a much slower rate from the third month of treatment. Acute toxic effects related to the administration of MPTP were never observed. The highest disability average score was reached during the tenth week of treatment, shortly before the end of it. After discontinuance of chronic MPTP administration, parkinsonian features gradually recovered for few months and remained stable afterwards (Fig. 4). Behavioural recovery was almost complete in two out of five monkeys (Albanese *et al.*, 1991).

The analysis of individual items of the disability scale showed that some scores were more commonly altered than others. Motility of the head and eyes, vocalisation, posture and the ability to react to external stimuli were constantly affected. On the other hand, tremor was constantly absent, and balance, alertness and self-caring (as shown by fur conditions) were always rated normal.

In this group of animals, the biochemical study showed no significant differences between controls and MPTP-treated animals; the amount of dopamine, HVA and DOPAC contained in the caudate nucleus and in the putamen and dopamine turnover (measured as the DOPACHVA/dopamine ratio) were unaffected (Table 3).

DISCUSSION

The present study shows that when marmosets are chronically exposed to MPTP a progressive parkinsonian syndrome occurs. The low doses, which are required for the chronic regimen, do not produce acute pharmacologic effects, which usually appear when repeated maximal doses of MPTP are administered (Jenner and Marsden, 1986). In addition to behavioural signs, chronic administration of MPTP also produces morphological alterations, which are non-linearly related with the total amount of MPTP given over time. Finally, the existence of individual susceptibilities to the toxic effects of MPTP must also be taken into account, as different clinical pictures are observed in monkeys belonging to a single experimental group.

Table 3. Biochemical analysis of the forebrain in marmosets exposed chronically to MPTP, which had recovered over the following eight months

	Control	MPTP-treated
Caudate nucleus		
DA	87.77± 6.04	89.35± 3.55
HVA	28.81± 1.84	30.54± 3.27
DOPAC	6.83± 0.79	6.46± 0.41
Turnover ratio	0.41± 0.02	0.42± 0.03
Putamen		
DA	87.46± 11.53	87.77± 10.56
HVA	71.47± 22.47	70.38± 9.
DOPAC	16.41± 5.79	10.36± 1.32
Turnover ratio	0.98± 0.23	0.82± 0.11

Bradykinesia was the clinical sign most clearly associated with chronic administration of MPTP. Bradykinesia gradually progressed as toxin administration was pursued. Vocalisation and eye movements were always not severely impaired. Balance on the perch was mildly impaired in some monkeys. Dystonia (including blepharospasm) was also observed occasionally; its severity was clearly not related to the total amount of toxin injected. Tremor was only observed in some animals treated with 1.25 mg/kg of MPTP. Postural abnormalities were never observed.

When our observations are compared to those of monkeys treated with acute or subacute regimens, it is concluded that the basic parkinsonian syndrome is quite comparable, while the severity of each clinical sign differs according to the experimental conditions. First, in the chronic regimen motor disabilities, although gradually progressive, impair motor behaviour without threatening vital functions (e.g., feeding and self-caring). Second, chronic MPTP treatment neither brings about acute toxic reactions nor it is associated with such self-damaging behaviours, which are commonly seen after repeated treatments with high doses of MPTP. As a consequence, higher cumulative doses of MPTP can be given with the chronic regimen. Such high cumulative doses are required in order to produce motor abnormalities comparable to those observed after acute MPTP administration. Taking all these considerations into account, it can be observed that, at variance with the syndrome produced by acute or subacute administration of MPTP, in chronically treated monkeys (as well as in the human idiopathic condition) a slow progression of signs and symptoms, which are mainly restricted to the motor system, is observed.

The existence of morphological abnormalities in the midbrain of marmosets treated chronically with MPTP is well in keeping with knowledge that similar alterations are usually found in idiopathic and in MPTP-induced parkinsonism. The present data show that, after chronic treatment, loss of TH staining without cell shrinkage may occur in midbrain dopamine-containing neurons. As shown by Lams *et al.* (1988), the lack of staining for a metabolic marker

does not necessarily imply that non stained neurons are not viable; as a matter of fact, the derangement from a normal metabolic balance is reversible to a large extent.

Therefore, it may well be the case that a loss of TH staining in the midbrain of marmosets treated chronically with MPTP may be due to biochemical alterations brought about by uptake (and, possibly, build-up) of toxic compounds into such neurons. The existence of a metabolic reaction due to a direct effect of toxic compounds is also supported by the lack of a linear dose-response curve in our immunocytochemical data. As shown in Fig. 2, the density of TH-stained neurons located in the substantia nigra is significantly lower in treated monkeys than in controls, but it does not change according to the dose regimen of MPTP administration. This leads to the fundamental question as to whether the parkinsonian signs of chronically treated marmosets are related to the occurrence of cell loss in the substantia nigra or they are a transient pharmacologic effect.

The occurrence of a behavioural recovery after discontinuance of MPTP treatment is in keeping with earlier observations based on acute or subacute administration of MPTP to monkeys (Eidelberg *et al.*, 1986; Ueki *et al.*, 1989): it is widely accepted that recovery of parkinsonian signs is a common phenomenon, the causes of which are still unknown. In the second group of monkeys the study of dopamine and of its metabolites contained in the neostriatum showed that clinical recovery was associated with a normal biochemical picture, since eight months after the end of the MPTP administration there was no depletion of this neurotransmitter in the forebrain. Moreover, no difference has been found in its normal pattern of distribution between the caudate nucleus and the putamen.

The morphological study of the marmosets, which were treated chronically with MPTP for four months and which were followed-up for eight months, is expected to solve some still unanswered questions. All data obtained from the morphological analysis of midbrain dopaminergic neurons will be compared to those obtained in the dose-response study, in which marmosets were killed soon after MPTP administration was discontinued. This will probably allow to clarify to what extent a cellular damage produced by a chronic administration of MPTP for four months may be reversed by discontinuing the toxin.

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