

## FUNCTIONAL IMPAIRMENT OF NIGROSTRIATAL NEURONS PROGRESSES FOLLOWING WITHDRAWAL OF 1-METHYL-4-PHENYL-1,2,3,6-TETRAHYDROPYRIDINE

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**Abstract**—C57 BL/6 mice were rendered severely parkinsonian by exposure to high doses of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. The fluorescent retrograde tracer Fast Blue was injected into the neostriatum one (group A) or five weeks (group B) following exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. Neurons located in the substantia nigra pars compacta and in the centre median-parafascicular complex were analysed. There was no variation in the number and distribution of Fast Blue-labelled perikarya located in the centre median-parafascicular complex, which are insensitive to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. No variation was seen in the number of Nissl-stained perikarya located in the substantia nigra pars compacta, indicating that neurons had not degenerated. The number and the density of Fast Blue retrogradely-labelled neurons located in the same region were decreased in group A by 41% and in group B by 55%. Fast Blue labelling provided a measure of functional impairment in viable neurons. The Fast Blue-to-Nissl cell ratio was 55% in controls and declined to 20% in group A and to 17% in group B mice.

The present study shows that (i) functional inactivation of viable neurons can be measured by using a fluorescent retrograde tracer following exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine and (ii) inactivation of retrograde axonal transport progresses from one to five weeks following withdrawal of the toxin. Fluorescent retrograde probes may be used to measure the anatomical substrate of recovery induced by drugs or by brain grafts in parkinsonian animals. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

**Key words:** axonal transport, retrograde fluorescent tracers, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, mouse, substantia nigra, thalamus.

Accidental or experimental exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) brings about parkinsonian signs in man and animals.<sup>23,29</sup> Exposure to MPTP activates a cascade of events producing a degeneration of dopaminergic nigrostriatal neurons. There is evidence that, following exposure to MPTP, the time-course of cell degeneration differs in dopaminergic perikarya and terminals.<sup>18</sup> In addition, the neurotoxic aftermath of MPTP exposure differ according to the animal species, to the total dose injected, to the administration route and schedule, and to age.<sup>3,7,14,31</sup> In mice, sub-lethal doses of MPTP produce a transient depletion of dopamine in the neostriatum but no appreciable cell loss in the substantia nigra.<sup>8,17</sup>

Fast Blue is a fluorescent retrograde tracer used as a tract-tracing tool,<sup>1,2,4,22</sup> that yields long-lasting labelling of the neural cytoplasm.<sup>20</sup> Since it is actively taken-up by nerve terminals and not by the fibres-of-passage through the injection site,<sup>19</sup> Fast Blue acts as a probe specifically measuring nerve terminal uptake.

The aim of this study was to measure how many nigrostriatal neurons can uptake and retrogradely transport Fast Blue following exposure to MPTP. In order to detect variations in time of such functional capabilities of nigrostriatal neurons, retrograde labelling was studied one and five weeks following MPTP withdrawal.

### EXPERIMENTAL PROCEDURES

Eighteen C57 BL/6 mice (Jackson) of either sex aged between eight and 12 weeks were used. The animals were housed at room temperature in cages containing six mice each, with food and water *ad libitum*.

#### *Treatments and surgery*

The animals were divided in three experimental groups of six mice each. Group A and group B mice were treated subcutaneously with MPTP, dissolved in saline solution, at the dose of 40 mg/kg i.p. twice-a-day for two days.<sup>27</sup> Group C animals served as controls: they were injected i.p. with 0.2 ml saline solution using the same administration schedule. The behaviour was observed before and after each injection for at least 5–6 h; group B mice were observed at least twice-a-week during the survival period. Surgical procedures were performed under general anaesthesia (sodium pentobarbitone 40 mg/kg i.p.). Each mouse received an intracerebral injection (0.2 µl) of a 2% aqueous solution containing the fluorescent tracer Fast Blue. All the injections were placed stereotactically in the right striatum; the

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**Abbreviations:** CMP, centre median and parafascicular complex; MPP<sup>+</sup>, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; SN, substantia nigra pars compacta.

Table 1. Density of Nissl-stained cells located in the substantia nigra pars compacta (mean  $\pm$  S.E.M.)

|                      | Substantia nigra | Medial substantia nigra | Lateral substantia nigra | Medial vs lateral |
|----------------------|------------------|-------------------------|--------------------------|-------------------|
| Controls             | 536.0 $\pm$ 21.4 | 584.0 $\pm$ 26.8        | 488.0 $\pm$ 29.1         | 19.67% $P < 0.05$ |
| Group A (one week)   | 559.2 $\pm$ 22.8 | 603.7 $\pm$ 24.7        | 514.7 $\pm$ 35.5         | 17.29% $P < 0.05$ |
| Group B (five weeks) | 499.2 $\pm$ 21.8 | 551.7 $\pm$ 17.1        | 446.7 $\pm$ 35.8         | 23.51% $P < 0.05$ |

No between-group differences were observed.

needle was placed 2 mm lateral and 1 mm anterior to the bregma, at a depth of 3 mm from the pial surface. Group A mice received the intrastriatal injection one week after MPTP intoxication was discontinued; group B mice were injected five weeks after the last MPTP dose. Group C mice underwent surgery a few days after being treated with saline solution.

#### Histological study

Three days after the stereotactic injection, the animals were perfused transcardially with isotonic saline solution, followed by 4% phosphate-buffered paraformaldehyde. Perfusion was performed under deep general anaesthesia (sodium pentobarbitone, 40 mg/kg). The brains were removed from the skull and stored in 30% buffered sucrose for at least 24 h. Coronal sections were cut at 40  $\mu$ m intervals by a freezing microtome. Two alternate series of sections through the forebrain, the diencephalon and the midbrain were collected; sections of the first series were mounted immediately on slides from saline solution, air dried and cover-slipped without prior dehydration; sections of the alternate series were mounted on slides and counterstained with Cresyl Violet. Unstained sections were observed with an epifluorescence microscope (excitation wavelength 360–390 nm). Charts of the Fast Blue injection area were first analysed. Only the cases displaying an injection site of a standard size (i.e. located laterally and medially in the anterior third of the neostriatum), negligible necrosis around the needle track and no glial or neuropil staining in the outer zone (see below) were further processed. Based on these criteria, several mice were discarded in order to obtain three groups of six mice each. Charts of the Fast Blue injection area, of the thalamus and of the midbrain were plotted by means of a computer-assisted bidimensional plotter. Counterstained sections were fed into a microcomputer-based image analyser. The whole histological analysis was performed blindly by a single observer.

Average neural density was measured in Nissl-stained sections by random sampling 0.2 mm<sup>2</sup> areas located in the medial and in the lateral part of the substantia nigra. Fluorescent retrogradely-labelled neurons located in the substantia nigra pars compacta (SN) and centre median and parafascicular complex (CMP) were counted on charts obtained from three sections equally spaced throughout the rostral-to-caudal extent of the CMP and from a similar series of thirteen sections throughout the ventral midbrain tegmentum. All perikarya that could be visually detected with the 25 $\times$  lens under oil immersion were charted. The following data were collected from paper charts of Fast Blue-labelled neurons: (1) the total number of Fast Blue-labelled neurons located in the SN; (2) the total number of Fast Blue-labelled neurons located in the CMP; (3) the SN/CMP ratio of Fast Blue-labelled neurons; (4) the density of Fast Blue-labelled neurons, by sampling two areas of approximately 0.79 mm<sup>2</sup> each (1 mm diameter) on each chart (one sampling area was located in the medial SN, the other in the lateral SN). Statistical evaluations were performed using Student's *t*-test. The least significance level was  $P < 0.05$ .

## RESULTS

### Behaviour

Soon after the first MPTP injection, group A and group B mice showed the behavioural signs, that are commonly associated with the administration of high doses of MPTP. They did not feed themselves for up to 8 h following each MPTP dose. Whole body piloerection, tachypnea, limb tremor, scialorrhoea and increased motor activity were observed shortly after each MPTP injection and lasted for approximately 1 h. The initial increase in motor activity was followed by a decrease, lasting for about 3 h. The overall mortality rate was 55%. Surviving MPTP-treated animals showed complete behavioural recovery few days after the intoxication was discontinued. Controls injected with saline solution (group C) did not present behavioural alterations.

### Morphology

In all mice, Nissl-stained sections of the midbrain allowed the observation of clusters of neurons located in the SN and few neurons scattered in the ventral tegmental area. Morphometric analysis performed in the SN showed that the density of midbrain neurons of group A and group B mice did not differ from that of controls (Table 1). In all the experimental groups the density of Nissl-stained neurons was higher medially than laterally ( $P < 0.05$  in each case). No between-group difference was observed either for the medial or for the lateral density (Table 1).

Fast Blue injection sites were highly reproducible and have been schematically outlined in Fig. 1. Charts of the injection sites allowed to observe in all cases a small necrotic zone at the tip of the needle track, a surrounding zone with intensely-fluorescent glial and neural nuclei and an outer zone with fluorescent neural perikarya, but no glial or neuropil staining. Fast Blue retrogradely-labelled neurons located in the thalamus and in the midbrain were clearly recognized, due to their intensely fluorescent, blue cytoplasm and the unstained nucleus. Labelled neurons located in the CMP appeared as small, round perikarya, with some dendritic processes abutting the fasciculus retroflexus (Fig. 2). Nigrostriatal neurons appeared as large, polymorphic perikarya

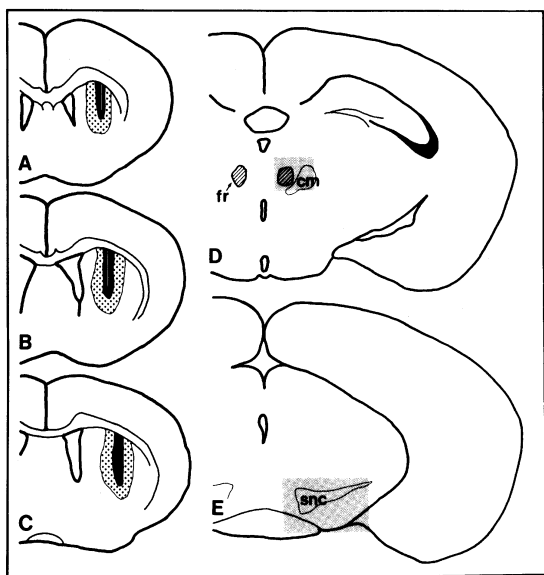


Fig. 1. Coronal sections through the mouse brain are shown in rostral-to-caudal order. A-C) Three forebrain levels displaying a representative injection site. The needle track is white, the intensely-fluorescent inner zone is black, the outer zone with fluorescent neural perikarya is dotted. D) Representative section crossing the CMP. E) Representative section crossing the SN. Shaded areas in D and E refer to Figs 2 and 3, respectively. cm, centre median-parafascicular complex; fr, fasciculus retroflexus; snc, substantia nigra pars compacta.

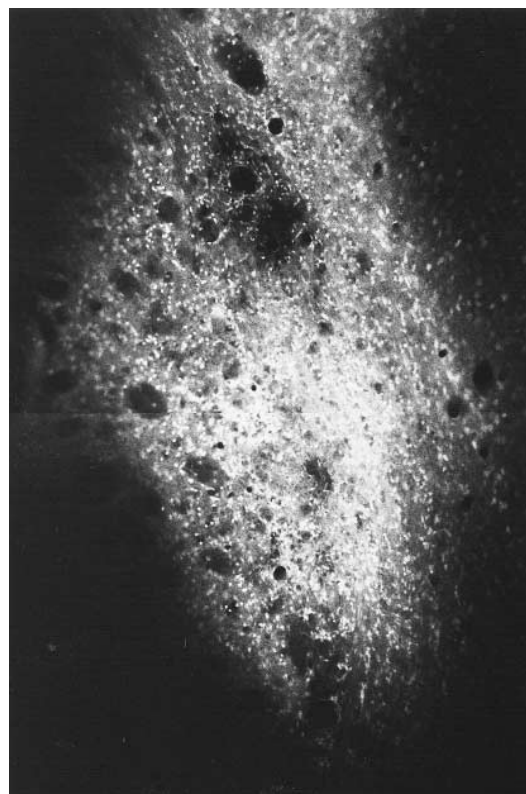


Fig. 2. Representative Fast Blue injection site corresponding to level C of Fig. 1.

with lightly-stained dendritic processes, that were equally distributed throughout the rostral-to-caudal extent of the SN (Fig. 4).

Counts of Fast Blue-labelled neurons located in the SN showed that the number of fluorescent perikarya progressively decreased from controls to group A and group B. As compared to controls, a 40.95% decrease in the total amount of Fast Blue-labelled neurons located in the SN occurred in group A ( $P < 0.0005$ ); a 55.26% decrease occurred in group B ( $P < 0.0005$ ). As compared to group A, group B mice had a 24.24% reduction of Fast Blue-labelled neurons ( $P < 0.05$ ; Table 2). Counts of Fast Blue-labelled neurons located in the CMP did not reveal between-group variations: as compared to controls, no significant differences occurred in the total number of Fast Blue-labelled neurons in the CMP of group A and group B mice. The SN/CMP labelling ratio of controls was 42.82%; this ratio declined in group A to 22.54% and in group B to 16.29% (Table 2).

Sampling performed on midbrain charts allowed the estimation of the density of Fast Blue-labelled neurons located in the medial and in the lateral portions of the midbrain (Table 3). As compared to controls, the average density of SN Fast Blue-labelled neurons was decreased by 39.45% in group A and by 54.26% in group B mice. As compared to group A, the average nigral density was reduced by 24.45% in group B. In control mice, the density of

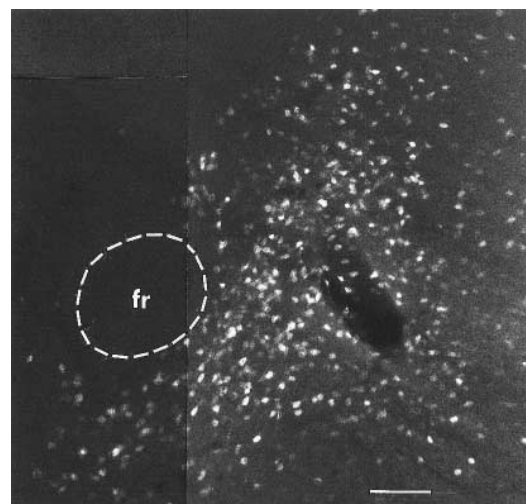


Fig. 3. Fast Blue-labelled perikarya located in the centre median parafascicular complex of a control mouse. The picture corresponds to shaded area of Fig. 1D. fr, fasciculus retroflexus. Scale bar = 150  $\mu$ m.-

Fast Blue-labelled neurons located medially was lower than that of the lateral SN ( $-29.75\%$ ,  $P < 0.05$ ). In group A and group B mice, instead, no medial-to-lateral variation was detected, due to a more severe decrease of retrograde labelling in the lateral than in the medial SN.

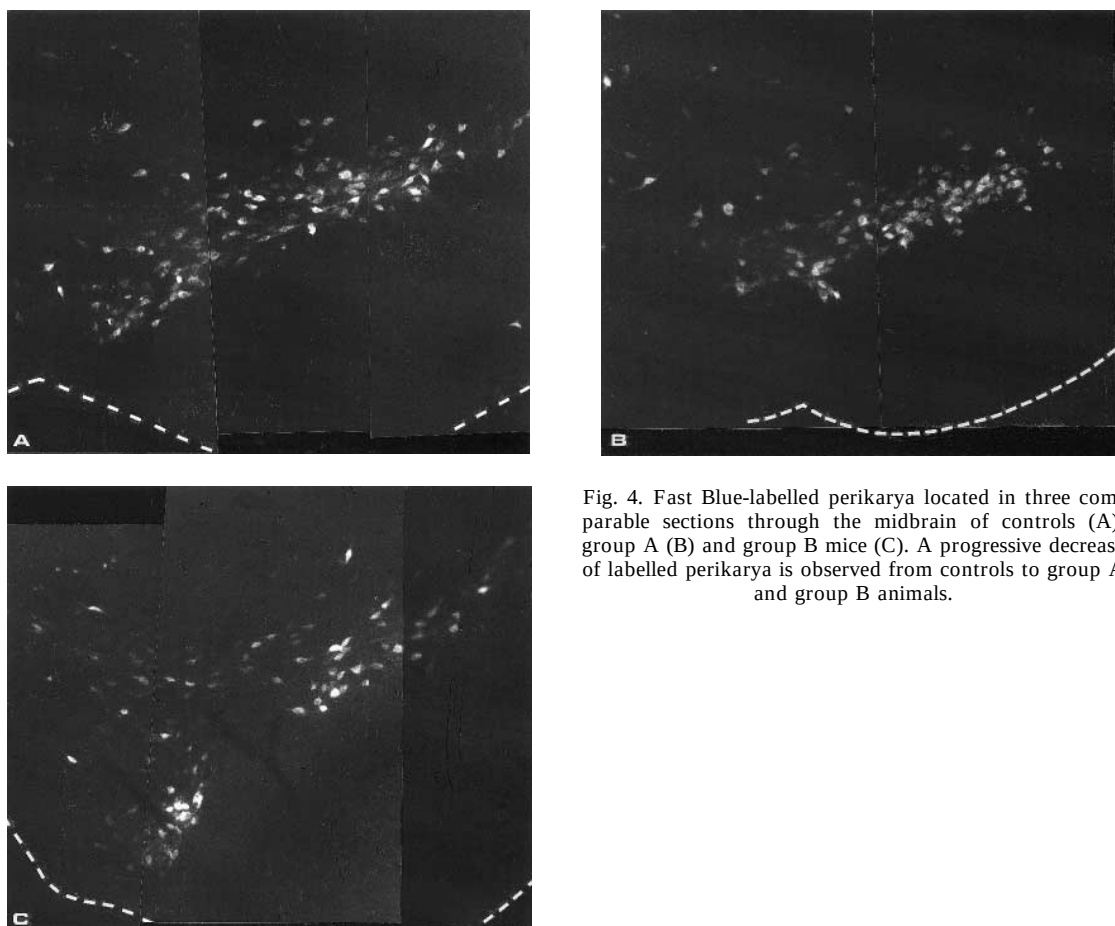


Fig. 4. Fast Blue-labelled perikarya located in three comparable sections through the midbrain of controls (A), group A (B) and group B mice (C). A progressive decrease of labelled perikarya is observed from controls to group A and group B animals.

The topography of retrogradely-labelled neurons depended on the localization of the neostriatal Fast Blue injection site, and could be appreciated in sagittal and transversal planes. The analysis of rostral-to-caudal series of sections through the midbrain of control mice showed that the bulk of Fast Blue-labelled neurons was concentrated at levels 5–8, i.e. intermediate from the rostral edge to the caudal border of SN. In group A and group B mice, instead, the reduction of Fast Blue cell density was particularly severe at such levels, thus producing a hollow, rather than a bell-shaped, rostral-to-caudal plot (Fig. 5). In control mice it was observed that Fast Blue labelling occurred both in the medial and in the

lateral half of SN, being prevalent laterally (Table 3).

Variations of the ratio between Fast Blue cell density and Nissl cell density provided a measure of the number of viable nigrostriatal neurons that were functionally impaired. In controls, the mean Fast Blue/Nissl ratio was 35.02% (45.18% in the lateral SN, 26.52% in the medial SN); it dropped to 20.32% (25.66% in the lateral SN, 16.14% in the medial SN) in group A mice, and to 17.2% (21.66% in the lateral SN, 14.09% in the medial SN) in group B mice. Based on these data, it was reckoned that 41.98% of viable neurons were functionally impaired one week following MPTP exposure and that a further

Table 2. Total number of Fast Blue-labelled neurons located in the substantia nigra pars compacta or in the centre median and parafascicular complex (mean  $\pm$  S.E.M.)

|                      | Substantia nigra            | Thalamus         | SN/CMP ratio |
|----------------------|-----------------------------|------------------|--------------|
| Controls             | 122.4 $\pm$ 8.5             | 285.8 $\pm$ 31.7 | 42.82%       |
| Group A (one week)   | 72.3 $\pm$ 7.0 <sup>a</sup> | 320.6 $\pm$ 33.1 | 22.54%       |
| Group B (five weeks) | 54.8 $\pm$ 4.3 <sup>b</sup> | 336.1 $\pm$ 29.3 | 16.29%       |

<sup>a</sup>Significantly different from controls ( $-40.95\%$ ;  $P < 0.0005$ ). <sup>b</sup>Significantly different from controls ( $-55.26\%$ ;  $P < 0.0005$ ), and from group A ( $-24.24\%$ ;  $P < 0.05$ ).

Table 3. Density of Fast Blue-labelled neurons located in the substantia nigra pars compacta (mean  $\pm$  S.E.M.)

|                      | Substantia nigra              | Medial substantia nigra      | Lateral substantia nigra       | Medial vs lateral   |
|----------------------|-------------------------------|------------------------------|--------------------------------|---------------------|
| Controls             | 187.7 $\pm$ 13.0              | 154.9 $\pm$ 14.0             | 220.5 $\pm$ 21.1               | - 29.75% $P$ < 0.05 |
| Group A (one week)   | 113.7 $\pm$ 10.9 <sup>a</sup> | 97.5 $\pm$ 12.2 <sup>b</sup> | 132.17 $\pm$ 17.9 <sup>c</sup> | n.s.                |
| Group B (five weeks) | 85.9 $\pm$ 6.3 <sup>d</sup>   | 77.7 $\pm$ 9.3 <sup>e</sup>  | 96.7 $\pm$ 8.6 <sup>f</sup>    | n.s.                |

<sup>a</sup>Significantly different from controls (-39.45%;  $P$  < 0.00005); <sup>b</sup>Significantly different from controls (-37.09%;  $P$  < 0.005); <sup>c</sup>Significantly different from controls (-40.10%;  $P$  < 0.005); <sup>d</sup>Significantly different from controls (-54.26%;  $P$  < 0.00005) and from group A (-24.45%;  $P$  < 0.05); <sup>e</sup>Significantly different from controls (-49.81%;  $P$  < 0.005); <sup>f</sup>Significantly different from controls (-56.12%;  $P$  < 0.005). n.s., not significant.

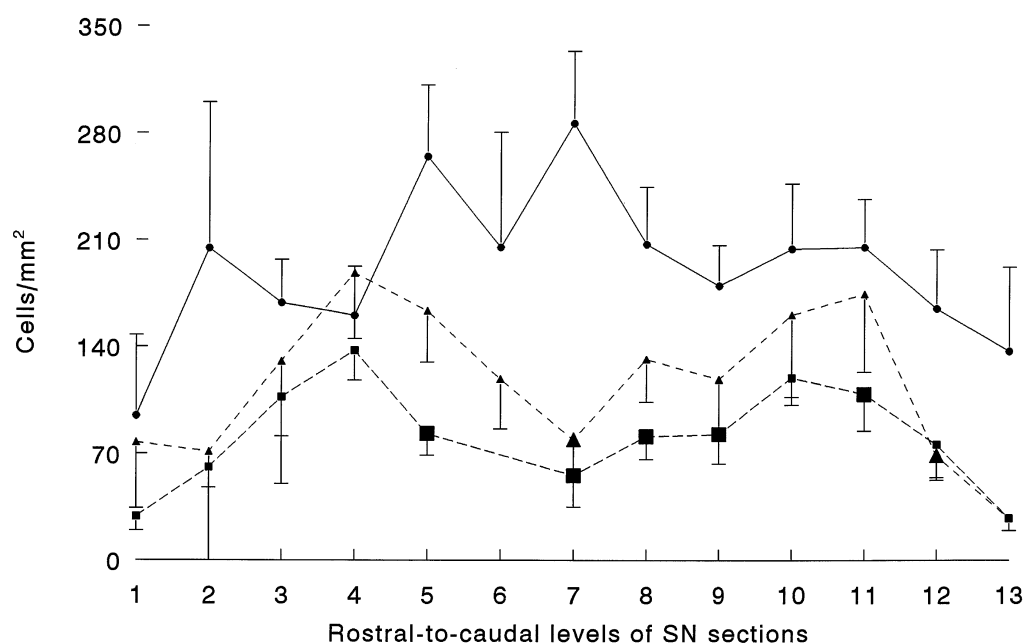


Fig. 5. Density of Fast Blue-labelled perikarya (mean  $\pm$  S.E.M.) in thirteen rostral-to-caudal series of sections through the SN (the rostral-most level being 1). In control mice (circles) it is observed that the density of retrogradely-labelled neurons is higher in sections 5–8 which correspond to the greatest amount of innervation of the striatum at the point of Fast Blue injection; the plot fits with a bell-shaped curve. In group A (triangles) and group B (squares) mice, the number of Fast Blue-labelled neurons is particularly reduced in the intermediate sections. Values significantly different from controls are indicated by larger plot markers. Significance values are:  $P$  < 0.05 for levels 7 (group A), 11 and 12;  $P$  < 0.01 for levels 7 (group B), 8 and 9;  $P$  < 0.001 for level 5. Group A and group B values differed only at level 5 ( $P$  < 0.05).

15.35% were impaired five weeks following MPTP exposure.

## DISCUSSION

The present study is in keeping with a number of observations demonstrating that the neostriatum receives afferent projections from the SN and from the CMP. Both tracts constitute rich projections that are arranged topographically. Since the first, but not the latter, is affected by MPTP,<sup>6</sup> thalamostriatal labelling served as an internal control of the injection size and topography, and as a measure of the uptake and retrograde transport of the fluorescent tracer. The injection sites and thalamic labelling were highly consistent in all the experimental groups; thus, the observed between-group variations depended ex-

clusively on exposure to MPTP and on the time differences between groups.

The present data are also in keeping with earlier evidence that, at variance with higher species, in the mouse MPTP does not produce a degeneration of SN neurons.<sup>16,17,26</sup> Our data showed that the density of Nissl-stained nigral neurons was not reduced in group A and group B mice. Appreciable cell degeneration can be produced in the mouse when MPTP is administered in combination with diethyldithiocarbamate or acetaldehyde;<sup>9,10,24</sup> both drugs delay the disappearance of MPP<sup>+</sup> from the brain and the latter also enhances MPP<sup>+</sup> neurotoxicity.<sup>21,33</sup> These combined treatments were not performed in the present study, that was aimed at evaluating the true effect of MPTP poisoning.

The MPTP dose injected to group A and group B mice was above LD<sub>50</sub>, since 55% of the mice died.

This regimen depletes striatal dopamine by 70–90% on the third day following the last MPTP dose; recovery of striatal dopamine levels toward control values occurs after 6–8 months.<sup>8</sup> It has also been reported that, in the mouse, MPTP destroys dopaminergic nerve terminals located in the neostriatum but spares dopaminergic perikarya in the SN.<sup>26</sup> The recovery of dopamine levels in the neostriatum is thought to be caused by a regeneration of dopaminergic nerve fibres, possibly associated with collateral sprouting of undamaged axons.<sup>26,31</sup>

The fluorescent tracer labelled only the SN neurons that were not functionally impaired by MPTP. The present study shows that neurones labelled by Fast Blue were viable and functionally intact, as they were capable of taking up the fluorescent tracer and of carrying it to their perikaryon by fast retrograde axonal transport. Neurons located in the SN of MPTP-treated mice that were not labelled by Fast Blue, but stained for Cresyl Violet, were viable although unable either to take up or to transport the retrograde tracer. The observation of a progressive decrease of Fast Blue-labelled, but not of Nissl-stained, nigrostriatal neurons from one week (group A) to five weeks (group B) following MPTP intoxication revealed that functional impairment of nigrostriatal neurons progressed after MPTP administration was discontinued. Interestingly, it has been shown recently in man that short-term exposure to MPTP leads to a protracted decline in nigrostriatal dopaminergic function, as observed by fluorodopa positron emission tomography scan.<sup>32</sup>

Active uptake and fast retrograde transport are energy-dependent processes.<sup>13</sup> MPTP is known to impair energy metabolism by blocking complex I of the mitochondrial energy chain,<sup>25</sup> a process which brings about irreversible cell degeneration in pri-

mates, but not in C57 mice. Energy impairment following the exposure to MPTP may cause an impairment of Fast Blue endocytosis or of fast retrograde transport; this would reduce the number of Fast Blue-labelled neurons before a degeneration of terminals may take place. The present data show that the functional impairment of nigrostriatal neurons proceeds after exposure to MPTP is discontinued, and that such impairment increases from one to five weeks following exposure. This is in keeping with recovery studies, showing that dopamine depletion in the mouse is still appreciable several months after exposure to MPTP is discontinued.<sup>8,26</sup>

## CONCLUSION

Retrograde labelling of nigrostriatal neurons following MPTP exposure provides a new tool for the identification and sorting out of viable, but functionally impaired, neurons from those that are viable and functionally intact, without considering the ones that have degenerated. This tool may prove particularly useful to measure the anatomical substrate of recovery induced by drugs, by growth factors<sup>11,12,15,28</sup> or by intracerebral grafts<sup>5,30</sup> in MPTP-treated animals. In summary, the use of fluorescent retrograde tracing may allow us to obtain quantitative information on the processes leading to neural degeneration and regeneration in the MPTP model of Parkinson's disease.

*Acknowledgements*—The authors are indebted to A. Granato and to G. Macchi for helpful suggestions on the experimental procedures. This research was supported in part by CNR (Consiglio Nazionale delle Ricerche) grant 94.02345.04.

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(Accepted 5 June 1996)