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DEGENERATIVE AND COMPENSATORY FEATURES IN THE STRIATUM OF MPTP-TREATED MICE

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Masters of Science

by

Reza Ehsanian

May 2008

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04/30/08

ABSTRACT

DEGENERATIVE AND COMPENSATORY FEATURES IN THE STRIATUM OF MPTP-TREATED MICE

By Reza Ehsanian

This study focuses on the time course of damage and compensatory ultrastructural changes associated with Parkinson's disease, as induced by a 35 mg/kg dose of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in C57BL/6 mice. The results suggested continued deterioration of the striatum up to 7 days post-injection of the MPTP, with notable recovery at 21 days post-injection. Dark terminals (DTs) were observed at 3, 7, and 21 days post-injection, with the highest frequencies occurring at 7 days post-injection. The morphology of DTs indicates that they may contribute to the recovery of the animal. At 21 days post-injection, a point when behavioral and neurochemical recovery is expected to be nearly complete, there was a dramatic increase in healthy axons. The increase in axonal frequency at 21 days is indirect structural evidence for dopaminergic neuronal sprouting or some other regenerative process that would likely allow the mouse brain to compensate for previously eliminated neurons.

ACKNOWLEDGEMENTS

Thank you to Alireza Ehsanian, Steven Goldschmidt, and Beth Fernandez, for your help in tissue processing. Thank you to Dr. Michael Sneary for your critical reading and input. Special thank you to Dr. Richard Boyle and Dr. David Bruck for your mentorship and friendship.

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INTRODUCTION

History

Although, the most famed description of Parkinson's disease (PD) is attributed to James Parkinson, who described the disease in 1817, it was recognized long before then. Ancient Egyptians (1350-1200 BC) suggested the occurrence of parkinsonism in old age (Forno, 1996), while later observations of the disease were also documented by Leonardo da Vinci sometime between 1489 and 1506 (Calne et al., 1989). Charcot's lectures and observations give detailed descriptions of the manifestations of the disease (Charcot, 1877). These early observations undoubtedly led to the work by the British physician Parkinson, who gave a nearly complete description of the disease he termed "*paralysis agitans*" (shaking palsy) (Parkinson, 1955), now called PD.

Disease Frequency

PD is the most common cause of parkinsonism, a term that refers to a syndrome of muscle rigidity, tremor, bradykinesia, and akinesia. PD is one of the most commonly encountered neurological disorders in clinical practice (MacDonald et al., 2000). From birth, the lifetime risk of developing PD is about 2% for men and 1.3% for women, while that for parkinsonism is slightly higher (4.4% for men and 3.7% for women); for both, the risk factor increases with age (Elbaz et al., 2002). The annual incidence of PD ranges

from 110 to 330 per 100,000 individuals over 50 years of age (Bower et al., 1999). For individuals over 65, the prevalence of PD has been estimated at 1800 per 100,000 individuals. The incidence in those 85 to 89 years old increases 4.3 times to 2600 per 100,000 over that in individuals 65 to 69 years old, in whom incidence is 600 per 100,000 (de Rijk et al., 2000). When these statistics are considered in light of our increasing life spans, the need is amplified for basic scientific research investigating the degenerative and compensatory mechanisms involved in PD in hopes of finding potential targets for therapy.

Clinical-Pathological Description

PD is clinically defined as a distinctive progressive disorder characterized by asymmetric onset of the cardinal motor signs of resting tremor, rigidity, bradykinesia, and postural instability. A number of other clinical findings, such as masked face and festinating gait, occur less consistently (Calne, 1992; Forno, 1996; Gibb & Lees, 1988; Hughes, 1992; Larsen, 1994; Ward & Gibb, 1990). Nonmotor clinical features include dementia (Brown et al., 1984; Cummings et al., 1988; Mortimer et al., 1985; Rajput et al., 1992), depression (Mayeux, 1992; Tandberg et al., 1996), hallucinations (Celesia et al., 1972), and autonomic dysfunction (Quinn, 1989; Tanner et al., 1992) as well as abnormalities in olfactory and visual perception (Langston, 2006). These signs of PD are accompanied by progressive neuropsychological impairment (Dubois & Pillon, 1997; Langston, 2006) that includes abnormal emotion processing (Benke et al., 1998; Blonder

et al., 1989; Borod et al., 1990, Breitenstein et al., 2001; Langston, 2006; Pell, 1996). The average duration of illness is nine years (Hely et al., 1999). Patients with late onset PD and/or those whose disease is accompanied by dementia have short survival periods. Patients with tremor-dominant disease have a longer survival period than those with the akinetic form (Jellinger et al., 2002).

The pathology responsible for the clinical conditions is accompanied by a reduction in dopamine levels in the striatum (Hornykiewicz, 2008; Kish et al., 1988; Langston et al., 1992; Tetrud & Langston, 1989) and severe degeneration of dopaminergic neurons in the substantia nigra *pars* compacta (Forno, 1969; Langston et al., 1992; Tetrud & Langston, 1989). The cardinal symptoms are observed once the striatal dopamine levels decrease by 60-80% (Bernheimer et al., 1973; Hornykiewicz and Kish, 1987). The fact that patients show few symptoms despite extreme dopamine reduction (< 60% of normal) suggests the existence of compensatory mechanisms in the remaining dopaminergic neurons (Bezard and Gross, 1998; Hornykiewicz, 1993, 2008). Therefore, it is clear that an understanding of the mechanisms of compensation in an accurate animal model that mimics this loss of dopaminergic neurons will lead to a better understanding of the progression of the disease. Cell losses also extend into extranigral sites, such as the locus coeruleus, the cholinergic nucleus basalis of Meynert, and frequently in the dorsal motor nucleus of the vagus (Forno, 1996; Forno et al., 1986; Markham & Diamond, 1993). Although these sites may play a role in the development of symptoms, our focus remained on the site most directly implicated in the development of PD, the striatum.

Lewy Bodies

Formation of Lewy bodies and Lewy neurites within the substantia nigra is considered a pathological marker for the clinical condition in humans (Jellinger, 1987; Lewy, 1912; Lowe et al., 1997). Lewy bodies are also observed within the dorsal motor nucleus of the vagus, hypothalamus, cholinergic nucleus basalis of Meynert (Lewy, 1912, 1923; Ohma & Ikuta, 1976), locus coeruleus, Edinger-Westphal nucleus in the midbrain, raphe nuclei, cerebral cortex (Forno, 1996; Hughes et al., 1993), olfactory bulb (Reyes et al., 1992), cranial nerve motor nuclei, and central and peripheral divisions of the autonomic ganglia (Jager & Bethlem, 1960; Iwanaga et al., 1999; Wakabayahi & Takahashi, 1997).

In 1997, a mutation was identified in the α-synuclein gene in families with autosomal dominant PD (Polymeropoulos et al., 1997). This form of PD (familial PD) is a variant of age-related, apparently nonhereditary (sporadic) PD that attacks people as early as in their 30s and has a strong hereditary component. This discovery, along with an earlier confirmation of the presence of Lewy bodies accompanying neural degeneration in individuals with familial PD (Golbe et al., 1990), led to pathohistological and molecular pathological identification of PD. Abnormal filaments in Lewy bodies are recognized by antibodies against α-synuclein in both familial and sporadic PD (Arima et al., 1998; Baba et al., 1998; Irizarry et al., 1998; Spillantini et al., 1997; Taeka et al., 1998; Wakabayahi et al., 1997; Wakabayashi et al., 1998). While Lewy bodies are considered to be reliable markers of neurodegeneration, they may not be present in the substantia nigra of all PD sufferers (Forno, 1996). In addition, the status of Lewy bodies as markers for

presymptomatic PD has been brought into question by reports documenting the presence of Lewy bodies in postmortem specimens from asymptomatic individuals (Gibb, 1988; Gibb, 1993; Forno, 1996). Therefore, although Lewy bodies do not occur in the mouse model of PD, the importance of the model cannot be disregarded due to the absence of Lewy bodies.

MPTP-Mouse Model

The MPTP model (Langston, 1995) has been one of the most accurate models of human idiopathic PD and has contributed to the understanding of the course and cause of PD. MPTP is a neurotoxin accidentally developed in humans (Forno, 1996) that targets the dopaminergic neurons within the nigrostriatal tract and produces an array of clinical and pathological features that nearly duplicates that of idiopathic PD.

MPTP induces parkinsonian symptoms in various animals, including the strain of mice used in this study (Langston et al., 1984, 1992; Lewin, 1986; Tetrud & Langston, 1989). Of the different studies implementing the mouse model (Edwards, 1993; Irwin et al., 1992; Riachi & Harik, 1Y988; Sundstrom et al., 1988), older mice (>8 weeks) of the strain C57BL/6 appear to be the most responsive to the initial neurotoxic effects of the MPTP. The mouse brain is purged of MPTP in a shorter period of time than in the brain of the primate (Edwards, 1993; Riachi & Harik, 1988). This reduction in neurotoxic insult may allow for increases in synaptic output (Cochiolo et al., 2000) and regrowth of injured nerve fibers (Ricaurte et al., 1986; Sundstrom et al., 1987) to restore dopamine

production to near normal levels (Hallman et al., 1985; Reinhard et al., 1988b; Ricaurte et al., 1986; Sundstrom et al., 1990) and allow for the behavioral recovery observed in mice (Hallman et al., 1984; Willis and Donnan, 1987). This clearing may also explain the fact that mice in general are more highly resistant to MPTP than are primates, requiring higher doses for comparable effects (Edwards, 1993). Due to the mouse's robust nature and ability to recover from MPTP-induced parkinsonism, the mouse model is one of particular interest in the study of compensatory pathways arising after MPTP treatment.

As in human PD, the MPTP-treated mouse has been shown to suffer substantial depletion of striatal dopamine (Irwin, Langston, & DeLanney, 1987; Irwin et al., 1992; Reinhard et al., 1988b). Several laboratories have reported severe lesions of the substantia nigra in mice, with cell deaths comparable in number to those in primates (Cochiolo et al., 2000; Heikkila et al., 1984a; Sundstrom et al., 1987, 1988, 1990). In the most critical aspects of true parkinsonism, the neurochemical and neuropathological syndrome induced in the C57BL/6 strain of older mice by MPTP corresponds to that arising from human PD. Therefore, the MPTP-treated C57BL/6 mouse older than 12 weeks serves as an adequate model to study the degenerative and compensatory effects of MPTP-induced neurodegeneration.

Site and Mmode of MPTP Action

The site and mode of action of MPTP have been heavily investigated. Evidence indicates that MPTP enters the glial cells in the striatum or the substantia nigra, where it

is cleaved by the monoamine oxidase-B isozyme to form MPP^+ (1-methyl-4-phenylpyridinium), the neurotoxic metabolite (Chiba et al., 1985; Giovanni et al., 1991; Javitch & Snyder, 1985; Ransom et al., 1987; Riachi & Harik, 1988). Striatal MPP^+ is then taken up through the dopamine transporter of dopaminergic neurons and routed in a retrograde fashion to the cell bodies (Chiba et al., 1985; Markham & Diamond, 1993). There is little doubt that MPP^+ is imported into mitochondria where it binds to NADH dehydrogenase in complex I of the oxidative electron transport chain (Mizumo et al., 1989; Parker et al., 1989; Ramsay & Singer, 1986; Ramsay et al., 1991) inhibiting mitochondrial respiration (Ramsay et al., 1986, 1987). Oxidative stress is also increased through the production of toxic free radicals (Adams et al., 1986; Corsini et al., 1985; Cleeter et al., 1992; Hasegawa et al., 1990; Johannessen et al., 1986; Perry et al., 1985; Rossetti et al., 1988; Sinha et al., 1986).

Compensatory Increase in Dopamine Output in Response to Nigrostriatal Lesioning

Since the early postmortem analyses of dopaminergic neurons and dopamine levels in PD, a causal relation has been proposed between the extended preclinical phase of the disease and the elevated ratio of dopamine metabolites to dopamine. Dopaminergic metabolites, such as homovanillic acid (Bernheimer and Hornykiewicz, 1965; Hornykiewicz, 1993) and dihydroxyphenylacetic acid (Zigmond and Stricker, 1977; Zigmond et al., 1984), increase in striatal extracts in relation to dopamine in the context of lowered cell numbers, reflecting an increase in dopamine production in the surviving

dopaminergic neurons and implying an increase in DA release (Hornykiewicz, 1966, 1993; Zigmond et al., 1990, 1993). Several studies have indeed shown an increase in dopamine production by the spared dopaminergic neurons (Alter et al., 1987; Hefti et al., 1980; Onn et al., 1986; Uretsky and Iversen, 1970; Zigmond et al., 1984). Of particular note is an increase in the ratio of tyrosine hydroxylase activity to dopamine levels as well as an increase in dopamine synthesis in spared dopaminergic neurons (Acheson and Zigmond, 1981; Agid et al., 1973; Altar et al., 1987; Bloom et al., 1969; Onn et al., 1986; Uretsky and Iversen, 1971; Wolf et al., 1989; Zigmond et al., 1984). The increased dopamine production and release in diminished cell numbers led to the conclusion that a major compensatory mechanism involves increased dopamine output by existing terminals (Zigmond et al., 1984) and/or by newly created terminals of surviving cells (Cochiolo et al, 2000). To our knowledge, our initial study (Cochiolo et al, 2000) was the first to present ultrastructural evidence of increased synaptic output in spared neurons as a compensatory mechanism to increase dopamine levels in response to the neurotoxin, MPTP. This compensatory mechanism, by itself or in combination with other mechanisms, is effective in maintaining survival of the individual until dopaminergic neuronal death exceeds a critical threshold: 70-80% of striatal nerve terminals and 50-60% of substantia nigra *pars* compacta pericaryons (Bernheimer et al., 1973; Riederer and Wuketich, 1976).

Aims of the Time-Course Study of MPTP-Treated C57BL/6 Mice

The overall focus of the present time-course study was on the MPTP-induced ultrastructural damage and compensatory mechanisms that allow the C57 mouse to recover from the array of PD-like changes that occur in response to MPTP. It is likely that analogous compensatory mechanisms during the preclinical phase of human PD delay the appearance of the cardinal symptoms until a threshold is reached whereby these mechanisms are no longer effective. Hence, a better understanding of the mechanisms involved in mouse recovery may lead to the development of new detection and therapeutic approaches to stave off the debilitating symptoms of PD.

Our laboratory (Cochiolo et al., 2000) previously produced evidence for a structural and functional repatterning of dopaminergic neurons that allowed them to compensate for the loss of neighboring cells to effect long-term survival of the individual. Observations 3, 7, and 21 days following MPTP administration were made in the present investigation to search for the presence of dark terminals to determine whether they are indicative of degeneration or of recovery. Our results lend support to the notion that dopaminergic neurons spared from destruction compensate for the loss of other dopaminergic neurons by increasing signaling functionality through increased vesicle production in terminals and the production of new terminals. Indirect evidence was also accumulated that indicated regenerative events either via sprouting from or division of existing cells or the entrance of new dopaminergic neurons into the striatum.

MATERIALS AND METHODS

Middle-aged (6-8-month-old) male C57BL/6 mice, obtained from Simonsen Labs (Gilroy, California), were housed one per Plexiglas cage with free access to food and water in a colony room maintained at 23 ± 1° C. Control mice were injected with 0.85% saline. Mice were given a single subcutaneous injection of a 35 mg/kg dose of the hydrochloride salt of MPTP. MPTP-treated mice were sacrificed 3 (5 mice), 7 (5 mice), or 21 (2 mice) days post-injection. All animals were anesthetized subcutaneously with Phenobarbital and perfused via the left atrium with heparin (10 units/ml 0.85% saline) and then 2.5% (w/v) paraformaldehyde and 3% (w/v) glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4. The brains were immediately removed from their skulls and striatal tissues dissected out and diced. These tissue fragments were bathed in the primary fixative for 5 days and postfixed in 2% (w/v) osmium tetroxide and 1.25% potassium ferrocyanide in sodium cacodylate buffer, pH 7.4, for 2 hours. They were prestained *en bloc* with 2% aqueous (w/v) uranyl acetate, dehydrated in a graded series of ethyl alcohol, and infiltrated with and embedded in Epon 812 resin. The blocks were thin sectioned (100-120 nm) on a Leica Ultracut UCT ultramicrotome. Sections were post-stained with 2% uranyl acetate and Reynold's lead citrate and viewed on a Leo 912AB transmission electron microscope.

To distinguish between the effects of MPTP and preparation artifacts, fixation quality was assessed in each region by observation of mitochondrial intactness and maintenance of periplasmic space volume. When any two of these features were absent, the brain

tissues from which the section was taken were discarded and the section was excluded from the study.

RESULTS

Degenerative Changes

Severe deterioration of myelin sheaths was observed at each stage of the time course (Fig. 1A-C,E, 2A-C, 3A-D). Gaps were detected between the plasma membrane and myelin sheath due to cytoplasmic shrinkage, the plasma membrane receding from the sheath, and the sheath pulling away from the axon (Fig. 1A,C, 2A, 3A). Separation of the laminae of the myelin sheath (Fig. 1A,E, 2A-C, 3A) was accompanied by electron-dense particles (Fig. 3D,E) that appeared to be myelin breakdown products. In axons that were clearly undergoing degeneration, these particles were also present in areas where unraveling had not yet occurred (Fig. 3D). Demyelination, observable at early stages as interruptions in the myelin sheath, exposed the plasma membrane to the neuropil (Fig 1A, 3A-D). Presumptive microglial processes were seen in close proximity to degenerating axons (Fig. 1A).

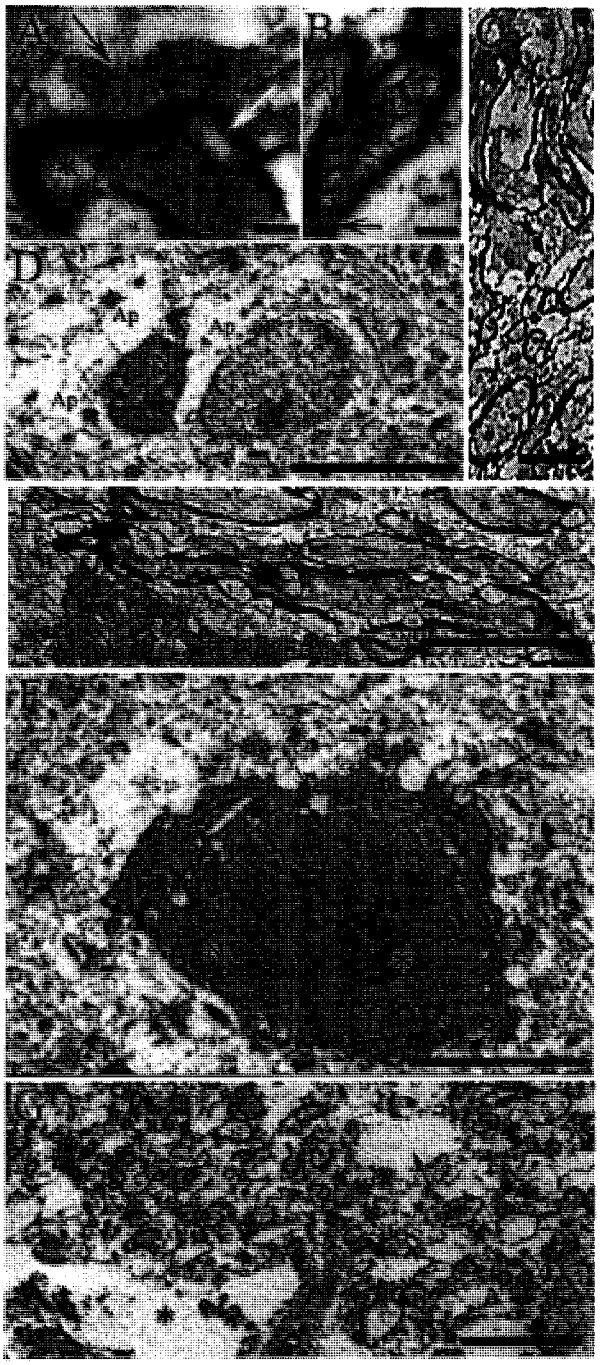


Fig. 1. 3 days post-MPTP.

A. Myelin sheath pulling away from axonal plasma membrane (*) and separation of laminae of myelin (white arrow). A microglial process occurs at the black arrow. B. Compression and tangling of cytoskeletal elements within an axon displaying slight myelin damage at arrows. C. Severe cytoplasmic shrinkage (*) and myelin breaks. D. Low density astrocytic processes (Ap) surrounding an interneuron (N) that displays cytoplasmic condensation and vacuolation. E. Damaged axons (Ax) showing unraveling of myelin adjacent to a degenerating interneuron (N). F. An interneuron (N) demonstrating nuclear (*) and cytoplasmic condensation, vacuolation (V), and peripheral blebbing (unlabeled arrow). The surrounding neuropil shows disruption and clearing. G. Axonal ghost (arrow) and more extensive region of clearing. Scale bar = 0.2 μm in A and B; 2 μm in C; and 5 μm in D-G.

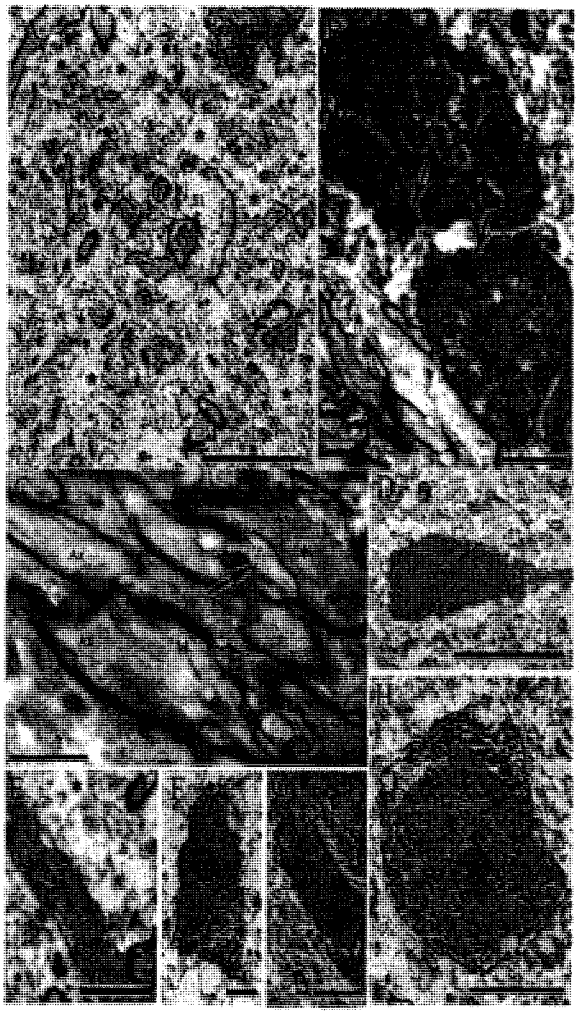


Fig. 2. 7 days post-MPTP.

A. Extensive cytoplasimc clearing in the neuropil (*) with damaged axons throughout. Enlarged space (S1) contains remnants of cytoplasm and is surrounded by myelin, indicating an early stage of cellular clearing. The space at S2 is similar but has been subjected to a greater degree of demyelination so that only a faint boundary of the original degenerating neuron is visible. B. Degenerative neurons with dark inclusions (large arrows) and large lysosomes with dense cores (small arrows). C. Impaired axons (Ax) displaying disruption of cytoskeletal elements (surrounding *), myelin unraveling (black arrow), and dense particles at the sites of unraveling (white arrow). Mitochondrial damage is also observed (M). D. A cell with morphology clearly identifying it as a degenerating interneuron (N) with condensed chromatin and small vacuoles. Cleared regions around the neuron are prevalent. E. Higher magnification view of the axonal segment contiguous with the axonal hillock of the interneuron in the previous figure. The configuration of the cytoskeletal elements clearly indicate that the cell is a neuron. Cleared regions surround the axon (*). F.-H. Examples of apoptotic appearing interneurons. Scale bar = 5 μm in A; 2 μm in B; 1 μm in C; 10 μm in D-E, 1 μm in F, and 5 μm in G-H.

MPTP-induced displacement and clumping of neurofilaments and microtubules occurred in conjunction with myelin unraveling (Fig. 2C) as well as in axons with apparently intact myelin, at least as revealed in the plane of section of the figure (Fig. 1B). Mitochondrial swelling was common (Fig. 1E, 2C) but was more frequent at 7 days post-MPTP injection than at 21 days.

Electron-translucent areas occurred at all stages of the time course, maximally at 7 days (Fig.1G) and generally much less so at 21 days (Fig. 3A-C), as deduced through consistent scans (not all shown) of large numbers of axonal and synaptic fields. Most of

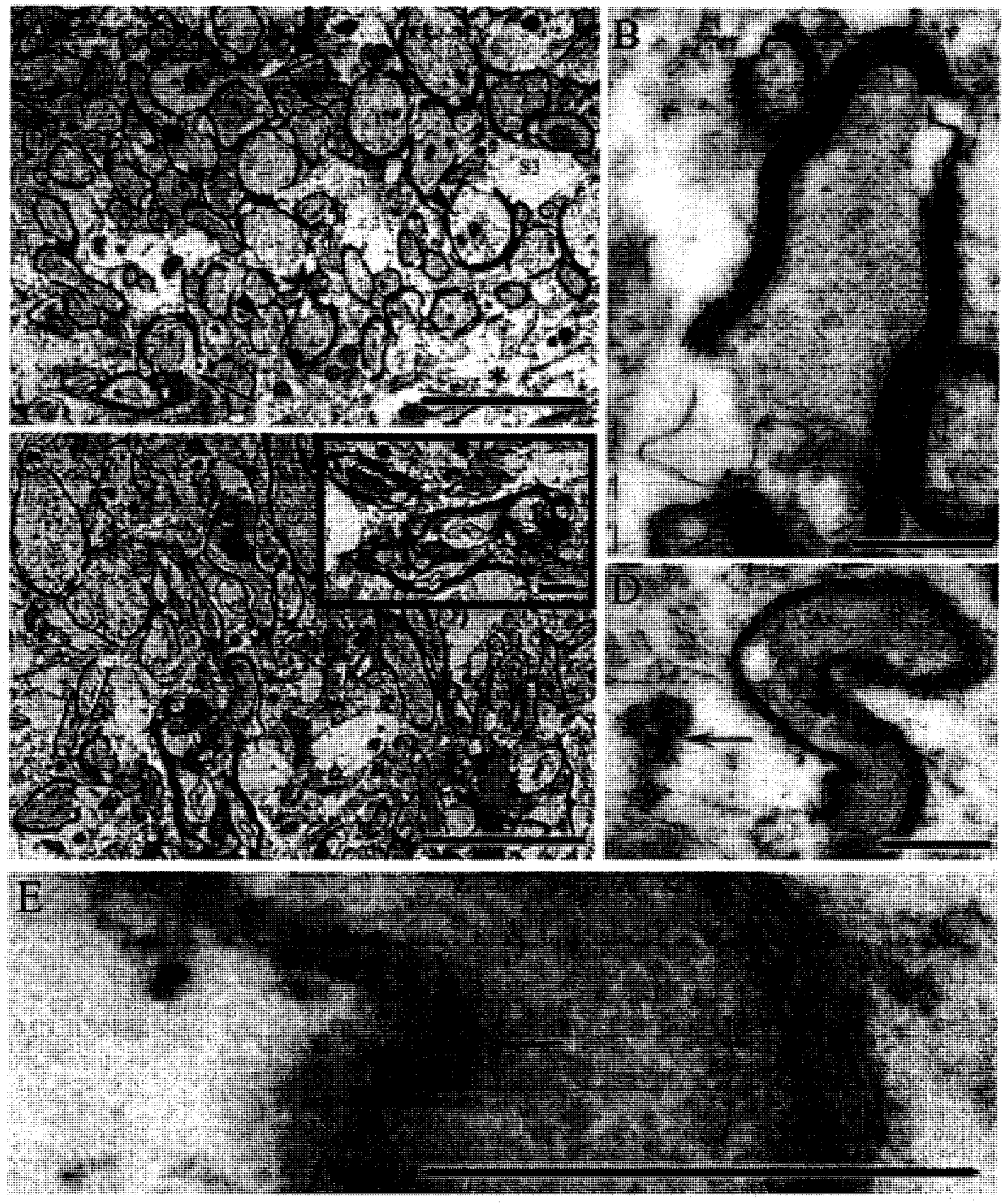


Fig. 3. 21 days post-MPTP.

A. Clearing in the neuropil at advanced stage of cellular elimination, where myelin is no longer visible around the space (S3); the original cell shape has still been maintained producing a cellular ghost. Also present are breaks in the myelin sheath (arrows) and disorganization and disappearance of axonal contents (arrowhead). B. Breaks in and unraveling of the myelin sheath and disruption of the cytoskeleton in region of cytoplasmic vacuolation. C. Damaged axons displaying all forms of degeneration. Accumulation of electron-dense deposits and membranous structures in the axon (inset). D. Electron-dense particles (arrow) in nearly completely reabsorbed cell. Nearby is a degenerative axon (Ax) showing myelin interruption, cytoplasmic shrinkage, and cytoskeletal disruption (arrowhead). E. Higher magnification view of an area in Fig. 3D revealing congealment of the myelin sheath and subsequent granulation. Scale bar = 5 μm in A; 0.5μm in B; 5 μm in C; 1 μm in C inset; 0.5 μm in D-E.

these areas were clearly the result of progressive neuronal degeneration, as various stages of formation of cellular "ghosts" were evident (Fig. 1G, 2A,E, 3A,C,D). For example, in Fig. 2A, the cell at S1 displays obvious protoplasmic and myelin degradation, but the myelin is still visible enough to make out the cell outline. The cell at S2 of Fig 2A progressed further, deteriorating nearly to the point of complete cellular clearing. The cell at S3 in Fig. 3A vanished entirely, but the maintenance of the shape of the cleared region indicates the former presence of a cell. Some of these ghosts still contain remnants of myelin (Fig. 3D), in the form of the electron-dense particles mentioned above, lending support to our designation of the spaces as cellular in origin. Some electron-transparent regions, however, appear to be occupied by astrocytes, whose processes have intruded into regions vacated by cellular clearing (Fig 1D). Our methods of assessing the degree of degeneration within the large striatal expanse may be prone to undersampling, but our observations are consistent with those of other studies employing immunohistochemical and biochemical methods.

Extremely dense cell bodies of questionable identity occurred in highest number at 7 days and declined in frequency by 21 days. Through observations of alternative planes of section, we were able to capture images of unmistakable neuronal morphology in cells of a similar nature (Fig. 2D). These cells bore axonal processes and thus were concluded to be interneurons undergoing degeneration. Degenerative processes included various stages of chromatin condensation in (Fig. 1F, 2D, 2F-H) and vesiculation of (Fig. 2F-H) their large nuclei . The cytoplasm was in a condensed state containing distended and dense lysosomes (Fig. 2B) and swollen and distorted mitochondria with disrupted cristae

and cleared matrices both within the cell body (Fig. 2B) and the axon (Fig. 4A,B). Axonal cytoskeletal displacement was also evident (Fig. 4A,B).

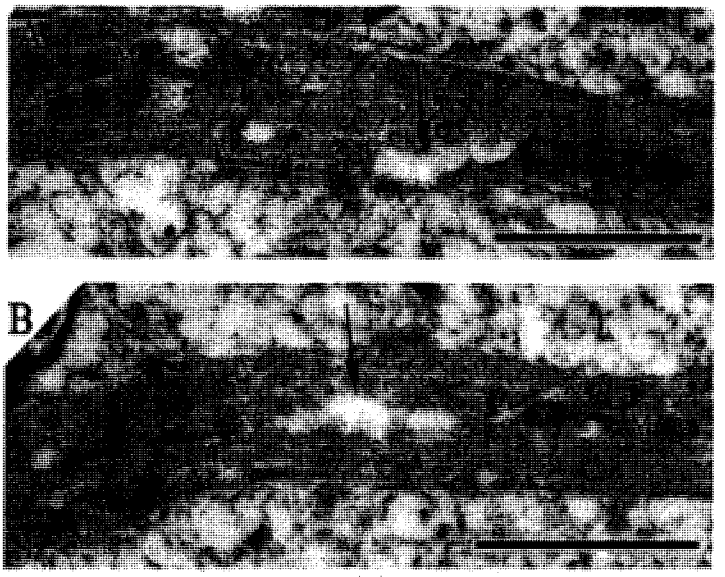


Fig. 4. Degenerative axon of interneuron at 7 days post-MPTP.

A, B. Damaged mitochondrion (M) and cytoplasmic clearing (arrows) in the axon of a degenerating interneuron. Scale bar = 2 μm.

Cytoplasmic deterioration progressed to an even more highly condensed condition lacking any recognizable organelles and with especially dense blotches (Fig. 2B). Vacuolation was extensive on the periphery of the cells (Fig. 2F-H). Astrocytic processes (Fig. 1D) and areas of clearing occurred both interior (Fig. 2D-E) and exterior to these cells.

Dark Axon Terminals and Interneurons

Axon terminals of dopaminergic neurons were darkened as a result of their high density of synaptic vesicles (Fig. 5-10). They were positioned extensively *en passant.* Darkened *en passant* boutons were most abundant in our sections in the spared fibers of

the 7-day samples (Fig. 5-8, 10), but they were still present in the 21-day samples (Fig.

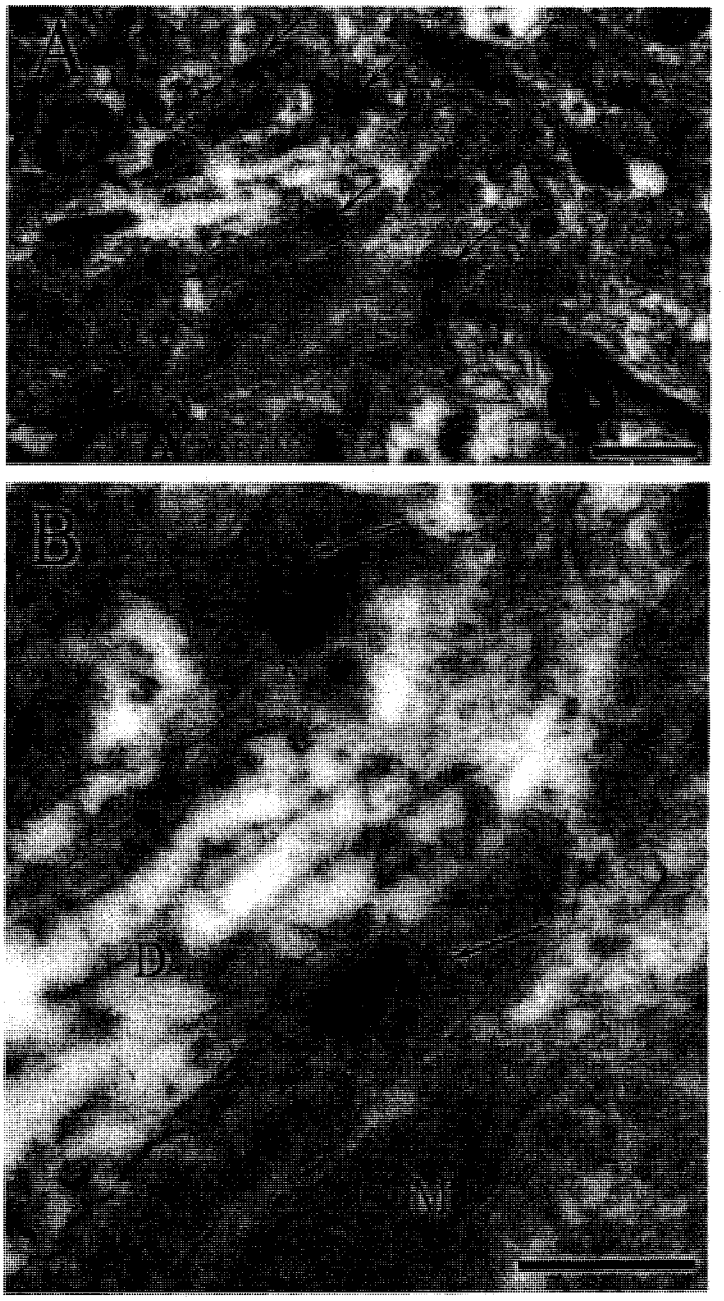


Fig. 5. Dark terminals at 7 days post-MPTP.

A. Low magnification view of dark terminals (arrows). B. Higher magnification view of two of the dark terminals (arrows) in Fig. 5A, in which dense vesicles are visible. The labeled mitochondrion (M) has maintained its integrity and appears to be part of the same cell as the lowermost dark bouton, which is synapsing on the dendrite at D. Scale bar = 5 μm in A; 0.5 μm in B.

9A,B). Multiple (mostly paired) and perforated synaptic densities occurred in the dark terminals (Fig. 8A,B). We made an effort to visualize the axons that the dark boutons flanked to determine whether these boutons were associated with healthy or damaged

axons. The extreme curvature of axons in the vicinity of these boutons made their detection challenging. Several were identified and revealed to have dark boutons associated with intact axons lacking any of the morphological features that characterized injured axons (Figs. 6A,B). A convincing example is shown in Fig. 6A where a transverse section through a parent axon includes a dark terminal. It is clear that the axon is healthy, lacking any shrinkage, autolysis, or microtubule and neurofilament disarrangement or tangling.

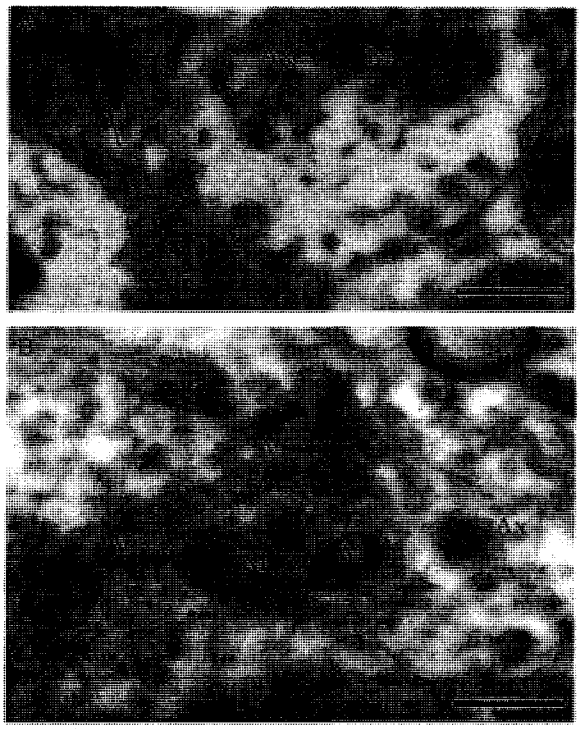


Fig. 6. Dark terminals at 7 days post-MPTP.

A. Dark *en passant* bouton with arrow pointing to synaptic vesicles within an axon (Ax) containing an undamaged mitochondrion (M). The region of extreme electron density is a face view of the pre-synaptic density. B. Two populations of vesicles (V) forming electron-dense regions within probable separate dark terminals of a single healthy axon (Ax) bearing an undisrupted cytoskeleton and mitochondria (M). Scale bar = 0.5 μm.

As we observed previously, parallel arrays of microtubules appeared to traverse the neuropil (Fig. 7A). Because confining cell membranes could rarely be seen, these microtubules seemed to be located external to cells. In agreement with our previous study, some of these arrays were seen in association with *en passant* boutons, confirming that they were located in axons (Fig. 6A). Unrecognized previously, however, some of

these arrays were clearly in interneuronal dendrites synapsed on by dark terminals (Fig. 5B, 7B).

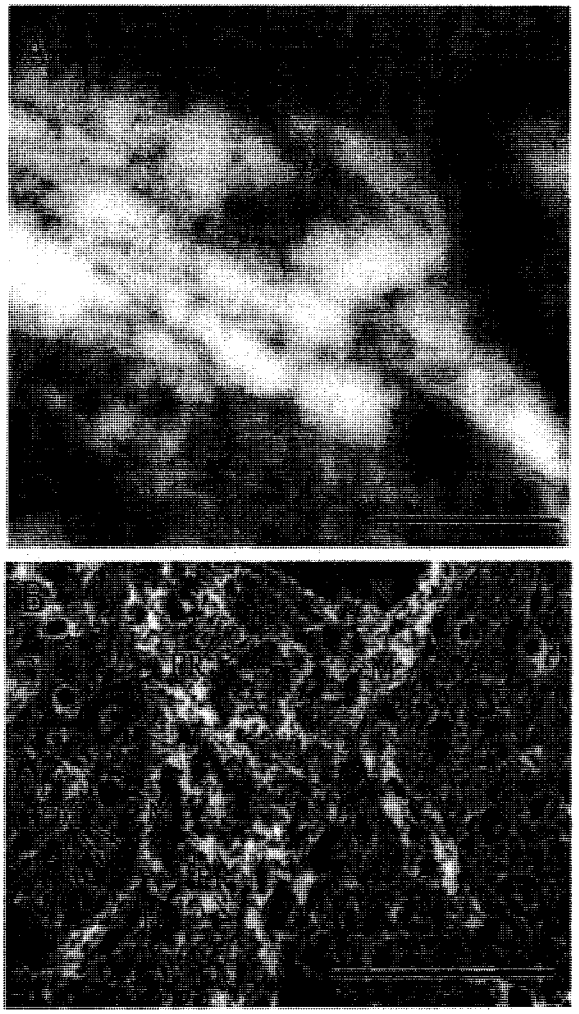


Fig. 7. Dark terminals synapsing on healthy interneuron at 7 days post-MPTP.

A. Cytoskeletal elements seemingly running through region with unidentifiable cellular boundaries. This structure resembles the dendrites of healthy interneurons as well as the axons from which dark terminals branch. B. Healthy interneuron (N) with abundant endoplasmic reticulum (ER) arranged both in parallel array and in a more disorganized fashion. Healthy mitochondria (M) are located throughout. Dark terminals (DT) are synapsing proximal to the cell body onto a primary dendritic process (D). The dark terminals were identified by viewing them at higher magnification. The neuropil surrounding this healthy neuron shows no clearing or damage. Scale bar = 0.5 μm in A; 5 μm in B.

We observed many cases of the darkened *en passant* terminals synapsing on dendrites of healthy (spared) interneurons (Fig. 7B). Spared interneurons were relatively large with abundant parallel and randomly arranged rough endoplasmic reticulum (Fig. 7B,10). The cells also contained healthy mitochondria (Fig. 7B,10). Hence, it is reasonable to assume that the majority of dendrites on which dark terminals synapsed but whose cell bodies were not visible in section also belonged to healthy interneurons.

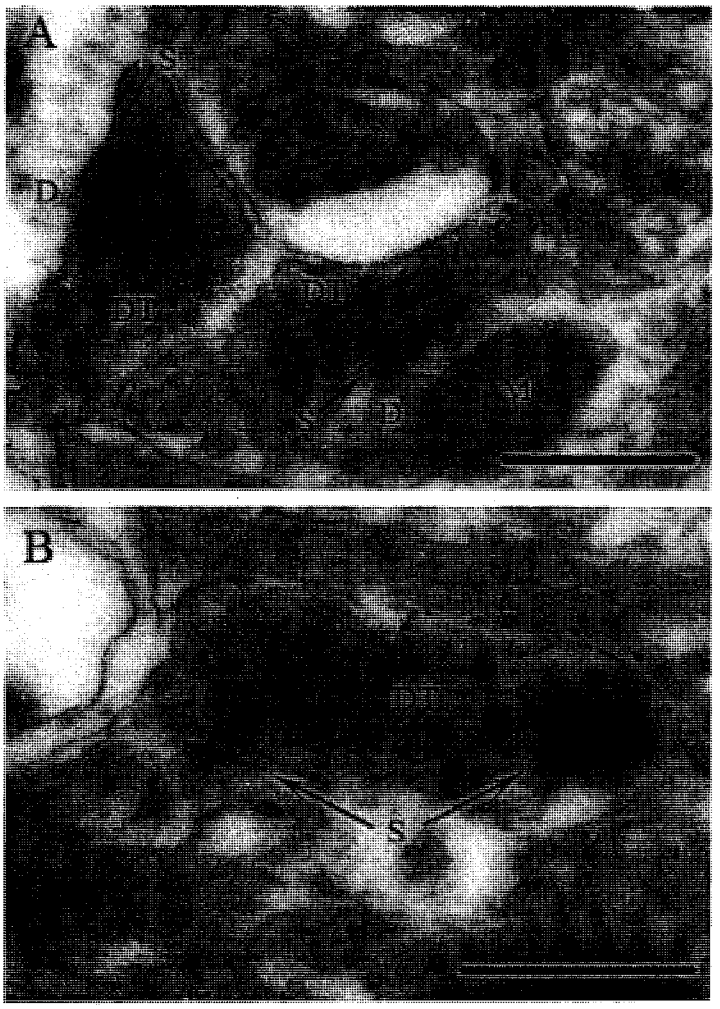


Fig. 8. Dark terminals synapsing on dendrites at 7 days post-MPTP.

A. Dark terminal (DT) with densely packed vesicles. The terminal is synapsing (S) on a dendrite (D) with darkly staining post-synaptic density. M, mitochondrion. B. Dark terminal with a perforated synapse (S) composed of deeply staining post- and pre-synaptic densities. Scale bar = 0.5 μm.

21-Day Axonal Fields

There was a dramatic increase in fields of healthy axons at the 21-day stage (Fig. 11). Evident was a jigsaw puzzle pattern of abutted axons with minimal extracellular space. The myelin sheath of the axons appeared to be tightly wound, although with slight disruptions attributable to imperfect fixation. Fields of more narrow, unmyelinated axons were also most abundant in the 21-day samples.

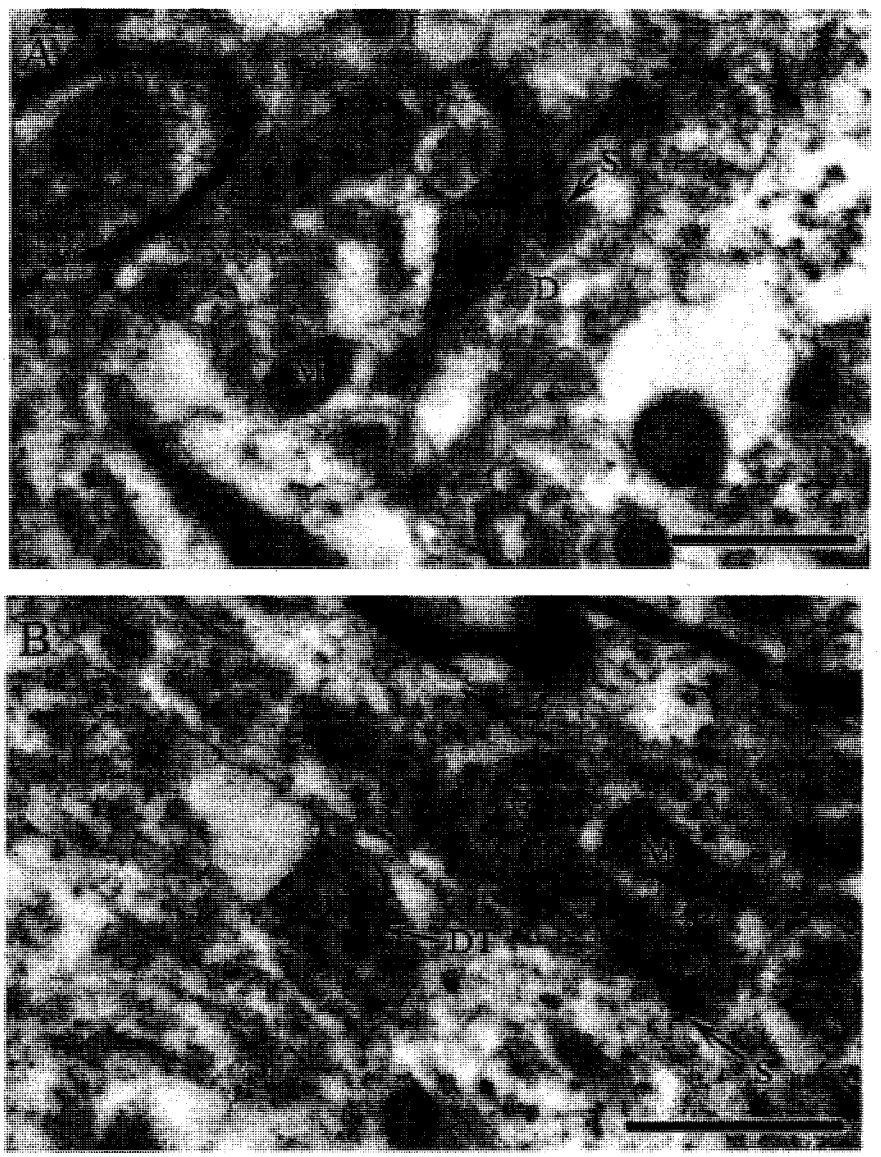


Fig. 9. Dark terminals synapsing on dendrites at 21 days post-MPTP

A. Dark terminal (DT) with densely packed vesicles synapsing (S) on the dendrite at D. M, mitochondrion. B. Dark *en passant* terminals with densely packed vesicles (V) an d healthy mitochondrion (M). The synapse (S) of the uppermost terminal is clearly perforated. Scale bar = 1 μm.

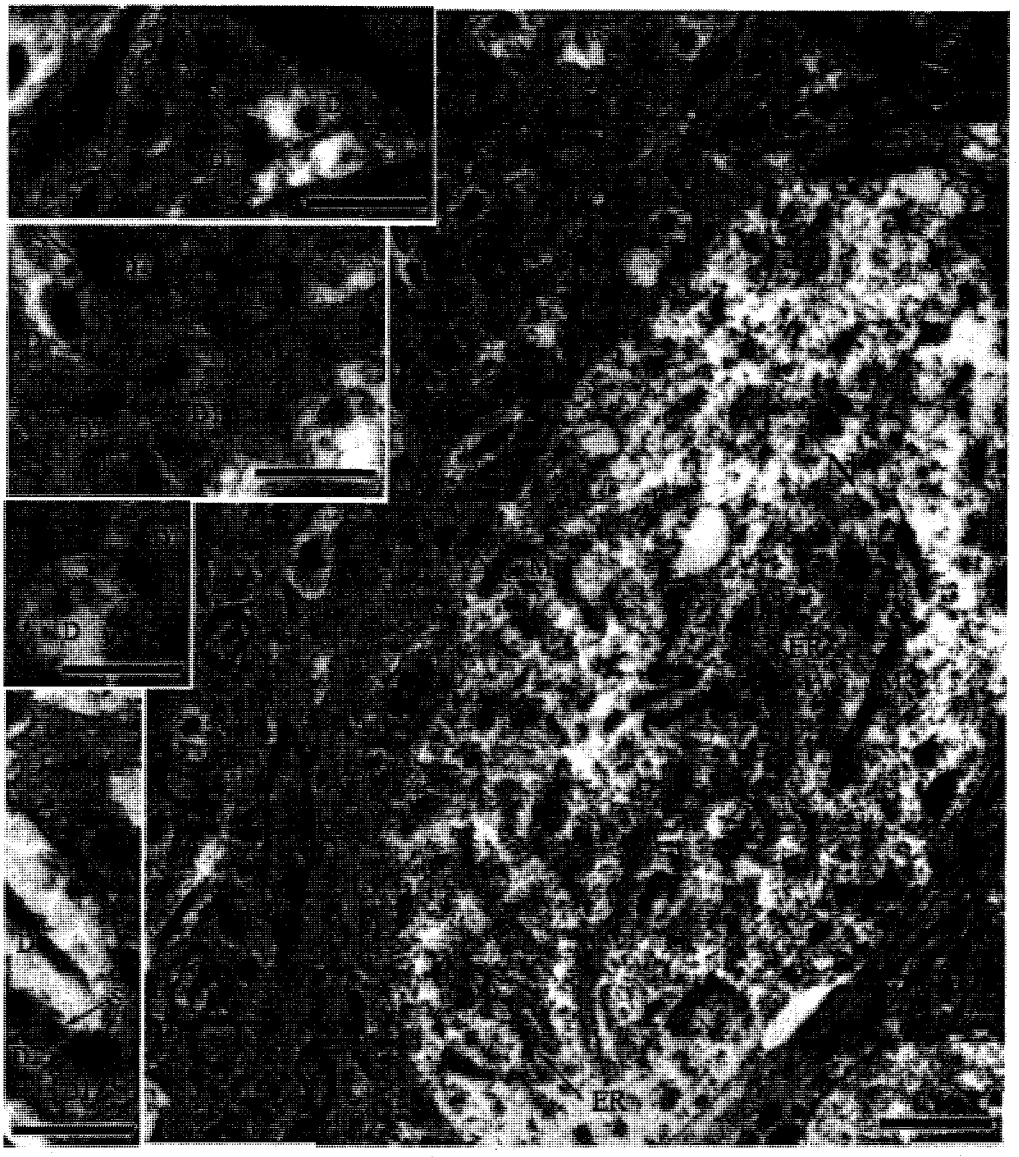


Fig. 10. Dark terminals synapsing on interneuron at 7 days post-MPTP.

Healthy interneuronal cell body (N) with ER and mitochondria (M) throughout the cell. A high number of dark terminals (DT) are synapsing (S) onto dendrites (D) in close proximity to the cell body of the interneuron. Insets are of higher magnification views of portions of the montaged image on the right. Scale bar = 2 μm in large image and 1 μm in insets.

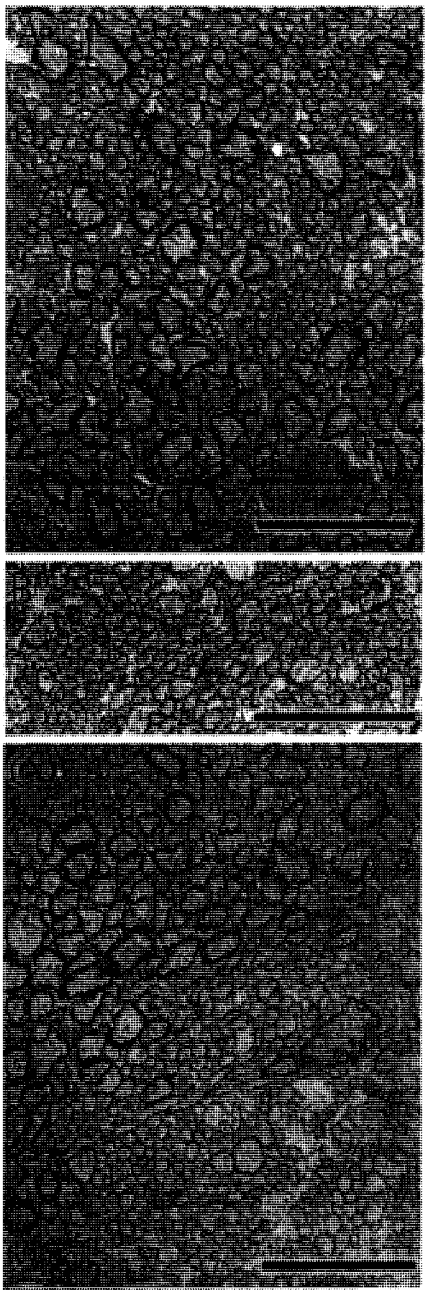


Fig. 11. Axon fields at 21 days post-MPTP.

A-E. Fields of healthy axons with minimal clearing. Relative to other stages, there is an increase in the number of small diameter fibers. Scale bar = 10 μm.

DISCUSSION

Damage Induced by MPTP

Axonal degeneration was evident at all stages of the time course in the form of myelin separation, demyelination, localized cytoplasmic shrinkage, mitochondrial disruption, or microtubule and neurofilament disturbance. The MPTP-induced striatal degeneration observed in response to MPTP insult was comparable to that reported in other ultrastructural studies (Forno et al., 1994; Lewandowska et al., 1999; Rapisardi et al., 1990). The changes observed 3 days post-MPTP injection were similar to those at the 1-day post-injection stage observed in our earlier study (Cochiolo et al., 2000). By 7 days, more extensive degeneration occurred, including a greater frequency of mitochondrial damage, of axonal ghosts, and of degenerative interneurons. Our findings at the 7-day stage correlate with the progressive disappearance of TH-positive fibers in the striatum, which Sundstrom et al. (1987, 1988) found to reach maximal levels 7 days after treatment. Although areas of damage and clearing can be located at 21-days post-injection, the striatal ultrastructure appears to be much healthier than that observed at 3 and 7 days.

Our finding of maximum mitochondrial degeneration at 7 days is in accord with studies in which mitochondrial dysfunction, particularly of complex I of the electron transport chain, has been implicated to contribute to the pathological progression of PD (Shults, 2004). MPTP has been shown to accumulate within the mitochondria as MPP^+

(Ramsay et al., 1986), which, through its interaction with complex I, causes a reduction in mouse striatal and midbrain ATP levels (Chan et al., 1991). This reduction in conjunction with the increased generation of reactive oxygen species (Cleeter et al., 1992; Hasegawa et al., 1990; Rossetti et al., 1988) most likely results in the ultrastructural aberrations that befall mitochondria and the rest of the cell. The existence in the same thin section of both unaltered mitochondria in healthy cells and comprised mitochondria in damaged cells indicated that the damage observed was not a fixation artifact.

Dark Terminals

In our previous study, we concluded that the abundant and densely vesiculated boutons, called dark degenerative boutons by others (Linder, 1987, 1995), were associated with large, healthy axons and, therefore, were unlikely to be degenerative (Cochiolo et al., 2000). The association of the terminals with healthy axons and the increased number and packing of vesicles 1 day post-MPTP injection led us to deduce that these surviving terminals increased their dopaminergic output to compensate for the overall loss of neurons. It could be argued that these darkened vesiculated terminals were an initial step in a degenerative process leading to darkened boutons lacking vesicles, as reported by Linder et al. (1987, 1995). However, the "condensed" terminals at the 1-day post-injection stage reported by Adams et al. (1989) contained dense and packed vesicles, similar to our findings at that stage (Cochiolo et al, 2000). Similarly, darkened vesiculated terminals persisted until 21 days in our present time course, failing to reveal

any vesicle elimination. Throughout the time course, these terminals were associated with healthy axons and synapsed on healthy dendrites, supporting the conclusion that they are not degenerative. We saw not a single case in which dark terminals synapsed on unhealthy neurons. Instead they appeared to be part of a compensatory increase in signaling efficacy responsible for the initial recovery of the mouse from MPTP toxicity.

If the dark terminals were indicators of neuronal degeneration, one would expect the time course to reveal an increase in dark terminals associated with degenerative axons Instead, we found in this and our previous study (Cochiolo et al., 2000) that the axons in continuity with the dark terminals were undamaged. Moreover, one would also expect rapid elimination of dark terminals as cells died and were cleared away. The persistence of dark terminals in the midst of striatal degeneration and clearing argues against the degeneration alternative. Cells clearly had died leaving substantial spaces, presumably as a result of reabsorption of cellular contents by microglia, while dark terminals remained intact. We, therefore, continue to maintain that the darkening processes are agents of recovery rather than of degeneration.

Early and Late Compensatory Mechanisms

We hypothesized that neurons spared from MPTP-induced death increased dopamine levels high enough to maintain signaling. Within the first 2-3 days post-injection, mice exhibit clear behavioral deficits; thereafter, the deficits diminish and the animals regain normal behavior (Hallman et al., 1985; Reinhard et al., 1988; Ricaurte et al., 1986;

Sundstrom et al., 1990). Ultrastructural abnormalities, however, persisted in our mice for three weeks, while evidence of sprouting or regeneration was not observed until this point. This distinction suggests that both acute and longer term compensatory mechanisms are involved in the recovery process.

There is strong evidence that after neurotoxin lesioning of the mouse substantia nigra *pars* compacta (SNc), dopaminergic neurons from the SNc or other regions of the brain sprout to reinnervate the dorsal striatum (Anglade et al., 1996; Blanchard et al., 1995, 1996; Finkelstein et al., 2000; Ho and Blum, 1998; Ingham et al., 1996, 1998; Kerns et al., 1992; Leonard et al., 1993; Mitsumoto et al., 1998; Parish et al., 2001; Pickel et al., 1992; Thomas et al., 1994; Wong et al., 1997). In approximately 4 months, most of the dopaminergic terminals in the striatum were found to be newly formed (Finkelstein et al., 2000; Stanic et al., 2003) and to bear features, including amassing synaptic vesicles, that signified a greater ability to produce, store, and release dopamine in comparison to the predeath capacity of the terminals that did not survive lesioning (Stanic et al., 2003). Regeneration became apparent in the striatum by the identification of hypertrophic dopamine transporter-immunoreactive terminals about 30 days after lesioning (Stanic et al., 2002, 2003), and it continued for up to 7 months (Blanchard et al., 1996). Because C57 mice recover behaviorally within seven days (Donnan et al., 1987; Tillerson and Miller, 2003; Willis and Donnan, 1987) and increase striatal dopamine levels significantly within the first 30 days (Hallman et al., 1985; Reinhard et al., 1988; Ricaurte et al., 1986; Sundstrom et al., 1990), some other mechanism must exist to allow survival following the loss of dopaminergic neurons before the reinnervation period. Likely

candidates, as reported here, are the formation of new *en passant* terminals and the elevation in dopamine production by existing terminals, as indicated by heightened synaptic vesicle density.

Nigrostriatal synaptic terminals most commonly form contacts with dendritic spines and shafts (Anglade et al., 1996; Descarries et al., 1996; Freund et al., 1984; Groves et al., 1994; Hanley and Bolam, 1997; Ingham et al., 1998; Zahm, 1992). Of the new contacts made following lesioning, the majority are proximal to the cell body (Ingham et al., 1996, 1998). The dark terminals in our investigation also synapsed proximal to interneuronal cell bodies. More proximal synapses are thought to have a more substantial impact in target neurons than distal synapses (Pickel et al., 1992). Hence, not only are the dark terminals apparently increasing dopamine release, their proximal location enhances the efficiency of signaling.

It is our conclusion that the dark vesiculated terminals contribute to mouse recovery from MPTP by increasing the efficacy of dopamine signal transmission in the spared neurons. Increased functional capacity of the synapse due to an increase in synaptic vesicles in boutons has been observed in other studies (Lnenicka et al., 1991; Pierce and Lewin, 1994; Sasaki and Iwata, 1996). Other researchers have found that spared dopaminergic neurons of the SNc did not increase their firing rate or pattern of firing (Hollerman and Grace, 1990; Pucak and Grace 1991). Hence, the increase in dopamine production by spared cells is due to an increase in the net amount of dopamine released in response to each action potential (Stachowiak et al., 1987; Synder et al. 1990). Evidence for a functional significance of increased dopamine output comes from studies

elucidating the ability of dopaminergic neurons to inhibit cholinergic interneurons after insult to the dopaminergic pathway (Grewaal et al., 1974; Guyenet et al., 1975; MacKenzie et al., 1989).

Our ultrastructural results as well as the immunocytochemistry results of others (Jackson-Lewis et al., 1995) support the role of increased synaptic output in surviving neurons as an early compensatory response. At 7 days post-injection, when recovery in animal behavior has been reported (Rozas et al., 1998) in spite of significant depletion of dopamine (70-90%) (Rozas et al., 1998; Sundstrom et al., 1988), we observed an increase in the frequency of dark terminals. This set of results is consistent with our hypothesis that the increased frequency of dark terminals aids the animal in its recovery despite significant ultrastructural deterioration.

Our results indicating a lack of sprouting at 7 days correlate with studies reporting sprouting to occur between 14 days (Ho and Blum. 1998) and 210 days post-injection (Bezard et al., 2000), making it unlikely for sprouting to play a role in the acute phase of recovery. At the 7-day post-injection point, when sprouting was absent and the degenerative effects of MPTP were most apparent in this and other studies (Sundstrom et al., 1988), dark terminals were most frequent and associated with healthy axons. From these results, it is logical to infer that the dark terminals act to stabilize the intact circuitry until sprouting can occur. The notion that dark terminals are agents of the compensatory response that allows the animal to survive the MPTP challenge until sprouting can occur is further supported by studies reporting no change in TH-positive cell bodies in the SNc

and statistically significant changes in TH-positive fiber densities, and thus the number of axons in the striatum, only at 24 days post-injection (Mitsumoto et al., 1998).

It is likely that the increase in synaptic output guides the synaptic remodeling that later occurs (Meredith et al., 2000; Meshul and Allen 2000; Meshul and Tan 1994; Stantic et al., 2003). A marked decrease in the volume of cleared regions, which occurred at the 21-day stage, is most readily explained by sprouting and/or regeneration. Sprouting and regeneration are likely to play a prominent role in longer term compensation, whereas the increase in dopamine output per cell by spared (or newly formed) boutons plays a role in early stage compensatory responses. This model of recovery is based on the decline of dark terminal frequency at 21 days accompanied by a conspicuous increase in healthy fields of axons as well as by the well documented increase in overall tissue dopamine levels at this time (Hallman et al., 1985; Reinhard et al., 1988; Ricaurte et al., 1986; Sundstrom et al., 1990).

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