MPTP-induced increase in c-Fos- and c-Jun-like immunoreactivity in the monkey cerebellum

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The transcription factors c-Fos and c-Jun have been described to be overexpressed following many pathological stimuli, but whether they are required for neurodegeneration or neuroprotection is still open. In the present report, we analyzed the role of c-Fos and c-Jun proteins in Purkinje cell degeneration caused by the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in the monkey cerebellum, and determined the neuroprotective effect of the antioxidant drug a-dihydroergocryptine (DHEC), whose prior and simultaneous administration reduced the MPTP-induced neuronal loss in the substantia nigra. Immunocytochemistry for c-Fos- and c-Jun-like proteins showed persistent increased staining in Purkinje cells of MPTP-treated monkeys. The staining was greatly reduced in animals receiving DHEC. Similar results were observed in white matter glial cells after immunoreaction for c-Fos. The results suggest that, at least as far as the cerebellum is concerned, the increase in c-Fos and c-Jun expression correlate with cell damage, rather than with preservation.

Key words: immediate early genes, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP, apoptosis, cerebellum.

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MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a neurotoxin whose administration to non-human primates and rodents replicates most of the behavioral and clinical symptomatology of Parkinson's disease, as well as the main biochemical and pathological hallmarks of the disease (Burns *et al.*,1983; Langston *et al.* 1984; Burns *et al.*, 1986; Crossmann *et al.*, 1987; Linder *et al.*, 1987; Ricaurte *et al.*, 1987; Mori *et al.*, 1988; Beale *et al.*, 1990; Date *et al.*,1990). Although the mechanism of cytotoxicity is not fully understood, it is known that MPTP is converted to the toxic metabolite 1-methyl-1,4-phenylpyridinium ion (MPP^+) in a reaction catalyzed by monoamine oxidase B (Singer *et al.*, 1986; Trevor *et al.*, 1986; Kinemuchi *et al.*, 1987). MPP^+ is selectively taken up by dopaminergic cells through an active transporter that is normally involved in dopamine reuptake, and accumulates in neurons of the substantia nigra (Javitch *et al.*, 1985). Inside cells, MPP+ inhibits mitochondrial complex I activity of the electron transport chain leading to impaired ATP production (Singer and Ramsay, 1990; Wu *et al.*, 1990; Ali *et al.*, 1994), formation of superoxide radicals (Gass *et al.*, 1993) and release of cytochrome c from mitochondria (Cassarino and Bennet, 1999). Besides the substantia nigra, MPTP induces neuronal death in other catecholaminergic nuclei as well, such as the locus coeruleus, the ventral tegmental area and nucleus A13 (Crossman *et al.*, 1987; Seniuk *et al.*, 1990). On the contrary, damage to the cerebellum, where the dopaminergic pathway is poorly represented (Ross *et al.*, 1990), has been only sporadically reported (Takada *et al.*, 1988; Vignola *et al.*, 1994). The degeneration of cerebellar neurons, which can account for some locomotor symptoms of Parkinson's disease, may be secondary to the cell loss in the locus coeruleus and substantia nigra, since it is known that the locus coeruleus sends catecholaminergic fibers to the Purkinje cells (Ross *et al.*, 1990), and Purkinje cells

project to the substantia nigra via cerebellar nuclei (Snider *et al.*, 1976; Chan-Palay, 1977; Snider and Snider, 1977; Nieoullon *et al.*, 1978).

Apoptotic cell death has been found in the substantia nigra of Parkinsonian patients (Mochizuki *et al.*, 1997) and shown to be caused by MPTP in a variety of cell lines (Desole *et al.*, 1997; Sheehan *et al.*, 1997) and in mouse brain (Tatton and Kish, 1997).

Expression of the immediate early response genes c-fos and c-jun and their proteins c-Fos and c-Jun has been described in different brain regions following many pathological stimuli such as seizure, hypoxia, stress (Walker and Carlock, 1993; Kaminska *et al.*, 1994; Dragunow and Preston, 1995) or cytotoxic agents, including MPTP (Hashimoto *et al.*, 1997; Kaina *et al.*, 1997; Nishi, 1997; Araki *et al.*, 1998). In particular, increased immunocytochemical expression of *c*-Jun has been found in the substantia nigra after MPTP treatment in mice (Nishi, 1997). In addition, after chronic treatment, a *c*-Fos-like protein family termed chronic Fos-related antigens (FRAs) has been described to be responsible for the cellular response by means of a persisting expression lasting even for several months (Chen *et al.*, 1997). C-Fos and c-Jun are transcription factors that form heterodimers or homodimers to regulate gene expression and are thought to be involved in the process of apoptosis (Jenkins *et al.*, 1993; Estus *et al.*, 1994; Dragunow and Preston, 1995; Pruschy *et al.*, 1995). On the other hand, whether they are required for neurodegeneration or neuroprotection is still a matter of debate.

To address to this question, we have undertaken a study of the *c*-Fos and the Fos-like protein family (including Fos related antigens 1 and 2; i.e. Fra1 and Fra2) and c-Jun protein expression in the cerebellum of adult monkeys after MPTP treatment. To this aim, we considered the neuroprotective effect on the cerebellum by prior and simultaneous administration of α-dihydroergocryptine (DHEC). DHEC is an ergot alkaloid with dopaminomimetic (Poli *et al.*, 1986; Fiore *et al.*, 1987) and antioxidant activities (Benzi *et al.*, 1988; 1989); and we previously demonstrated reduced neuronal loss in the pars compacta of the substantia nigra of MPTP-treated monkeys given DHEC (Bernocchi *et al.*, 1993).

Materials and Methods

Animal experiments were carried out at RBM Laboratories (Ivrea, Italy). The Institute is fully authorized by the Veterinary Health Authorities. Experiments were conducted in accordance with the fundamental principles of the ethical code promulgated by the CIOMS (Recueil de Legislation Sanitaire, 36(2), 1985, Geneva), the provisions of the EEC Council (Directive 86/609) and the Italian law 116/92.

Treatment and tissue processing

Eleven male *Macaca fascicularis* (Charles River, U.K.) weighting 3.5-5.5 kg were purchased from Charles River Primates Corporation, Port Washington, NY, USA. The animals, caged singly in stainless steel cages, were kept in the following ambient conditions: 24±2°C temperature, about 12 air changes per hour, 60±20% relative humidity and artificial lighting in a 12 hour cycle. The animals were allocated at random to 3 groups: group 1 consisted of MPTP-treated monkeys (3 animals), group 2 MPTP plus DHEC-treated monkeys (4 animals), group 3 controls (4 animals). Group 1 was given 2 mL/kg deionized water orally, twice a day for 15 days and 0.3 mg/kg/day MPTP (as free base dissolved in saline) i.v. from the 5th to the 10th day (5 days of treatment). Group 2 was administered 6 mg/kg DHEC, orally, twice a day for 15 days and MPTP as in group 1. Control monkeys received vehicles alone.

The animals were bled to death through the femoral arteries under sodium pentobarbital anesthesia (50 mg/kg i.v.) 15 days after the beginning of the experiment; brains were immediately excised and the cerebella fixed by immersion in Bouin solution and embedded in Paraplast X-tra.

Ten-µm-thick sagittal sections of cerebellar vermis and paravermis were used for immunocytochemical detection of c-Fos and c-Jun proteins, glial fibrillary acid protein (GFAP) and in situ nick end labeling (TdT-mediated Fluorescein-dUTP nick end labeling, TUNEL) for revelation of apoptotic cells.

Immunohistochemistry

After removal of the paraffin, the sections were immersed in 10% methanol in phosphate-buffered saline (PBS) with 3% H_2O_2, pretreated with block-

ing serum and incubated with the primary polyclonal antibodies against c-Fos (2 µg/mL, SantaCruz Biotechnology, CA), c-Jun/AP-1 (2 µg/mL, SantaCruz Biotechnology, CA, USA) and GFAP (200 µg/mL, Sigma, MO, USA) overnight at room temperature. The sites of antigen-antibody reaction were revealed with biotin-conjugated goat anti-rabbit IgG and streptavidin conjugated with peroxidase (Histomark Kit, Kirkegaard & Perry Laboratories, ML, USA) according to the kit protocol. The immunostaining was visualized by using 0.05% 3,3′-diaminobenzidine tetrahydrocloride with 0.01% H_2O_2 in 0.05 M Tris/HCl buffer, pH 7.6, for 30 min. The c-Fos antibody is raised against the amino acids 128-152 of human c-Fos p62 and recognizes c-Fos, FosB and Fra1 and Fra2.

For the reaction control, some slides were reacted as above, with the omission of the primary antibodies. These slides showed a slight diffuse background only.

In situ nick end labeling

For the TUNEL method, the sections were pretreated with 20 µg/mL proteinase K in Tris/HCl for 10 min at room temperature. After endogenous peroxidase bleaching with 10% methanol in phosphate-buffered saline (PBS) with 3% H_2O_2 for 7 min, the DNA fragmentation was revealed by a fluorescein Apo-Direct Kit (Phoenix Flow Systems, San Diego, CA, USA), according to the manufacturer instructions. The fluorescein labeling was revealed by an antibody anti-fluorescein conjugated with peroxidase (Boehringher Mannheim, D) for 30 min at 37°C and diaminobenzidine as above.

Cell counting

Cell counting was performed by two separate investigators in sections which had been stained with hematoxylin and eosin, or stained for GFAP. For each animal, at least 10 sections were evaluated. To reduce potential subjective bias, slides were numbered randomly, without indication whether they belonged to MPTP, MPTP plus DHEC-treated or control monkeys. Images of the cerebellar cortex and white matter were recorded with an Olympus Camedia C-2000 Z digital camera operating on an Olympus BX50 microscope, and reversed on a Macintosh computer. The number of stained cells was determined on the digital photographs using an NIH Image program, set as follows: threshold limits in the LUT window between 100 and 240; minimal particle size 50 pixels. For Purkinje cells, the number was obtained by counting cells over 1,000 µm length of convolution. GFAP-positive cells were scored in areas of 1,000 μm^2. At least 10 fields per section were analyzed. In order to evaluate possible differences among animals of the same experimental group and among cerebellar lobules and areas, a multiple way analysis of variance was performed. Since differences were not significant, the estimations from each experimental group were pooled and compared by Student's *t*-test. Results are expressed as means ± standard deviation (SD).

Results

As we had previously reported (Vignola *et al.*, 1994), there was loss of Purkinje cells in the MPTP-treated monkeys. DHEC prevented the loss of Purkinje neurons (Figure 1). After GFAP immunoreaction, the packing density of GFAP-positive glial cells was found to be reduced in the white matter of MPTP-treated monkeys as compared with the control group. In the MPTP plus DHEC group, the density of GFAP-positive glial cells remained at the control levels, although the immunopositive cells were unevenly distributed, as indicated, too, by the higher SD (Figure 2).

After immunoreaction for c-Fos family proteins in the cerebellum of control monkeys, light reactivity was present in the cytoplasm of large neurons, i.e. Purkinje cells (Figure 3A) and neurons of cerebellar nuclei. In the white matter, glial cells were slightly stained (Figure 3B) and, since these cells were also immunopositive for GFAP (*not shown*), they could be identified as astrocytes. In the MPTP-treated monkeys, c-Fos-like immunoreactivity was greatly increased (Figure 3C). Staining was present not only in the cytoplasm, but also in the nucleus of all large neurons (Figure 3C, inset). Astrocytes of the white matter also appeared strongly positive (Figure 3D). DHEC treatment reduced the immunostaining: most Purkinje cells showed immunoreactivity similar to that of control monkeys (Figures 3E, F) and only a few had strongly stained cytoplasm and nucleus. In the white matter, cells were similar to those of controls (Figure 3F).

Immunoreaction for c-Jun slightly labeled the cytoplasm of a few Purkinje cells of control animals (Figure 4A). On the contrary, in MPTP-treated monkeys, all Purkinje cells were stained (Figure

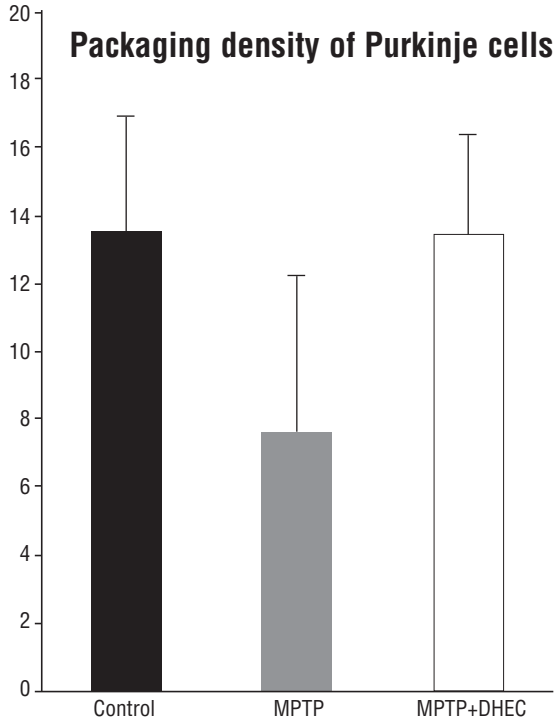

Figure 1. Number of Purkinje cells per 1,000 μm length of convolution. Bars are the means ± SD of 10 sections per animal in which cells were counted according to the procedures indicated in the Materials and Methods section. Differences between controls and MPTP-treated animals and between MPTP plus DHEC and MPTP-treated animals are highly significant ($p < 0.001$) as determined by multiple way analysis of variance and Student's *t*-test.

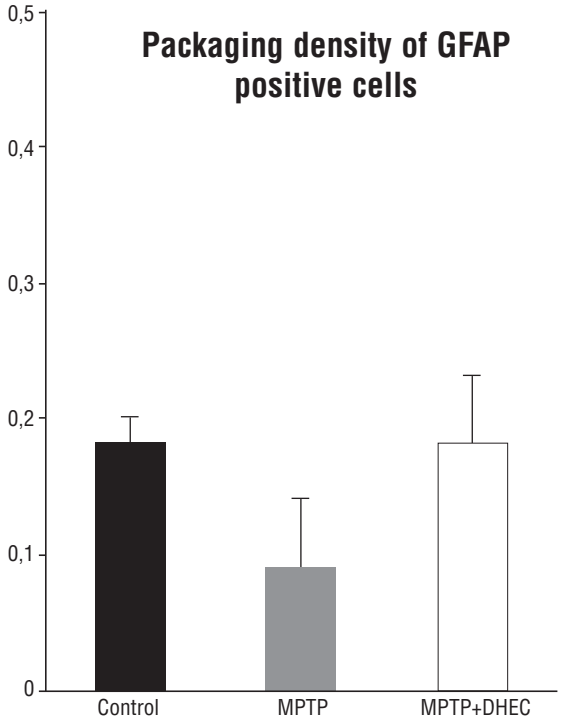

Figure 2. Number of GFAP immunoreactive cells per 1,000 μm^2 area of white matter. Bars are the means ± SD of 10 sections per animal in which cells were counted according to the procedures indicated in the Materials and Methods section. Differences between controls and MPTP-treated animals and between MPTP plus DHEC and MPTP treated animals are highly significant ($p < 0.001$)) as determined by multiple way analysis of variance and Student's *t*-test.

4B). The intensity of the reaction was greatly increased and immunoprecipitates were present also in the nucleus of several Purkinje cells (Figure 4B, inset). In MPTP plus DHEC-treated animals all Purkinje cells were labeled in their cytoplasm with different intensity (Figure 4C). Nuclei were stained.

After the TUNEL method for detection of apoptotic cells, no labeling was present in control monkeys. In MPTP-treated animals, several cells of medium size were labeled in the granule cell (Figure 5) and molecular layers. No apoptotic cells were found in MPTP plus DHEC-treated animals (*data not shown*).

Discussion

In the cerebellum of MPTP-treated monkeys, *in situ* nick end labeling revealed the presence of medium sized apoptotic cells located immediately beneath the Purkinje cell layer. On the contrary, Purkinje cells were unlabeled, though there was a loss of Purkinje cells after MPTP treatment. Probably, damage and loss of Purkinje cells occurred early following MPTP treatment and dead cells were immediately phagocytosed by scavenger glial cells. In the immature cerebellum of rats administered with the cytostatic drug cis-dichlorodiammineplatinum at 10 days of life, apoptotic cells were seen engulfed by glial cells as soon as 4h after the treatment (Scherini *et al.*, 1987; Biggiogera *et al.*, 1990). MPTP-treated monkeys were killed 5

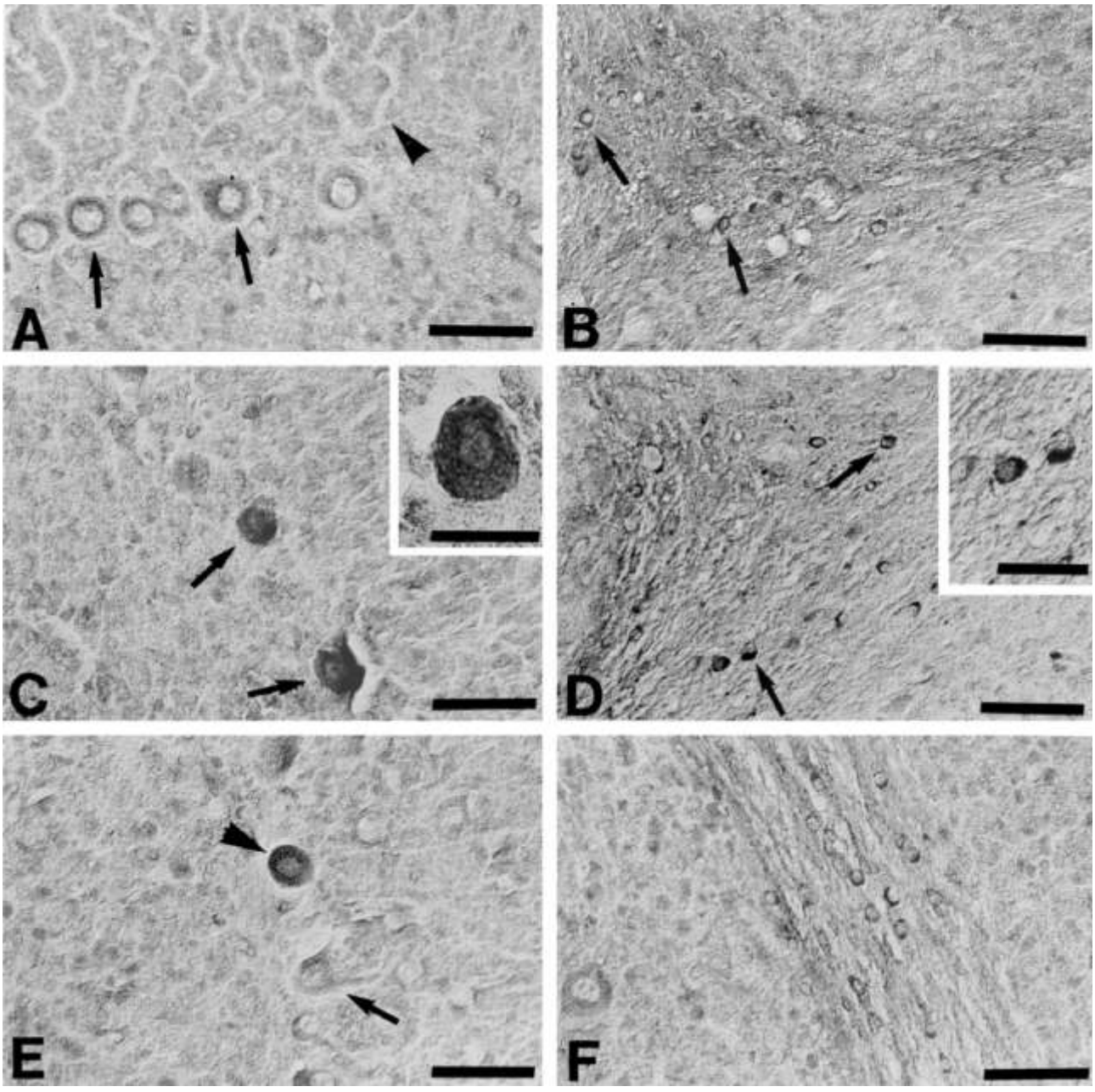

Figure 3. Immunoreactivity for c-Fos family proteins in the monkey cerebellum. A) Controls, cerebellar cortex. Faint staining is visible in Purkinje cell cytoplasm (arrows) around the unstained nucleus. The periphery of the perikaryon and dendritic trees appear negative (arrowheads). Scale bar: 50 μm; B) Controls, white matter. Some medium sized cells (astrocytes) have stained cytoplasm (arrows). Scale bar: 50 μm; C) MPTP-treated animals, cerebellar cortex. Purkinje cells have strongly stained cytoplasm (arrows). Scale bar: 50 μm. Inset: in most Purkinje cells also the nucleus is immunoreactive. Scale bar: 25 μm; D) MPTP-treated animals, white matter. Astrocytes show more intense cytoplasmic staining (arrows) in comparison with controls. Scale bar: 50 μm. Inset: a particular showing two intensely labeled astrocytes. Scale bar: 25 μm; E) MPTP plus DHEC treated animals, cerebellar cortex. The immunoreactivity of Purkinje cell cytoplasm ranges from that of controls (arrows) to that of MPTP alone treated animals (arrowhead). Scale bar: 50 μm; F) MPTP plus DHEC treated animals, white matter. The staining pattern is similar to that of controls. Scale bar: 50 μm.

days after the end of the treatment and 10 days after its beginning.

Strong immunoreactivity for both c-Fos family and c-Jun proteins was observed in Purkinje cells and large neurons of cerebellar nuclei of MPTP-treated monkeys. The immunoreactivity was present in both the cell cytoplasm and nucleus. C-Fos and c-Jun can dimerize and bind to the activator protein (AP-1) DNA consensus site, which is essential for both a basal and stimulated transcription of a series of genes (Sassone-Corsi *et al.*, 1988; Sonnenberg *et al.*, 1989) and has been implicated

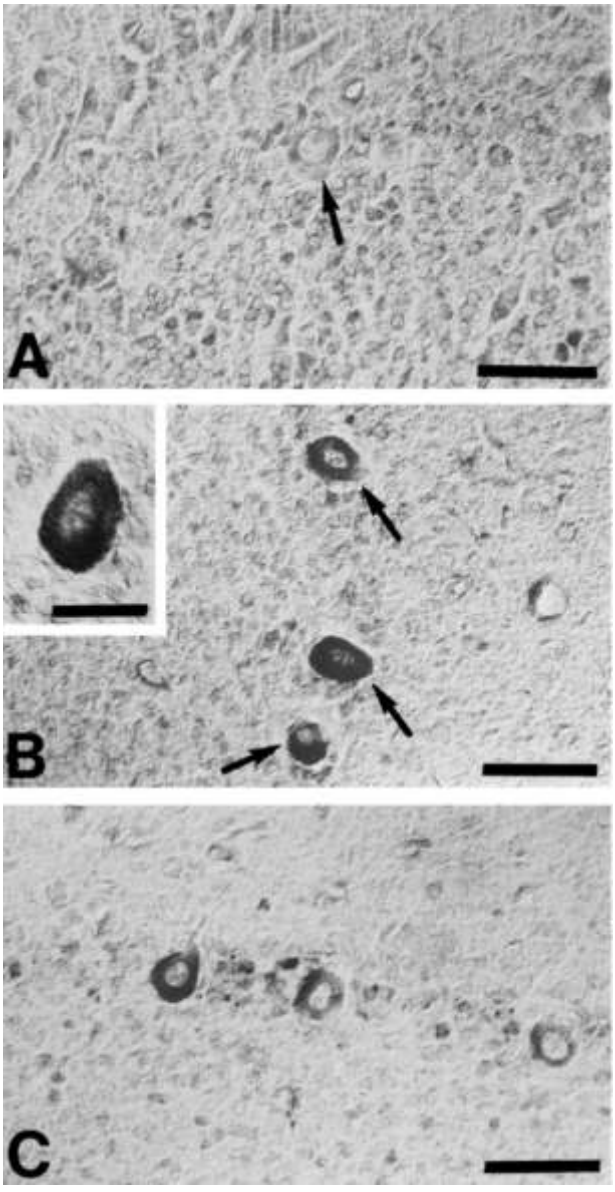

Figure 4. Immunoreactivity for c-Jun protein in the monkey cerebellum. A) Controls. Purkinje cells have unstained or slightly stained cytoplasm (arrow). Scale bar: 50 µm; B) MPTP-treated animals. Purkinje cells have intensely stained cytoplasms (arrows). Scale bar: 50 µm. Inset: some Purkinje cells show immunoreactivity in the nucleus. Scale bar: 25 µm; C) MPTP plus DHEC-treated animals. The staining of the cytoplasm of most Purkinje cells shows different intensity. The nucleus is never stained. Scale bar: 50 µm.

in the process of cell death (Ferrer *et al.*, 1995). Induction of c-fos and c-jun mRNAs occurs in lymphoid cell lines following growth factor deprivation (Coppi, 1995) and in neurons deprived of NGF undergoing apoptosis (Estus *et al.*, 1994). Increased c-Jun and c-Fos expression has been found also in dopaminergic neurons early after MPTP treatment and has been interpreted as due to stress caused by administration of MPTP (Nishi, 1997). Late overexpression of the two proteins has been, on the contrary, regarded as related to axonal damage rather than to be due to the action of MPTP itself.

This seems to be the case here, too, since MPTP is considered to be a neurotoxin specific for dopaminergic neurons. The delayed expression of the proteins must occur, therefore, in relation with the partial deafferentiation of the cerebellum due to the degeneration of neurons in the locus coeruleus. This interpretation is also supported by changes previously observed in some cytoskeleton components of MPTP-treated monkey cerebellar neurons, which is reminiscent of those following deafferentiation (Vignola *et al.*, 1994).

In addition, the long-term presence of c-Fos-like immunoreactivity may be due to the labeling of Fra1 and Fra2 proteins by the antibody used. Fras are constitutively expressed in the cerebellum (Herdegen and Leah, 1998), in which they show cytoplasmic localization in Purkinje cells (Alcantara and Greenough, 1993). Their expression, however, may be enhanced in response to chronic perturbations, such as electroconvulsive seizures, psychotropic drug treatment and lesions (Chen *et al.*, 1997). Fras, once induced, are relatively stable proteins, persisting in the brain for periods lasting up to several months and have been hypothesized to play a role in synaptic remodeling (Chen *et al.*, 1997). Also the persisting presence of c-Jun has been related to an attempt of cells to regenerate (Hughes *et al.*, 1999). In fact, c-Jun expression persists till regeneration of axotomized neurons is completed (Dragunow and Preston, 1995). Therefore, the persistent presence of the protein in Purkinje cells of MPTP-treated monkeys might be due to circuitry remodeling, as observed in the cerebellum of mutant mice (Sotelo, 1982), or after cis-dichlorodiammineplatinum treatment of immature rats (Scherini and Bernocchi, 1994). On the other hand, this interpretation is contradicted by the results obtained in monkeys treated with MPTP and DHEC. In fact, the prior and contemporary treatment of MPTP-induced Parkinsonian monkeys with DHEC drastically reduced the damage to the cerebellum, since much more Purkinje cells were spared by the treatment and apoptotic cells were not present. DHEC, therefore, seems to

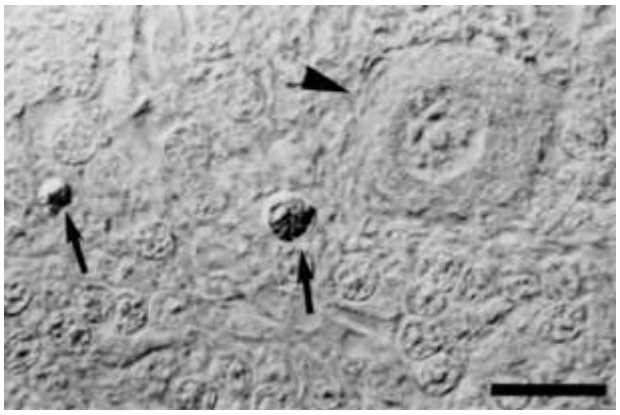

Figure 5. *In situ* nick end labeling (TUNEL) in the cerebellar cortex of MPTP treated monkeys. Two apoptotic nuclei (arrows) are visible in the granule cell layer, near Purkinje cells (arrowhead). Scale bar: 150 μm.

have neuroprotective effects against the action of MPTP in the cerebellum, as already observed by us in the substantia nigra pars compacta (Bernocchi *et al.*, 1993). For this reason, if c-Fos family and c-Jun proteins have a neuroprotective or regenerating role, an increase, rather than a decrease, in their immunocytochemical expression would be expected after DHEC administration.

In support of a role in neurodegeneration for the transcription factors, there is also the behavior of glial cells in the white matter. These resulted positive to c-Fos family protein immunocytochemistry. In comparison with controls, the stain was notably enhanced in MPTP-treated monkeys, but did not change when DHEC was given. In addition, the packing density of astrocytes, as revealed by GFAP immunocytochemistry, was reduced in MPTP-treated monkeys, while it increased in the animals treated with MPTP plus DHEC. Taking into account that astrogliosis may have a neuroprotective significance in injured areas (Aschner and Kimelberg, 1991; Martin, 1992; Norenberg, 1994), the increase in c-Fos immunoreactivity in glial cells, without any increase in their packing density seems to be correlated with cell death or disease.

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