Histol Histopathol (2012) 27: 89-94

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Nigral degeneration correlates with persistent activation of cerebellar Purkinje cells in MPTP-treated monkeys

Paola Heman[1,3], Carlos Barcia[1,2], Aurora Gómez[1,2], Carmen M. Ros[1,2], Francisco Ros-Bernal[1,2], José Enrique Yuste[1,2], Vicente de Pablos[1,2], Emiliano Fernandez-Villalba[1,2], María Rebeca Toledo-Cárdenas[3] and María Trinidad Herrero[1,2]

[1]Clinical and Experimental Neuroscience, [2]Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED), School of Medicine, Murcia, Spain and [3]Programa de Neurobiología, Instituto de Neuroetología, Universidad Veracruzana, Xalapa, Veracruz, México

Summary. In the present work we analyze the cerebellum of chronic parkinsonian monkeys in order to clarify whether chronic mesencephalic depletion is associated with long term activation of the cerebellar neurons in chronic Parkinsonism. In our study, we observed a persistent activation of Purkinje cells in the cerebellum of chronic parkinsonian macaques, characterized by the expression of c-Fos, which correlated with dopaminergic degeneration. These results are compatible with the results observed in fMRI in Parkinson's disease patients, and may contribute to the understanding of additional alterations in the brain circuitry in Parkinsonism.

Key words: Parkinson's disease, Cerebellum, MPTP, Purkinje cells, c-Fos

Introduction

Previous reports have shown increased activity of the cerebellum measured by fMRI in patients with Parkinson's disease (PD) (Rascol et al., 1997; Cerasa et al., 2006; Yu et al., 2007), and various studies performed in experimental models of PD have shown particular cerebellar alterations, which suggests that cerebellum activity may be related with dopaminergic neuronal loss and be implicated in parkinsonian symptoms. Previous studies in an MPTP-induced model in mice show neuronal degeneration in the cerebellum (Takada et al., 1993), characterized by the loss of Nissl-stained Purkinje cells and aggravated by the number of repeated MPTP injections. Other studies performed in monkeys have also reported the loss of calcium-binding positive (CB-D28k$^+$) Purkinje cells shortly after the MPTP insult (Vignola et al., 1994).

Previous studies analyzing the cerebellar alterations of parkinsonian monkeys and mice have also reported an increase in c-Fos expressing cells in the cerebellum of MPTP-treated animals but only showing acute effects of the systemic intoxication (Chen et al., 2001; Necchi et al., 2004). In fact, in both reports this increase in c-Fos was connected to apoptotic events occurring in the cerebellum shortly after the neurotoxin insult. However, it is necessary to ascertain whether this effect persists in a chronic model of Parkinsonism and whether it is correlated with dopaminergic neuronal loss. As previous findings in this matter have been based on acute models of Parkinsonism (Chen et al., 2001; Necchi et al., 2004), in the present work we have analyzed the brains of parkinsonian monkeys two years after the neurotoxin insult in order to elucidate whether the activation of Purkinje cells in the cerebellum may persist long-term, since it is known that chronic nigrostriatal denervation results in a decrease in the metabolic activity of thalamic neurons in the territories innervated by the cerebellum (Rolland et al., 2007). In this study, we quantified the number of Nissl stained Purkinje cells and the number of Calbindin D28k$^+$ Purkinje cells in the cerebellum using stereological methods. In addition, since it is not clear whether the loss of dopaminergic neurons correlates with alterations in the cerebellum, we performed a detailed analysis of the number of cerebellar Purkinje cells and their activity, as measured by c-Fos expression, and compared the findings with different levels of dopaminergic degeneration.

Offprint requests to: Dra. María-Trinidad Herrero, Clinical and Experimental Neuroscience (NiCE-CIBERNED), Department of Human Anatomy and Psychobiology, School of Medicine, Campus de Espinardo, University of Murcia, 30071 Murcia, Spain. e-mail: mtherrer@um.es

Material and methods

Stereological analysis

For the present study we used brain tissue from chronic parkinsonian macaques (*Macaca fascicularis*) which were studied previously in our Primate Unit (Barcia et al., 2004). All studies were carried out in accordance with the Helsinki Declaration, the International Primatological Society Guidelines and *the Guide for the Care and Use of Laboratory Animals* (National Research Council, NIH Guide revised 1996), and with the norms of the state members of the European Union (2003/65/CE), the Guidelines of the European Convention for the protection of Vertebrate Animals used for Experimental and other scientific purposes of the Council of Europe of 2006, and the European Communities Council Directive 2010/63/ECC. All efforts were made to minimize animal suffering and to reduce the number of animals used and to utilize alternatives to in vivo techniques. Six adult macaques (5 males and 1 female) were treated every other week during three months with low intravenous doses of MPTP (0.3 mg/kg) according to previous protocols (Herrero et al., 1993; Barcia et al., 2003). Each animal received a different number of doses according to the level of Parkinsonism and their susceptibility to MPTP (Fig. 1A). None of them received L-DOPA or dopaminergic agonists. Motor symptoms were assessed using a previously described rating scale (Herrero et al., 1993). The motor score of the animals used for this study ranged from 0 to 10 and their *postmortem* analysis revealed different degrees of dopaminergic depletion according to their motor score. The sacrifice, perfusion and fixation of the animals were performed as previously described (Barcia et al., 2011). Two years after the last MPTP dose animals were anaesthetized with an overdose with pentobarbital (100 mg/Kg) and transcardially perfused-fixed with 2-5 L of saline solution. Immediately afterwards, the animals were perfused with 4% paraformaldehyde to fix the tissue. Brains were removed and sectioned by microtome (Microm® HM400) into 40 μm thick sections. For the present experiment, we classified the animals according to the dopaminergic (DA) depletion of the SNpc: animals with preserved (PR) SNpc, those with moderate depletion (MD) (less than 50%) of DA neurons of the SNpc, and animals with severe depletion (SD) (more than 50%) of nigral DA neurons (Fig. 1A,B).

The fixed cerebellum was sectioned into sagittal serial sections while the SNpc was sectioned into coronal sections. Series of sections regularly spaced at intervals of 1,440 μm were stained with thionin. Adjacent sections were immunostained to visualize Calbinding D28k (CB D28k) (mouse monoclonal antibody 1:500; Chemicon, Temecula, CA, USA) in order to quantify the Purkinje cells in the MPTP-intoxicated monkeys, c-Fos (rabbit polyclonal antibody 1:500; Santa Cruz, CA, USA) to analyze the neuronal activation in the Purkinje cells of parkinsonian monkeys, and tyrosine hydroxylase (TH) (sheep polyclonal antibody 1:1000; Chemicon, Temecula, CA, USA) in order to quantify the neuronal loss in the SNpc of MPTP-intoxicated monkeys. Sections from all the animals were stained simultaneously and under the same experimental conditions as previously described (Barcia et al., 2004). Sections of the cerebellum and the SNpc (40 μm) were used for immunohistochemistry to detect specific cells. Cerebellum and SNpc were defined according to the Monkey Brain Atlas (Paxinos et al., 2000). Nissl stained or DAB labeled cells were quantified in serial sagittal sections from each animal. The number of cells was estimated with an optical fractionator probe using a computer assisted image analysis system (Image J, © 2009 Burger and Burge; ScionImage, Frederick, Maryland, USA) with a Zeiss Axioplan 2 microscope connected to a digital camera. The area of interest was traced using a 1.25x objective. The number of cells was measured in 200x200 μm dissectors covering the surface of the analyzed area. Labeled cells were counted using the 40x objective and counting frames throughout the delineated area of the SNpc in the right and left hemisphere and in the cerebellar gyrus in each section via the optical fractionator. Serial sections of the monkey cerebellum were used for quantification of the number of Purkinje cells in the Purkinje layer of the cerebellar cortex. Data were expressed as an absolute number of positive cells in each anatomical area analyzed considering both hemispheres. The results are expressed as the mean ± SEM. The volume of the cerebellum was measured using scanned images of the Nissl stained series of sections following Cavalieri's method (Gundersen, 1986) (Fig. 1C). All the quantifications were done blindly by a researcher (P.H.) who did not know the degree of Parkinsonism of the animals.

Statistical analysis

Data are expressed as mean ± SEM. Since groups of 2 animals would be insufficient to assess statistically significant data, statistical analysis was performed using a Pearson correlation. The null hypothesis was rejected for an α risk equal to 5%.

Results

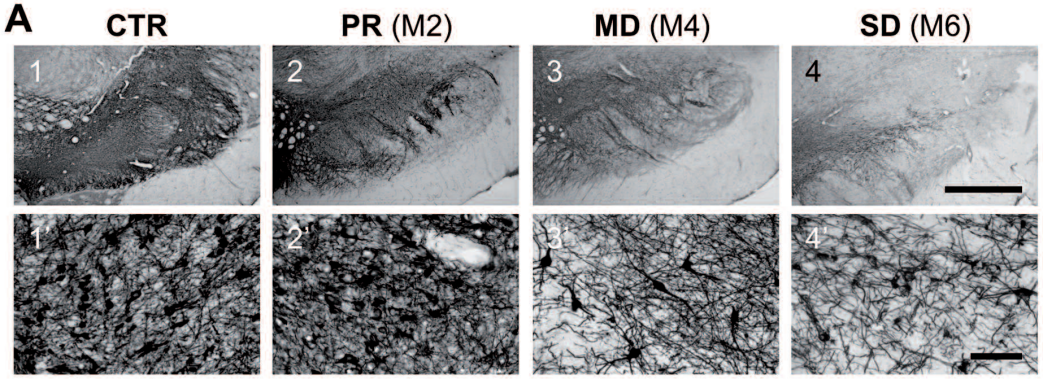
Cerebellar volume does not change with Parkinsonism in MPTP-treated monkeys

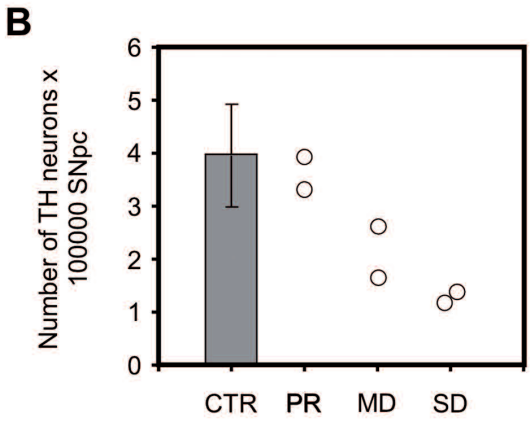
We analyzed animals with different degrees of dopaminergic neuronal loss: PR, MD and SD monkeys (Fig. 1A,B). In order to analyze changes in the cerebellum according to the dopaminergic cell loss, we first analyzed the volume of the cerebellum. Since the density of neurons could vary with changes of the total volume of the nucleus, the total volume of each cerebellum was measured and no apparent differences

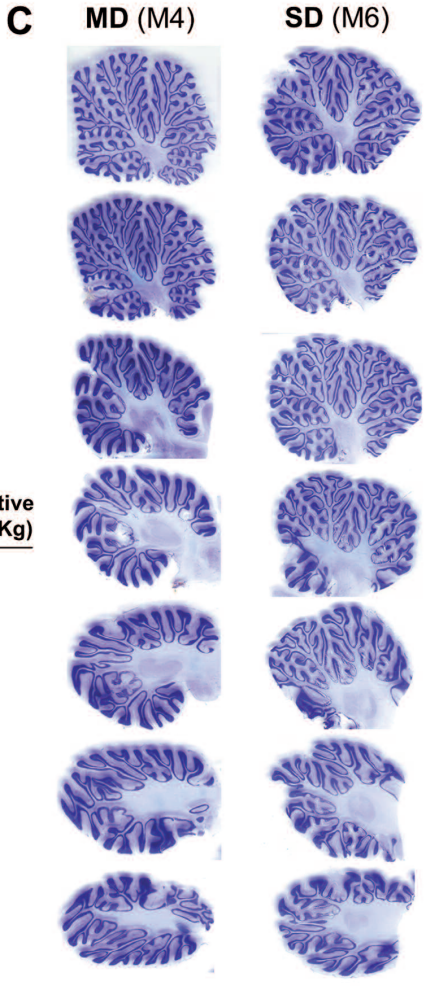
were detected according to the level of DA depletion (Pearson coefficient R^2= -0.3) (Fig. 1D).

MPTP –treated monkeys show no clear correlation between the loss of Purkinje cells and DA depletion

The number of thionin$^+$ Purkinje cells was quantified in the cerebellum with an optical dissector (optical fractionator) and compared with the degree of Parkinsonism (Fig. 2A top chart). Although a trend of correlation was seen, no significant differences were found between the loss of the number of thionin$^+$ Purkinje cells and the degree of dopaminergic neuronal loss in the SNpc (Fig. 2B top chart).







D

Animal	Sex	Degree of Parkinsonism	Cerebellar Volume (mm^3)	MPTP Accumulative dose (mg/Kg)
M1	M	PR	1848	1.8
M2	M	PR	2001	1.8
M3	M	MD	2460	1.8
M4	M	MD	2005	1.8
M5	F	SD	1853	0.9
M6	M	SD	2101	1.8

Fig. 1. Study of the levels of dopaminergic neuronal loss in the SNpc of MPTP-treated macaques with different degrees of Parkinsonism. **A.** Pictures of TH immunostaining of the SNpc of an intact monkey (CTR), a PR monkey (M2), an MD monkey (M4) and an SD monkey (M6). The top row of pictures were taken at low magnification (1-4) and the bottom row of pictures were taken at higher magnification. Scale bar in 4: 1 mm; scale bar in 4': 100 μm. **B.** Graph shows the different degree of dopaminergic cell loss in the SNpc of monkeys treated with MPTP. The histogram shows the average of the number of TH+ neurons of the SNpc of 4 intact monkeys in the same conditions (n=4). **C.** Volume of Monkey Cerebellum does not change with the different levels of DA depletion. Series of sections of the cerebellum were stained with thionin and the total volume was measured by Cavalieri's stereological method and compared between the animals. No major differences were observed between the groups. Cerebellar serial sections of two representative animals are shown. **D.** Inserted table shows the volumes of the cerebellum calculated by Cavalieri's method in all the studied monkeys. No volume differences were observed between the animals according to the DA depletion. PR, Preserved SNpc; MD, Moderately depleted SNpc; SD, Severely depleted SNpc.

Since previous reports mentioned a decrease in CB-D28k$^+$ Purkinje cells in MPTP-treated monkeys shortly after the neurotoxin insult (Vignola et al., 1994), we also analyzed these neurons in order to see whether this change was correlated with the degree of parkinsonism. For this, serial sections of the cerebellum were immunostained with an antibody against CB-D28k, and CB-D28k$^+$ Purkinje cells were quantified with the same stereological method used for Nissl-stained (thionin$^+$) Purkinje cells (Fig. 2A, middle row). We also analyzed the percentage of the total number of Purkinje cells, revealed by Nissl staining, which express CB D28k. In all the groups of animals only 40% of the total number of Purkinje cells was able to express CB D28k. Purkinje cells were clearly present in the cerebellar cortex but no differences were noted with respect to the severity of Parkinsonism.

Persistent c-Fos expression in the Purkinje cells in parkinsonian animals

Since Purkinje cells are the putative cell type to be overactivated in Parkinsonism, and their activation may persist after mesencephalic dopaminergic degeneration, serial sections of the cerebellum were stained with an antibody against c-Fos, a well-known protein expressed when neurons fire action potentials, and commonly used as an indirect marker of neuronal activity (Luckman et al., 1994; Fields et al., 1997). Immunostaining of c-Fos in serial sections of cerebellum revealed some well-

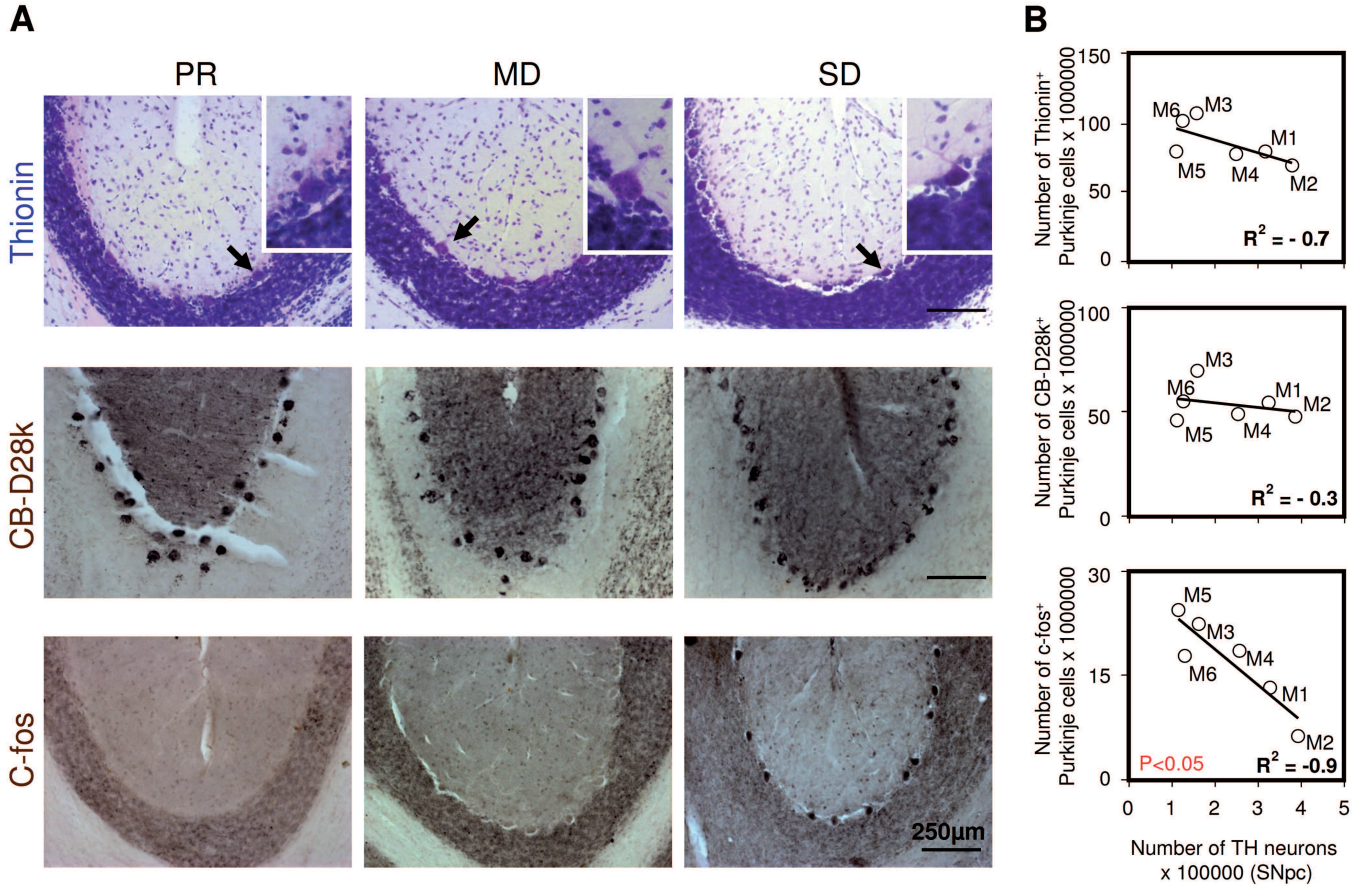


Fig. 2. The degree of persistent expression of c-Fos+ in Purkinje cells correlates with the degree of mesencephalic dopaminergic degeneration in parkinsonian monkeys. **A,** top row: Parkinsonian monkeys do not show clear correlation between thionin$^+$ Purkinje cell degeneration in cerebellum and mesencephalic DA depletion. **A.** Sections of the cerebellum were stained with thionin in PR, MD and SD monkeys. Inserts show pictures of higher magnification of thionin$^+$ Purkinje cells. Arrows indicate typical Purkinje cells magnified in the insert. The Purkinje cells were clearly observed between the granular and molecular layers in the cerebellar cortex (arrows) and quantified by stereological methods. **B,** top chart: There was no significant correlation between the number of Purkinje cells and the loss of dopaminergic neurons of the SNpc. **A,** middle row: The loss of Calbindin D28k expressing Purkinje cells does not correlate with the level of Parkinsonism. Sections of the cerebellum were immuno-stained for CB-D28k in monkeys PR, MD and SD. The Purkinje cells were clearly stained and quantified with stereological methods. **B,** middle chart: There was no significant correlation between the number of CB-D28k+ Purkinje cells and the loss of dopaminergic neurons of the SNpc. **A,** bottom row: The number of c-Fos expressing Purkinje cells strongly correlates with the degree of Parkinsonism. **A.** Sections of the cerebellar cortex were immunostained for c-Fos in monkeys with PR, MD and SD. The Purkinje cells were clearly stained and quantified with stereological methods. **B,** bottom chart: The correlation between the number of c-Fos$^+$ Purkinje cells and the loss of dopaminergic neurons of the SNpc revealed an increase of c-Fos in the animals with higher neuronal dopaminergic loss. The analysis revealed a very strong and statistically significant correlation between c-Fos expression in cerebellar cortex and the density of dopaminergic neurons in the SNpc.

stained c-Fos$^+$ Purkinje cells in the cerebellar cortex (Fig. 2A, bottom row). Using stereological methods we observed an increase in the number of c-Fos$^+$ Purkinje cells in the cerebellar cortex and the extent of the increase depended on the degree of mesencephalic dopaminergic depletion (Pearson coefficient R^2= -0.9, $p<0.05$) (Fig. 2B, bottom chart).

Discussion

In the present study, we performed a detailed histological analysis in brain slices of the cerebellum of chronic parkinsonian monkeys not treated with L-DOPA or with dopaminergic agonists. Purkinje cells presented a hyper-activation which correlated with the level of dopaminergic neuronal loss in the SNpc (Fig. 2), which may be crucial for the understanding of cerebellar alterations observed in Parkinsonism.

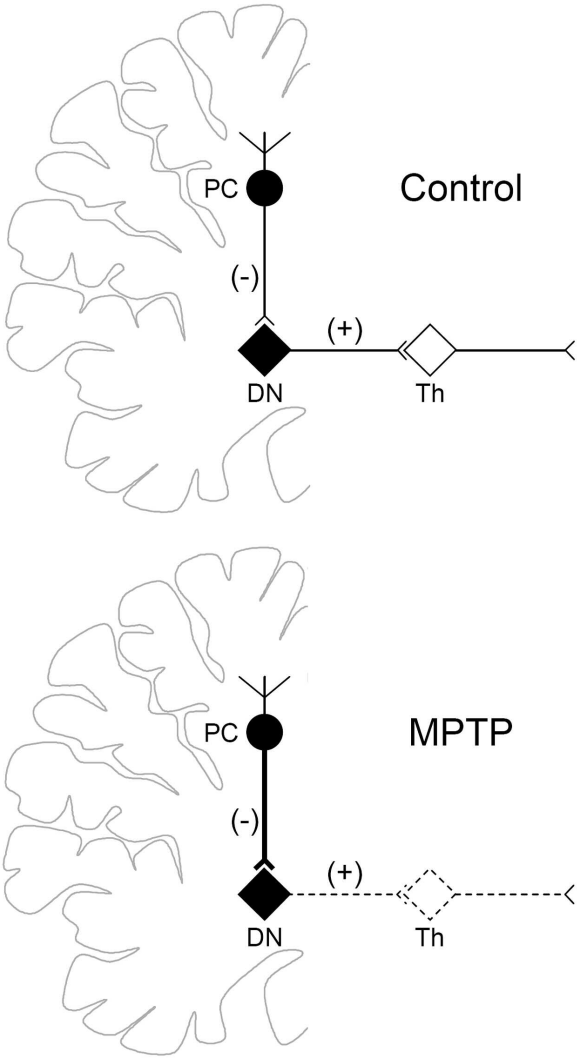


Fig. 3. Diagram of the putative alteration of the cerebellar circuit in Parkinsonism. In normal conditions Purkinje cells (PC) send inhibitory signals to the neurons of the nucleus dentatus (DN). The DN sends activation signals to the Thalamus (Th). As in Parkinsonism (MPTP model in non-human primates), PC appear hyper-activated, then they can highly inhibit the neurons of DN projecting to the Thalamus which may apply to a reduced activation over thalamic neurons.

Although not significant, we observed a certain correlation between the loss of the number of Nissl$^+$ Purkinje cells and the loss of dopaminergic neurons (Fig. 2). Probably a higher number of animals would be needed to reach statistical significance. Previous studies performed in MPTP-treated mice reported a loss of cerebellar Nissl-stained Purkinje cells that worsens with increasing doses of MPTP in mice (Takada et al., 1993; Vignola et al., 1994). Our data indicate that MPTP may have some effect on the cerebellum of animals with high degree of Parkinsonism but probably higher doses of MPTP may be needed to exert a massive Purkinje cell toxicity as occurs in mice.

A previous study performed in monkeys acutely treated with MPTP described degeneration of Purkinje cells, however they stained with calcium binding proteins (Vignola et al., 1994). Even known that calcium binding protein has been used as a classical marker for Purkinje cells in primates (Fortin et al., 1998), not all Purkinje cells are positive for calcium-binding protein. In our study only 40% of the Nissl-stained Purkinje cells were positive for CB-D28k. In fact, we do observed no evident correlation between CB-D28k$^+$ Purkinje cells and mesencephalic dopaminergic depletion. The results observed in Vignola's work may be due to the direct toxicity of the MPTP exerted over CB-D28k$^+$ Purkinje cells, although according to our results this phenomenon seems to be independent of mesencephalic dopaminergic degeneration.

We demonstrated that the cortex of the cerebellum presents hyper-activation of Purkinje cells, characterized by the persistent expression of c-Fos years after MPTP insult (Fig. 2A, bottom row). This activation of Purkinje cells in chronic parkinsonian monkeys certainly correlates with the level of neuronal degeneration of the SNpc (Fig. 2B, bottom chart), which may be due to a compensatory mechanism derived from the defective basal ganglia circuitry, as occurs in patients with PD (Rascol et al., 1997; Cerasa et al., 2006; Yu et al., 2007).

Previous reports have shown an increase of c-Fos in the cerebellum of mice and monkeys shortly after the MPTP insult (Chen et al., 2001; Necchi et al., 2004). The authors of both reports linked the increase in c-Fos with the induction of neuronal loss in the cerebellum. The work of Necchi and coworkers (2004) points to an increase in c-Fos in some Purkinje cells of the cerebellar cortex but also in astrocytes located in the white matter, which may be related with the immediate and general effect of the toxin. In contrast, in our study, Purkinje cells remained over-activated over a period of two years, probably caused by persistent dopaminergic mesencephalic loss. These results are compatible with previous results which show the hypoactivity of the cerebellar-thalamic pathways, which might therefore be implicated in the development of parkinsonian

symptoms (Rolland et al., 2007) (Fig. 3), as well as with observations made with fMRI in patients with Parkinson's disease (PD) (Yu et al., 2007). So, we suggest that the loss of dopaminergic neurons may have an effect on the activity of cerebellar neurons and have implications in the clinical syndrome and in the physiopathology of PD.

Acknowledgements. This work was supported by grants from: Spanish Ministry of Science (SAF 2004 07656 C02-02), Fundación Séneca (05662/PI/07; 15329/PI/10) and CIBERNED (Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas) and CONACyT (Consejo Nacional de Ciencia y Tecnología. México, 211521) which supported the scholarship of P. Heman. The authors would also like to thank Mr. P. Thomas for his valuable comments and language suggestions concerning this manuscript. Finally, the authors declare that there is no conflict of interests in the present work. Financial Disclosure: Paola Heman: Received salaries from the University of Veracruz and received a grant from the CONACyT (Consejo Nacional de Ciencia y Tecnología. México, 211521) to perform the present research. Carlos Barcia, Aurora Gómez and Carmen M. Ros: Received salaries from the Spanish Government (CIBERNED). Francisco Ros-Bernal: Received salaries from the Spanish Ministry of Science. Vicente de Pablos: Receives salaries form a Company not related to the subject of the study. Emiliano Fernandez-Villalba: Receives salaries form the Spanish Health System (Servicio Murciano de Salud; Hospital Morales Meseguer). María Rebeca Toledo-Cárdenas: Received salaries from the University of Veracruz. Maria-Trinidad Herrero: Received salaries from the University of Murcia and she holds grants related with the topic of this research from the Spanish Ministry of Science (SAF 2004 07656 C02-02), Fundación Séneca (05662/PI/07; 15329/PI/10) and CIBERNED. Author's role: M.T.H., C.B. and M.R.TC. designed the research, P.H., A.G., C.M.R., F.R-B, J.E.Y., V.dP. and E.F.V. performed the research, C.B. and M.T.H. wrote the manuscript.

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Accepted July 22, 2011