RESEARCH

Neuroprotective effect of the systemic administration of MK-801 on the pedunculopontine nucleus of hemiparkinsonian rats

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ABSTRACT

Glutamatergic antagonists were administered in rats, as part of the current pharmacological therapies for neuroprotection of patients with Parkinson disease, due to glutamatergic hyperactivity and the deleterious effects of this condition. The effect of the systemic administration of MK-801, an antagonist of N-methyl-D-aspartate (NMDA) receptors, was evaluated on the extracellular concentrations of glutamate (Glu) and gamma amino butyric acid (GABA), loss of dopaminergic cells and cell death in the pedunculopontine nucleus (PPN) of hemiparkinsonian rats. Five treatments were studied in Wistar rats: lesion in the *substantia nigra pars compacta* (SNpc) with 6-hydroxydopamine (6-OHDA)(n = 15); 6-OHDA lesion plus the systemic administration of MK-801 (0.5 mg/kg; n = 17); false lesion in the SNpc (n = 10), false lesion in the SNpc plus false systemic treatment (n = 10) and no treatment (n = 22). The extracellular concentrations of Glu and GABA were analyzed by cerebral microdialysis and high performance liquid chromatography coupled to fluorometric detection. The loss of dopaminergic cells and cell death processes in the PPN were assessed by immunohistochemistry for tyrosine hydroxylase and the TUNEL technique, respectively. The extracellular concentrations of Glu and GABA significantly decreased in the PPN after the MK-801 treatment, compared to untreated hemiparkinsonian rats. This treatment caused a decreased loss of dopaminergic cellular bodies in the tegmental ventral area of rats and prevented cell death in the PPN of hemiparkinsonian rats. These results suggest a neuroprotective effect mediated by a decreased glutamatergic tone in hemiparkinsoninan rats systemically treated with an antagonist of NMDA receptors.

Keywords: MK-801, pedunculopontine nucleus, Parkinson disease, 6-OHDA

Biotecnología Aplicada 2009;26:59-64

RESUMEN

Efecto neuroprotector de la administración sistémica de MK-801 en el núcleo pedunculopontino de ratas hemiparkinsonizadas. Como parte de las estrategias farmacológicas para la neuroprotección en pacientes con enfermedad de Parkinson se administraron antagonistas glutamatérgicos, ya que se conoce la hiperactividad glutamatérgica en esta afección y su consecuencia deletérea. Se evaluó el efecto de la administración sistémica de MK-801, un antagonista de los receptores N-metil-D-aspartato (NMDA), en las concentraciones extracelulares de glutamato (Glu) y ácido g-aminobutírico (GABA), la pérdida de células dopaminérgicas y el proceso de muerte celular en el núcleo pedunculopontino (NPP) de ratas hemiparkinsonizadas. Se evaluaron cinco grupos de ratas Wistar: ratas con lesión de la substantia nigra pars compacta (SNpc) con 6-hidroxidopamina (6-OHDA) (n = 15), lesionadas con 6-OHDA + administración sistémica de MK-801 (0.5 mg/kg de peso) (n = 17), con falsa lesión de la SNc (n = 10), lesionadas en SNc + falso tratamiento sistémico (n = 10) y ratas sanas (n = 22). Se estudió la concentración extracelular de Glu y GABA mediante microdiálisis cerebral y cromatografía líquida de alta resolución acoplada a detección fluorimétrica, así como la pérdida de células dopaminérgicas y el proceso de muerte celular en el NPP, mediante técnicas inmunohistoquímicas para tirosina hidroxilasa y TUNEL, respectivamente. Las concentraciones extracelulares de Glu y GABA en el NPP disminuyeron significativamente tras el tratamiento sistémico con MK-801, en comparación con las ratas hemiparkinsonizadas que no lo recibieron. La administración de este fármaco provocó menos pérdida de cuerpos celulares dopaminérgicos en el área tegmental ventral de las ratas tratadas, y previno el proceso de muerte celular en el NPP de las ratas hemiparkinsonizadas. Estos resultados sugieren un efecto neuroprotector por la disminución del tono glutamatérgico en ratas hemiparkinsonizadas tratadas sistémicamente con un antagonista de los receptores NMDA.

Palabras clave: MK-801, núcleo pedunculopontino, enfermedad de Parkinson, 6-OHDA

*I*ntroduction

Parkinson disease (PD) is a disorder affecting dopaminergic and non-dopaminergic structures that progressively degenerate neurons [1]. Although the death of the *substantia nigra pars compacta* (SNpc) dopaminergic cells is the neuropathological signature of PD, together with a subsequent dopaminergic deficiency, the function of other neurotransmitter systems (such as the glutamatergic system), the basal ganglion (BG) and other ganglia are also involved in this disease [2].

PD is currently treated with drugs intended to compensate dopaminergic deficiency, with L-dihydroxy-

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phenylalanine (L-DOPA) being the most effective treatment administered [3]. Nevertheless, its prolonged use leads to motor and psychiatric complications that have encouraged the search for, and the evaluation of, other neuroprotective pharmacological therapies [4]. Those treatments are focused on protecting or preventing the death of the neurons susceptible to the degenerative processes [5, 6].

In this sense, glutamate (Glu) is an excitatory amino acid that activates different types of receptors that are widely distributed throughout BG nuclei [7]. In PD, SNpc neuronal loss and the degenerated nigrostriatal network induce hyperactivity in efferent glutamatergic projections of the subtalamic nucleus (STN) towards the *globus pallidus medial* (GPm), *substantia nigra pars reticulata* (SNpr), the pedunculopontine nucleus (PPN), the SNpc and the tegmental ventral area (TVA)[8]. Thus, Glu becomes an endogenous toxin, provoking the massive entry of calcium into the cells and generating a series of reactions that are detrimental for survival [8].

Experimental evidence show a neuroprotective effect of strategies limiting the action of Glu on its receptors, by administering glutamatergic antagonists, or by attenuating the exposure of SNpc cells to this neurotransmitter through an early lesion of the STN [7-10]. Among them, the systemic administration of MK-801 (an N-methyl-D-aspartate receptor antagonist) prevents the toxicity of the direct injection of the 1-methyl-4-phenylpyridinium ion (MPP^+) into the SNpc in rats [11]. Other studies have documented the protection of SNpc dopaminergic neurons against degeneration, by the concomitant administration of MK-801 and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MTHP) in non-human primates [12].

Most of the studies on neurotransmission and cell death processes in PD experimental models have targeted the defective functioning of the SNpc [8]. However, PD-derived changes in other nuclei as the PPN, which is anatomically and functionally related to BG, have been insufficiently investigated [13, 14]. PPN is reciprocally connected to the STN through a glutamatergic projection and to the SNpr through a gabaergic projection, both of them are hyperactive in PD [8, 15]. Then, both excitatory and inhibitory stimuli converge at PPN from the motor circuitry network, underlying the relevance of studying the extracellular Glu and gamma-amino butyric acid (GABA) concentrations in this nucleus.

The aim of this study was to evaluate the effect of the systemic administration of MK-801 on the extracellular concentrations of Glu and GABA, cell death and loss of nigral dopaminergic cells.

Materials and methods

Experimental subjects

The study used adult male Wistar rats weighing from 200 to 250 g, provided by the Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Three rats were alloted per cage throughout the experiment, with a 12 h/12 h cycle of light and darkness, and water and food were offered *ad libitum*. The experimental procedures followed the Guideli-nes for the Care, Use and Reproduction of Laboratory Animals.

The SNpc lesion

The animals were anesthetized by the intraperitoneal (i.p.) administration of a chloral hydrate solution (420 mg/kg of body weight) and placed on a stereotactic surgery device for rodents (Stoelting, U.S.A). Three microliters of a neurotoxic solution containing 8 mg of 6-OHDA in 3 ìL of 0.9% physiological saline solution (PSS) plus 0.5 mg/mL of ascorbic acid were injected using a flow of 1 mL/min into the right SNpc, at the following stereotactic coordinates (mm) described in the Paxinos and Watson atlas [16]: AP = -4.9, L = 1.7, DV = 8.1 (according to Bregma). A control group with false SNpc lesions was obtained by the administration of an identical volume of PSS at the same coordinates.

Animal groups were formed according to the experimental treatment: the SNpc lesion (n=15), the SNpc lesion and the systemic administration of MK-801 (n=17), the SNpc lesion and the systemic administration of PSS, the false SNpc lesion (n=10) and healthy rats (n=22).

Rotational activity

One month after the injection of 6-OHDA, the rotational activity induced by D-amphetamine (5 mg/kg of body weight, i.p. route) was studied. Only the animals showing at least 7 turns per minute were included in the study. This variable was evaluated for 90 min using a LE 3806 electronic multicounter coupled to LE 902 sensors (PanLab, Barcelona, Spain) that detect the sense of rotation.

Systemic administration of MK-801

The rats received 3 i.p., daily injections of MK-801 (Sigma, St. Louis, USA; 0.5 mg/kg body weight), the third injection was immediately followed by surgery and the injection of the 6-OHDA solution. This schedule was repeated on days 14 and 21 after 6-OHDA administration. MK-801 was dissolved in 0.9% PSS. Control rats received the same treatment, but with PSS instead of MK-801.

In vivo microdialysis

Two weeks after the behavioral studies, a guide cannula was surgically implanted at the coordinates (mm) corresponding to the right PPN (AP = -8.00, L = 2.00, DV = 5.40; according to Bregma). The cerebral microdialysis experiments were performed 24 h after the implantation of this guide. Each rat was connected to a cerebral microdialysis infusion pump (CMA 100, CMA Microdialysis, Stockholm, Sweden) and the cannulae were then continuously perfused, at a flow of 2 µL/min, with a solution of artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 0.5 mM NaH_2PO_4, 5 mM Na_2HPO_4, 1 mM $MgCl_2$ x $6H_2O$, 1.2 mM $CaCl_2$ and 1.2 mM ascorbic acid, at a pH of 7.4 to 7.6. All the experiments were carried out while the animals were wide awake, as previously described [17].

Biochemical evaluation. Amino acid quantification

Amino acid concentrations in the dialysates were measured by High Performance Liquid Chromatography (HPLC) coupled to a fluorescence detector, via derivation with (OPA). Samples were analyzed in

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duplicates and procedures were carried out as previously described [17].

Evaluation of dopaminergic cell loss

After concluding the *in vivo* studies, the rats received a higher dose of chloral hydrate (480 mg/kg body weight, i.p.) and were then perfused via the ascending aorta with 500 mL of 0.9% NaCl and 500 mL of a fixing solution containing 4% paraformaldehyde, 0.1% glutaraldehyde and 15% picric acid in 0.1 M sodium phosphate, pH 7.4. The brains were then extracted, placed in fixing solution for 1 h, washed with 0.1 M sodium phosphate pH 7.4, cryoprotected in 7, 15 and 30% sucrose (24 h at each concentration), and frozen in liquid nitrogen. Coronal sections (20 mm) were obtained from the areas corresponding to the SNpc and placed on microscopy slides previously coated with gelatin-chrome alum. The immunohistochemical processing to visualize cells that are immunoreactive to the tyrosine hydroxylase (TH) enzyme was carried out as described elsewhere [18].

Methodology for the in situ *detection of cell death (TUNEL)*

The *in situ* detection of DNA fragmentation was performed using a kit that contains terminal deoxynucleotidyl transferase (TdT) according to the instructions from the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Experimental procedures were carried out as previously reported [17].

The occurrence of cell death was determined by contrasting sections with the pentahydrated Hoechst 33258, pentahydrate (bis-benzimide) - FluoroPure™ grade (Molecular Probes, USA). The sections were examined under a fluorescence microscope (excitation from 500 to 560 nm, detection from 515 to 565 nm; Leitz, Germany).

Data processing

The normal curve followed by the data was verified with the Kolmogorov-Smirnov test in every case. The homogeneity of variance was checked by the Levene test. Comparisons of Glu and GABA concentrations between experimental groups were done by a one-way ANOVA, followed by Tukey's test. The level of statistical significance of 0.05 was chosen for all analyses. The statistical software package Statistica CSS version 6.1 was employed throughout the study.

Results

Extracellular Glu and GABA concentrations at PPN

The comparison of the extracellular Glu concentrations revealed statistically significant differences among the experimental groups ($F_{(4, 39)} = 6.57$, $p < 0.05$), with the highest levels in the untreated hemiparkinsonian rats. The animals systemically administered with MK-801 showed an intermediate and significantly different behavior ($p < 0.05$), compared to untreated and SNpc-lesioned rats and to healthy control animals (Figure 1A).

Similarly, extracellular GABA concentrations were significantly different among experimental groups ($F_{(4, 37)}$=4.13, p<0.05). Once again, the highest values were detected in untreated hemiparkinsonian rats. Animals systemically treated with MK-801 showed significantly lower GABA concentrations that were statistically similar to those of healthy and false SNpc-lesioned rats (Figure 1B).

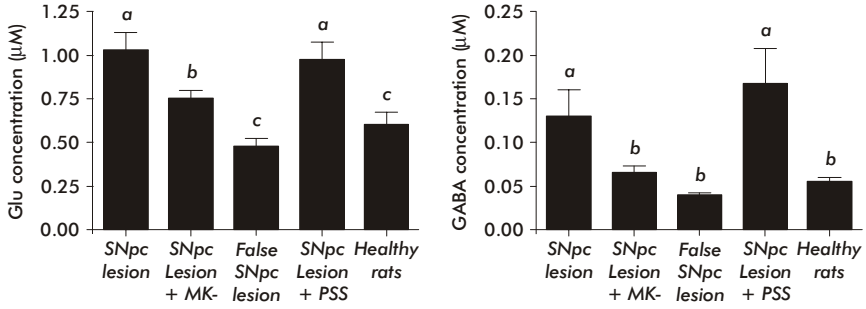

Figure 1. Effect of the systemic administration of MK-801 (0.5 mg/kg; i.p.) on the extracellular concentrations of glutamate (Glu) and gamma aminobutiric acid (GABA) in the pedunculopontine nucleus. A) Comparison of Glu concentrations among groups $F_{(4, 39)} = 6.57$; $p < 0.05$) *a* vs *b*, $p < 0.05$; *b* vs *c*, $p < 0.01$; *a* vs *c*, $p < 0.001$. B) Comparison of GABA concentrations among groups ($F_{(4, 37)} = 4.13$ $p < 0.05$) *a* vs *b*, $p < 0.01$. Experimental groups: rats with lesion in the *substantia nigra pars compacta* (SNpc; n = 9), rats with SNpc lesion + MK-801 (n = 17), rats with false SNpc lesion (n = 6), rats with SNpc lesion + physiological saline solution (PSS)(n = 6), and healthy untreated rats (n = 10). Data were analyzed by a one way ANOVA and Tukey´s test. Error bars represents the values ± standard error of the mean.

Presence of dopaminergic cells in the SNpc of hemiparkinsonian rats

The presence of cell bodies positive to the TH enzyme in the TVA of rats systemically administered with MK-801 demonstrates a decreased loss of dopaminergic cells in this zone, compared to untreated rats lesioned in the SNpc (Figure 2A and B). Nevertheless, the pharmacological treatment failed to protect them from loosing dopaminergic cells at the SNpc.

Study of cell DNA fragmentation at PPN

The TUNEL cellular fragmentation study showed TUNEL+ cells in the PPN of untreated hemiparkinsonian rats (Figure 3). On the other hand, the PPN of MK-801-treated hemiparkinsonian rats were weakly immunopositive to TUNEL, suggesting that this treatment prevents the development of cell death processes, but this effect is ipsilateral to the 6-OHDA injection (Figure 3).

Discussion

Considerations on the MK-801 administration schedule used in this study

Our results indicate that the administration schedule followed for the systemic delivery of MK-801 modifies the parkinsonian characteristics generated after the intracerebral injection of 6-OHDA, in agreement with previous reports [19, 20]. In general, hemiparkinsonian rats treated with MK-801 showed the poorest loss of dopaminergic cellular bodies in the TVA together with the lowest extracellular concentrations of Glu and GABA and cell death processes at the PPN.

This schedule corresponds to two effects reported for MK-801 in the literature: increased striatal expression of dinorphins [19] and modified discharge frequency of subthalamic glutamatergic neurons [20].

The administration of MK-801 prior to the 6-OHDA injection follows the principle of enhancing the striatal

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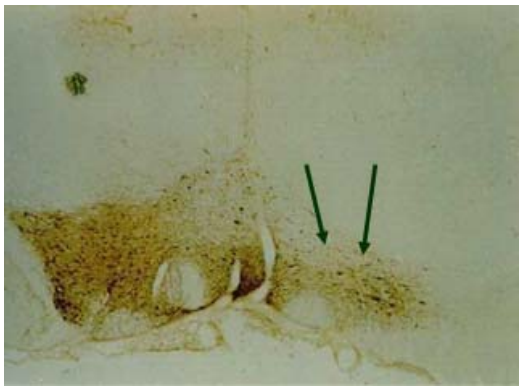
A



B

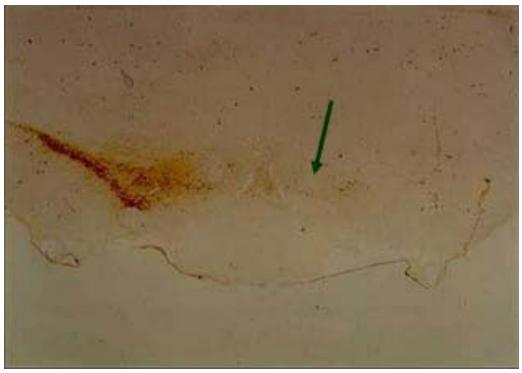


Figure 2. Immuno-detection micrograph of the tyrosine hydroxilase enzyme in a coronal section of the tegmental ventral area (TVA) and *substantia nigra pars compacta* (SNpc). A) Rat with a SNpc lesion and treated with MK-801 (10x). B) Rat with a SNpc lesion but without the MK-801 treatment (5x). Arrows indicate the presence of dopaminergic cellular bodies in the TVA area, which are absent in panel B.

gene expression of dynorphins in neurons of the direct network of the motor circuitry [19]. This effect would prevent the decreased striatal-nigral transmission that was established early in the 6-OHDA model [19, 21]. Nevertheless, the behavior during approximately 4 weeks described for the neurotoxic effect of 6-OHDA on nigral cells leads to the establishment of an irreversible motor asymmetry that characterizes this model [21]. During that period of time, glutamatergic activity increases gradually, the subthalamic glutamatergic activity peaking at day fifteen and preceding the appearance of PD symptoms [22, 23]. The second and third MK-801 administrations pursue the blockage of subthalamic hyperactivity in a critical period of subthalamic activation [24].

Effects of MK-801 administration on the loss of dopaminergic cells

Our results suggest that the MK-801 treatment can protect dopaminergic cell bodies in the TVA from neurotoxic damage. The TVA is part of the mesolimbocortical dopaminergic system and projects the *accumbens* nucleus [25]. There are several reports in the literature showing that the injection of 6-OHDA in coordinates targeting the SNpc similarly compromises the survival of TVA dopaminergic cells [26]. This contrasts with the effect of this neurotoxin when administered in the *striatum* (St) or in the nigral-striatal network where it produces its own retrograde transport, selectively killing SNpc dopaminergic cells and preserving TVA cells [27].

It is well known that at least some of the cascades of neurochemical changes triggered by dopaminergic denervation in BG are reverted by the administration of glutamatergic antagonists [28]. Moreover, the expression of NMDA receptors in TVA cells is higher than in SNpc cells, being this difference the possible morphophysiological support for the neuroprotective effect of MK-801 in these cells [29].

The reduced loss of dopaminergic cellular bodies in the TVA of rats treated with MK-801 can significantly influence the maintenance of St under dopaminergic control, at least partially [28]. This could attenuate synapse remodeling at St, ipsilateral to the lesion, and consequently decrease locomotor asymmetry in hemiparkinsonian rats [30, 31].

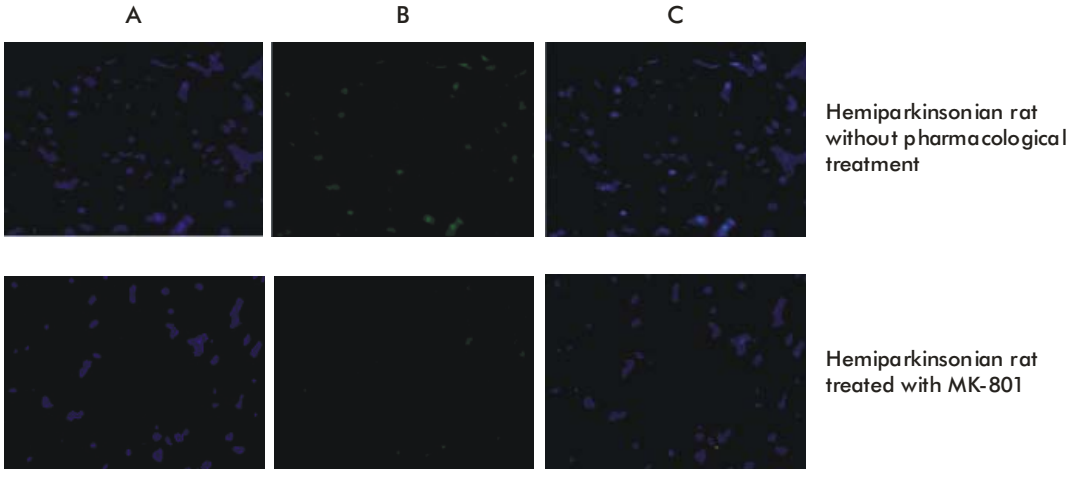


Figure 3. Micrograph representing the immunodetection of TUNEL+ cells in coronal sections of the pedunculopontine nucleus (PPN) contrasted with the Hoescht 33258 (RH) reagent (n=5). A) Stained microscopic field. B) Microscopic field similar to A but containing the TUNEL marker. Notice the immuno-positivity to TUNEL+ in hemiparkinsonian rats treated with MK-801. C) Double expression of staining with RH and the TUNEL marker. Brilliant blue cells are cells both stained and marked (40x).

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Extracellular Glu and GABA concentrations at the PPN

The decreased extracellular concentrations of Glu and GABA in rats treated with MK-801 after the 6-OHDA injection could result from the treatment by the attenuation of corticostriatal glutamatergic activity and the correction of misbalances of other neurotransmitters as dopamine.

The nigral dopaminergic network and cortical glutamatergic terminals converge at the striatal neuronal projection in a very fine synaptic interaction [32, 33]. In PD, a misbalanced estriatal neurotransmission and an increased cortical-striatal glutamatergic transmission coexist [32, 34]. Both effects convey, increasing the discharge of action potentials by striatal cells when depolarized after receiving the discharge through the presynaptic cortial-striatal terminal [35].

On the other hand, morphological studies show that dopaminergic denervation leads to structural changes at St, such as the remodeling of dendritic spines, which are able to interfere with cortical-striatal synaptic plasticity mechanisms [36, 37]. The systemic administration of antagonists of the NMDA glutamatergic receptors have multiple effects on the functioning of the BG nuclei [38]. However, there were no previous references on the effect of the MK-801 administration in the 6-OHDA model, specifically on Glu and GABA neurotransmission at PNN. Hence, our results are a contribution to this field of knowledge.

It was recently shown that the systemic administration of MK-801 reduces the rate of discharge and changes the pattern of electric activity of STN glutamatergic cells in hemiparkinsonian rats [22].

It is known that when locally administered in St, MK-801 generates distinct effects depending on the location of the intrastriatal injections and the topological distribution of NMDA receptors within this structure [38]. Subsequently, the perfusion of MK-801 into projection neurons of the St-GP1 network could generate PD-like effects by inhibiting this circuit. In contrast, MK-801 produces marked anti-parkinsonian effects when injected in neurons that originate the St-SNpr network [38].

It has been also reported that the excess of gabaergic inhibition in the PPN in PD can explain the PD-associated hypokinesia, based on the PPN projections through the reticulospinal tract towards the spinal cord interneuronal network [39].

Based on these previous findings, we speculate that the systemic administration of MK-801 used in this study could correct some of the effects of dopaminergic denervation on the corticostriatal excitatory neurotransmission. This will compensate the misbalance among BG motor circuitry networks. Thus, decreased extracellular concentrations of Glu and GABA in the PPN, where both motor circuits converge, may be a functional expression of such a correction.

Cell death at PPN

It is established that approximately 40 to 50% of PPN cholinergic cells are lost in PD [40]. In contrast, other authors have pointed out the absence of cell death processes in murine and non-human primate PD experimental models [41]. These latter findings have suggested that those neurons are less sensitive than dopaminergic SNpc cells to the exotoxicity processes triggered by the subthalamic glutamatergic activation [38]. Nevertheless, there are several, neurochemically different neuronal populations in the PPN. Besides, glutamatergic, gabaergic and peptidergic neurons have been identified at PPN in rats, in addition to the cholinergic ones [14, 42].

In previous studies, we demonstrated the occurrence of one cell death process at PPN, ipsilateral to the 6-OHDA injection [18]. The PPN neurons are exposed to an increased subthalamic glutamatergic activity in PD, together with a decreased nigropontine dopaminergic influence [43]. This misbalance could be associated to cell death-promoting events in some of these neuronal populations.

The results shown herein suggest that the treatment with MK-801 prevents cell death at PPN. This finding underline the relevance of therapeutic strategies intended to attenuate the glutamatergic hyperactivity on those nuclei innervated by such projections from the STN. Two main mechanisms are expressed in the literature as mediating this effect of MK-801: *i*) the direct blockage of the NMDA glutamatergic receptor; and *ii*) the inhibition of the tumor necrosis factor alpha, which enhances Glu toxicity and further inhibits recapture mechanisms of this neurotransmitter [10].

Due to the relevance of the PPN for controlling the voluntary movement of several parts of the body, the effect of the systemic blockage of the glutamatergic transmission on the cell death process is highly relevant. This control is exerted in close association with other brain nuclei [14]. Other studies highlight the role of PPN glutamatergic neurons to initiate conscious movements, while the activity of cholinergic neurons is associated to a steady and uniform walking pattern [44, 45].

In a recent study, 36% of the cholinergic and 27% of the non-cholinergic neurons were found to be lost in the PPN of PD patients [46]. Data also showed its highly significant negative correlation with the Hoehn and Yahr scores evaluating the axial symptoms of the disease, and its equilibrium and walking disorders. This means that the worse the handicap of the PD patient, the more profound the profile of cell death in the PPN [46].

Thus, cell death could be enhancing PPN malfunction in PD, being part of the morpho-physiological causes of motor disorders found in 6-OHDA-injected hemiparkinsonian rats.

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Received in October, 2008. Accepted for publication in March, 2009.