

## Changes in Glu and GABA extracellular concentrations and cell death process in the pedunculopontine nucleus of hemiparkinsonian rats

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Although the dysfunction of the pedunculopontine nucleus (PPN) has been related in the last decade to the pathophysiology of the Parkinson's disease (PD), the changes in the extracellular concentration of aminoacid neurotransmitters in this structure have not been extensively studied. This study focuses on the changes in glutamate (Glu) and  $\gamma$ -aminobutyric acid (GABA) of the PPN of hemiparkinsonian rats, and on the process of cellular death in this nucleus. Three groups of Wistar rats were examined: non-treated ( $n = 12$ ), lesioned with 6-hydroxydopamine (6-OHDA) ( $n = 11$ ), and sham-operated ( $n = 10$ ). In all groups, a microdialysis probe was implanted in the right PPN, ipsilateral to the injection of 6-OHDA. The dialyzates were analyzed using high performance liquid chromatography. The cellular death was studied by TUNEL immunohistochemistry. There were statistically significant increases in the concentration of Glu and GABA in the PPN of hemiparkinsonian rats ( $p < 0.001$ ) and the process of cell death was evidenced in the PPN ipsilateral to the *substantia nigra compacta* lesion. Higher Glu levels may be related to the excess of activity (hyperactivity) of the subthalamic nucleus-PPN pathway as well as the hyperactivity of the "indirect pathway" of the basal ganglia (BG). The increase in the glutamatergic tone may be the substratum for the cellular death events observed in the PPN. The increases in the GABA extracellular concentration in the PPN may be associated with the hyperactivity of the target nuclei in the BG. These results support the hypothesis concerning the involvement of the PPN in the pathophysiology of PD.

**Key words:** pedunculopontine nucleus, glutamate, GABA, cerebral microdialysis, cellular death.

### INTRODUCTION

Neurochemical changes occurring in the nuclei of the basal ganglia as a consequence of the degeneration of the *substantia nigra pars compacta* (SNc) that typically occurs in the Parkinson's disease (PD), have been

already studied (Zigmond *et al.*, 2002). There are well-established experimental models of parkinsonism using intracerebral injection of 6-hydroxydopamine (6-OHDA) in rodents and systemic or intracarotid delivery of 1-methyl, 4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in non-human primates that have been instrumental in the investigation of these changes (Blum *et al.*, 2001; Jenner, 2003). However, whereas

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the physiology of the nuclei of the basal ganglia in this setting has been the subject of intense research, this is not true for other closely related nuclei, such as the pedunculopontine nucleus (PPN) (Lee *et al.*, 2000; Winn, 2006).

It is also known that the PPN connects different nuclei of the basal ganglia, including the subthalamic nucleus (STN), the SNc and the output nuclei [the internal segment of the *globus pallidus* (Gpi) and the *substantia nigra pars reticulata* (SNr)] (Mena-Segovia *et al.*, 2004; Winn, 2008). The PPN also develops a projection through the medial reticulospinal tract to the interneurons of the spinal cord, and participates in the control of the axial muscles (Obeso *et al.*, 2000a).

A number of reports published during the last decade agree on considering the PPN as an important structure for the pathophysiology of the PD, based mainly on three factors: the location of the PPN in the locomotor mesencephalic area, its involvement in the control of posture and locomotion, and its anatomical and functional relationship with the basal ganglia (Lee *et al.*, 2000; Mena-Segovia *et al.*, 2004).

The physiological changes observed in the PPN of rodents and primate animal models of PD, have further emphasized the importance of this structure in processing motor information (Jenkinson *et al.*, 2004). An increase in the electrical activity of the PPN cells that is normalized upon the excitotoxic injury of the STN, has been described in the model of hemiparkinsonism in rats (Breit *et al.*, 2001). Other authors describe the reversion of akinesia that characterizes parkinsonian primates after injection of gabaergic antagonist drugs in the PPN (Nandi *et al.*, 2002). This last group has also developed significant motor improvements for parkinsonian primates subjected to electrical stimulation of the PPN, and the possibility of using this new target for improving akinesia of parkinsonian patients through deep brain electric stimulation has recently been considered (Jenkinson *et al.*, 2005).

The cerebral microdialysis technique has been used to study the release of neurotransmitters in the Central Nervous System. The fact that it provides information on the composition of the extracellular milieu in a particular nucleus *in vivo*, has made this methodology an essential tool for neurochemical studies during the last 10 years (Bianchi *et al.*, 2003).

In spite of the advances in cerebral microdialysis, there are reports on the patterns of release of amino acidic neurotransmitters [glutamate (Glu) and  $\gamma$ -ami-

no butyric acid (GABA)] in the PPN of parkinsonian animal models (Steiniger & Kretchmer, 2003). This is explained by the involvement of other nuclei, such as the SNc, in the motor dysfunctions that characterize the PD, which attracted the attention of most groups focused on the study of dopaminergic neurotransmission (Hurley & Jenner, 2006). Additionally, although the link between dopaminergic deficiencies and Parkinsonism has long been established, only recently the PPN is recognized as a key structure in the pathophysiology of the PD.

The aim of the present paper is to study the extracellular concentration of Glu and GABA on the PPN of hemiparkinsonian rats, and the cell death process in ipsilateral pontine cells after injection with 6-OHDA.

## MATERIALS AND METHODS

### *Experimental subject*

The animals used were adult male Wistar rats weighing from 200 to 250 g, from the Center for the Production of Laboratory Animals (CENPALAB, Havana, Cuba). Three animals were housed per cage throughout the experiment with a light-darkness cycle of 12-12 hours. Water and feed were offered *ad libitum*.

### *SNc lesion*

The rats were anaesthetized by intraperitoneal (i.p.) injection of a chloral hydrate solution (420 mg kg<sup>-1</sup> body weigh) and placed on a stereotactic surgery device for rodents (Stoelting, USA). Afterwards, the rats were injected with 3  $\mu$ l of 6-OHDA solution (St. Louis, USA, 8  $\mu$ g per 3  $\mu$ l of 0.9% NaCl and 0.5 mg ml<sup>-1</sup> ascorbic acid) at a rate of 1  $\mu$ l per min in the right SNc. The procedure used the following stereotactic coordinates (mm), as described in the Atlas by Paxinos & Watson (Paxinos & Watson, 1998): AP = -0.49; L = 0.17; DV = 0.81. One month after the SNc lesion, the rotational activity induced by D-amphetamine (5 mg kg<sup>-1</sup> body weigh, i.p. route) was studied according to the procedures described in Lezcano *et al.* (2008). The negative control group (false injured) was formed by animals receiving an injection of 0.9% of physiological saline solution (NaCl) with the same volume and stereotactic coordinates.

A total of three experimental groups were organized: untreated rats (n = 12), rats with SNc injury (n = 11) and sham-operated rats (n = 10).

### Microdialysis probe

The microdialysis probe was similar to that described previously by Robinson & Camp (1991). The dialysis tube (ID: 0.12 mm, OD: 0.22 mm) was prepared from polyacrylonitrile copolymer (Hospal Industrie Meyzie, Lyon, Francia) with a cut off 41 kDa.

### In vivo microdialysis

The surgical implantation of the guide cannulae was performed one month after the injection of 6-OHDA (Sigma, St Louis, USA) on the SNc. Using standard stereotaxic techniques, a stainless steel guide cannula was cemented to the cranium at the coordinates (mm) corresponding to the right PPN, ipsilateral to the SNc injury: AP = -8; L = 1.90; DV = 6.82 (adjusted to the cerebral microdialysis probe).

The animals were allowed to recover, and three days after the surgery, a microdialysis probe was introduced through the guide after removing its protective dummy cannula. Then, rats were tethered via a flexible stainless steel cable mounted on a counterbalanced arm located above the cage which allowed free movement within the cage.

The microdialysis probe was connected to an infusion pump (CMA 100, CMA Microdialysis, Stockholm, Sweden) and continuously perfused with a solution of artificial cerebrospinal fluid (aCSF) (125 mmol of NaCl; 2.5 mmol of KCl; 0.5 mmol of  $\text{NaH}_2\text{PO}_4$ ; 5 mmol of  $\text{Na}_2\text{HPO}_4$ ; 1 mmol of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ; 1.2 mmol of  $\text{CaCl}_2$ ; 1.2 mmol of ascorbic acid; pH 7.4–7.6) at a rate of  $2 \mu\text{l min}^{-1}$ . After stabilization period of 2 hrs, the samples were manually collected every 20 min.

Totally six dialyzates were collected per rat, which were immediately stored at  $-80^\circ\text{C}$  for further analysis.

### Amino acid analysis

Amino acid concentration in the dialysates was determined by high performance liquid chromatography (HPLC) coupled to a fluorimetric detector, after derivatization with ophthaldehyde (OPA) (Kilpatrick *et al.*, 1993). Ten  $\mu\text{l}$  of the samples were mixed with 10  $\mu\text{l}$  of the derivatizing reagent (10 mmol of OPA dissolved in 0.1 M sodium tetraborate buffer containing 77 mmol of 3-mercaptopropionic acid and 10% methanol at pH 9.3). The samples were placed on a shaker for 15 sec, and the reaction was stopped by adding 5% acetic acid after 45 sec. Twenty  $\mu\text{l}$  of this mix-

ture were loaded in the chromatography system using a Hamilton syringe. The derivatized amino acids were resolved on a reversed phase column (HR-80, 8 cm long  $\times$  4.6 mm internal diameter, ESA), with a pre-column having a similar stationary phase, using an isocratic chromatographic pump (Knauer K1001), and detected with a fluorescence detector set at  $\lambda_{\text{exc}}$  340 nm and  $\lambda_{\text{emis}}$  460 nm (Philips 4027). The chromatographic data were recorded using the CHROMATEPC software version 4.24 (Philips). The mobile phase was composed of 0.1 M  $\text{NaH}_2\text{PO}_4$  and 20% methanol. Each sample was analyzed twice.

### Morphological study

The animals were anaesthetized with chloral hydrate ( $480 \text{ mg Kg}^{-1}$  of body weight, i.p. route), and perfused through the ascending aorta. Animal brains were extracted and fixed with 10% formalin for 24 hrs. Coronal sections (20  $\mu\text{m}$  thick) were obtained (Leitz 1720 cryostat, Germany) from the areas corresponding to the SNc and the PPN. The sections were mounted on a gelled medium and the correct localization of the cerebral microdialysis probe into the PPN was checked by staining with cresyl violet.

The determination of the extent of nigral dopaminergic degeneration was carried out by the examination of the coronal sections of the SNc immunohistochemically stained for the tyrosine hydroxylase (TH) enzyme.

### Methodology for the in situ detection of death (TUNEL)

The animals with SNc lesions were anaesthetized with chloral hydrate ( $480 \text{ mg Kg}^{-1}$  of body weight, i.p. route) and sacrificed by decapitation. The brains were extracted immediately and submerged into 0.9% saline physiological solution at  $4^\circ\text{C}$ . After that, brains were fixed in phosphate buffer (SBF 0.1 M, pH 7.4) with 4% paraformaldehyde and 2% glutaraldehyde solution for 2 days at  $4^\circ\text{C}$ . After fixing, samples were submerged in solutions of increasing sucrose concentration (15%, 20%, 25%, 30%) for 2 hrs each (24 hrs for 30% solution). Coronal sections (10  $\mu\text{m}$  thick) of the PPN were obtained (Leitz 1720 cryostat, Germany), and placed on slides stored at  $4^\circ\text{C}$ .

The *in situ* detection of DNA fragmentation was performed using a kit containing terminal deoxynucleotidyl transferase (TdT), according to the instructions of the manufacturer (Roche Molecular Bioche-

micals, Mannheim, Germany). A permeabilizing solution was first used (0.1 Triton  $\times$  100, 0.1 sodium citrate) for 2 min at 4°C, followed by a wash with phosphate buffer and the addition of the TUNEL solution mix. The sections were incubated for 60 min at 37°C and then washed three times with phosphate buffer. In order to verify whether there is an apoptotic death, sections were stained with propidium iodide (Sigma, St Louis, USA) for 2 min, and, finally, covered with Vectashield mounting medium containing DAPI (a colorant fluorescent complex 4'-6-diamidino-2-phenylindole at 1.5  $\mu\text{g ml}^{-1}$ , Vector Lab, CA, USA). The mounted sections were examined under a fluorescence microscope (excitation from 500 to 560 nm, detection from 515 to 565 nm, Leitz, Germany) and a confocal microscope (Leica 2B).

#### Data analysis

The data were tested for normal distribution and homogeneity of variances using the Kolmogorov-Smirnov and the Bartlett tests. The Glu and GABA extracellular concentrations in the PPN of the experimental animals were compared using a single-classification analysis of variance, followed by the Tukey's test. Significance level for statistical analyses was set to 0.05. The data were processed and analyzed using the statistical software application package STATISTICA (StatSoft Inc., 2003).

## RESULTS

#### Morphological and immunohistochemical studies

The immunohistochemical studies confirmed the loss of dopaminergic cell bodies in the right SNc, according to the place where the neurotoxin 6-OHDA was injected (Fig. 1). At the same time, the morphological studies verified that cerebral microdialysis probe has been correctly placed on the coordinates corresponding to the PPN, located on the distal part of the superior cerebellar peduncle (Fig. 2).

#### Glu and GABA extracellular concentrations in the PPN

The comparison of the Glu extracellular concentration in the PPN revealed statistically significant differences between the experimental groups ( $F_{(2,33)} = 23.57, p < 0.001$ ), with a significant increase in the extracellular concentration of this neurotransmitter in rats with SNc injuries (Fig. 3A).

Likewise, the comparison of the GABA extracellular concentration in the PPN between the experimental groups again showed a significant increase in the PPN of rats with SNc injuries ( $F_{(2,31)} = 26.51, p < 0.01$ ) (Fig. 3B).

#### TUNEL studies

The DNA fragmentation studies (TUNEL) did not detect TUNEL+ cells in the left PPN, contralateral



FIG. 1. Coronal sections through the SNc of a rat 30 days after 6-hydroxydopamine (6-OHDA) injection processed for tyrosine hydroxylase (TH) immunohistochemistry. Injection of 6-OHDA induced cell loss in the right *substantia nigra* (indicated area) while the left *substantia nigra* remained intact ( $\times 10$ ).

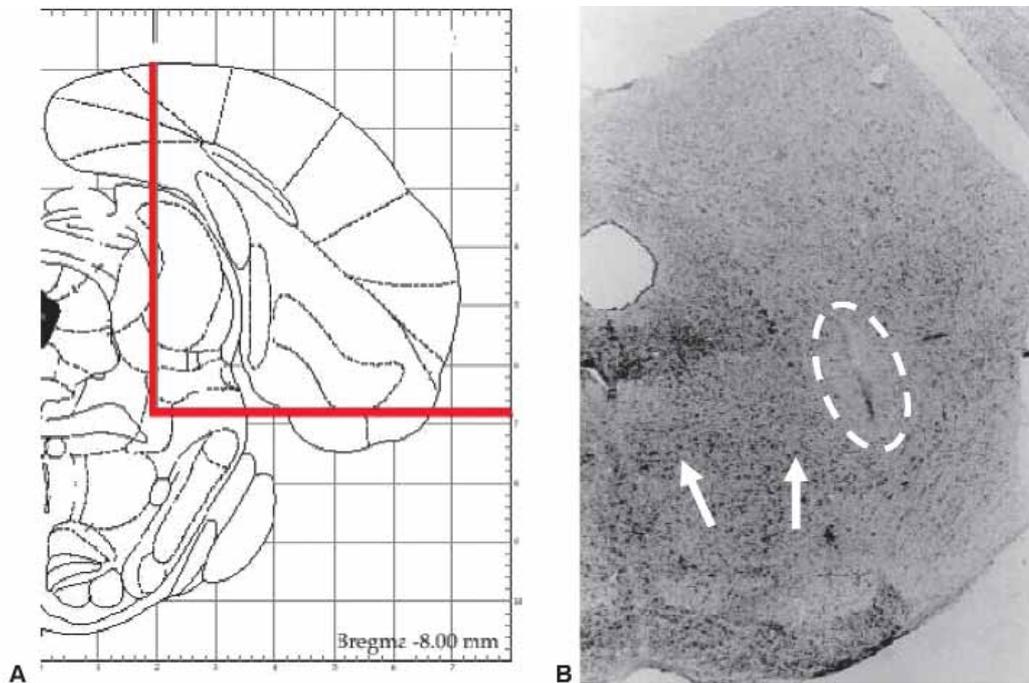


FIG. 2. A. Diagram that shows a coronal section of the PPN in the anteroposterior coordinate AP = -8 mm. Image modified for the best visualization of the structure. The intersection of black lines indicates the location of the probe in the cerebral tissue. B. Representative microphotograph of a coronal section stained with cresyl violet, illustrating the site where the cerebral microdialysis probe is located ( $\times 5$ ). The area in the dotted oval is the trace left by the probe in the tissue, at the distal part of the superior cerebellar peduncle. The white arrows indicate the fibers of the superior cerebellar peduncle.

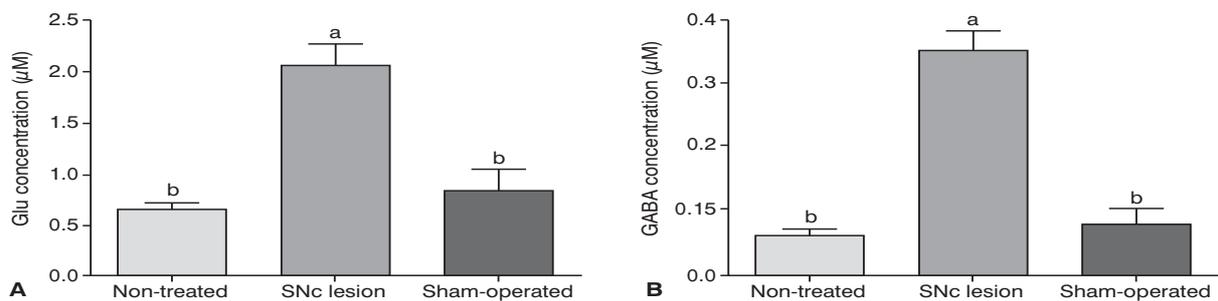


FIG. 3. Extracellular concentration of Glu and GABA in the PPN of non-treated, hemiparkinsonian and sham-operated rats. A. Inter-group comparison of Glu extracellular concentration. B. Inter-group comparison of GABA extracellular concentration. The groups were compared using one-way ANOVA, followed by Tukey's test. Different letters on the bars indicate statistically significant differences ( $p < 0.001$ ).

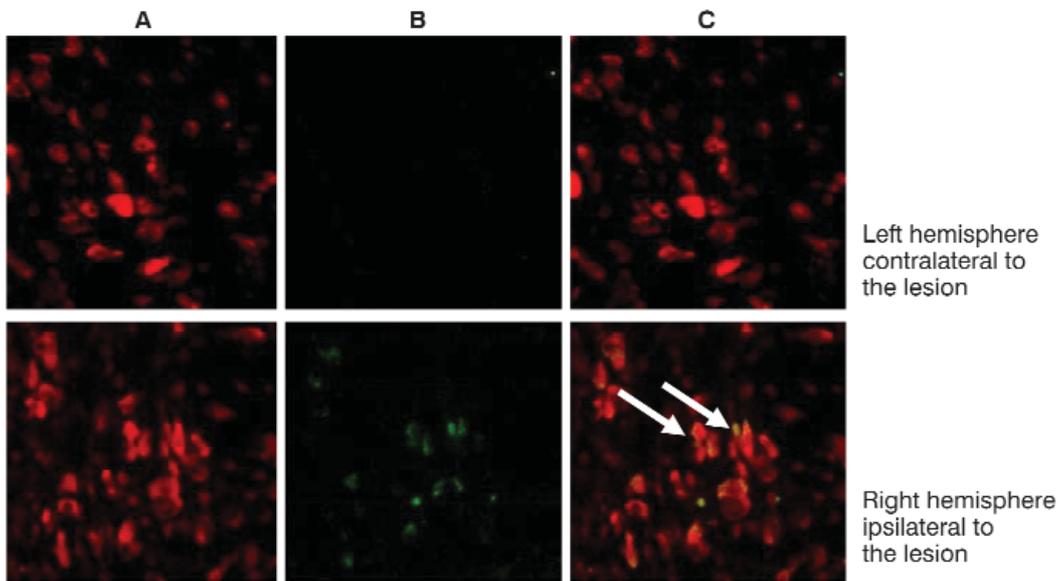


FIG. 4. Immunofluorescence illustrating the presence of TUNEL+ cells in coronal sections of the pedunculopontine nucleus counterstained with propidium iodide (PI). A. Stain PI in a field. B. TUNEL immunoreactivity in the same field. Note the total absence of TUNEL+ cells in left PPN. C. Show merged stain PI + TUNEL immunoreactivity. Note the cells expressing double stain (white arrows) in the right hemisphere (n = 5) ( $\times 40$ ).

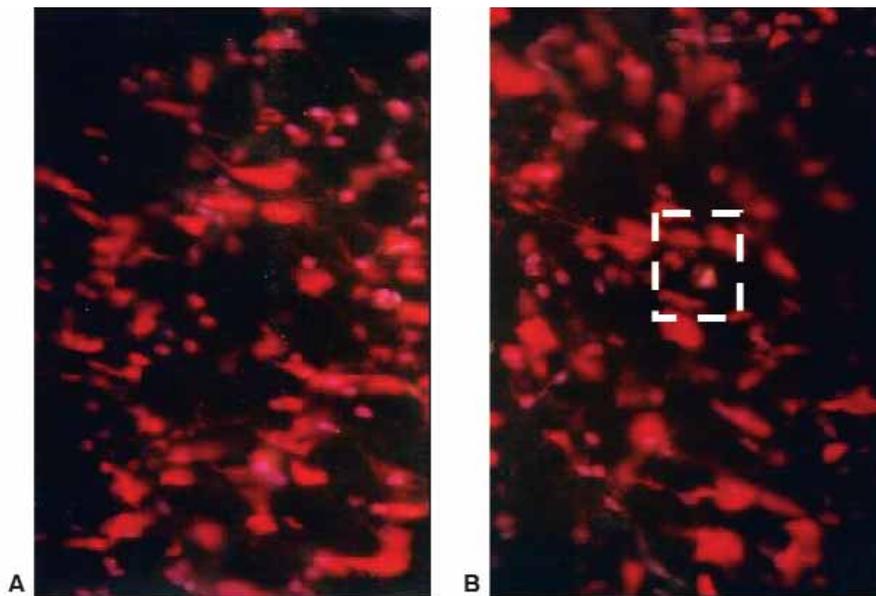


FIG. 5. Representative microphotograph ( $\times 40$ ) for a coronal section of the PPN, illustrating the results of the TUNEL and DAPI immunohistochemistry. A. PPN of the non-injured left hemisphere. B. PPN of the right hemisphere, ipsilateral to the SNc lesion, showing a cell with positive labelling for DAPI and TUNEL, which indicates apoptosis. Note that double-labelled cells are scarce.

to the site of the 6-OHDA injection into the SNc (Fig. 4). This result contrasts with the presence of TUNEL+ cells in the right PPN, representing the occurrence of cell death in the pontine cells ipsilateral to the SNc lesion (Fig. 4).

There were DAPI+ cells in the left PPN contralateral to the 6-OHDA injection, but none was immunoreactive to TUNEL (Fig. 5A). In the right PPN (ipsilateral to the lesion) there were very few cases of double DAPI + TUNEL + cells, indicating that the mechanism of cell death is not apoptotic in most of the cases (Fig. 5B).

## DISCUSSION

### *Effects of the SNc lesion on the extracellular concentrations of Glu and GABA in the PPN*

Most clinical and experimental studies that attempted to explain the motor dysfunctions associated to the parkinsonian syndrome, have focused on the classic motor cortex-striatum-globus pallidus-thalamus-motor cortex loop, without paying much attention to the projections from the basal ganglia to the pontine nuclei (Hamani et al., 2007; Redgrave & Coizet, 2007).

The death of dopaminergic cells leads to a loss of the inhibitory control exerted by dopamine through its D2 subtype receptor on the “indirect pathway” of the motor circuit in the basal ganglia (Gerfen, 2000). Consequently, there is an increase in neurotransmission through this pathway, which involves the STN (its cells express Glu as neurotransmitter) (Obeso et al., 2000b).

The increase in the glutamatergic activity in the PPN may contribute to the maintenance of the subthalamic hyperactivity. It is known that both structures (STN and PPN) are connected by a monosynaptic loop and project to the output nuclei in the SNr and the internal segment of the GP (Breit et al., 2001; 2005). The significant increase in the concentration of Glu in the extracellular milieu of the PPN may be a key element in the physiology of the nucleus itself under parkinsonian conditions. At the same time, it could have a fundamental role in the stimulation of dopaminergic cells surviving neurotoxic injury because the SNc receives from the PPN, cholinergic and glutamatergic projections (Anglade et al., 1995; Breit et al., 2005).

The increased extracellular concentration of GABA in the PPN of hemiparkinsonian rats may be explained by SNr and GPi hyperactivity. The glutamatergic neurons of the *pars dissipata* from the PPN receive a

gabaergic projection from these nuclei, whose hyperactivity constitutes a distinctive feature of the changes in the neurotransmission system during Parkinsonism (Groenewegen & Dongen, 2007; Androulidakis et al., 2008).

The increase of the electrical activity of the PPN cells in hemiparkinsonian rats is well known (Breit et al., 2001). Then, the output nuclei of the basal ganglia are under the effect of two glutamatergic projections, both hyperactive in parkinsonian conditions; one from the STN and the other from the PPN (Obeso et al., 2000b). This condition could perpetuate the gabaergic discharge from the output nuclei of the basal ganglia and the net result could be the increase of the GABA extracellular concentration in the PPN.

### *Cell death in the PPN of hemiparkinsonian rats*

Some studies have documented a loss of approximately 40% or even 50% of cholinergic cells on the lateral part of the PPN of deceased parkinsonian patients (Zweig et al., 1989; Rinne et al., 2008). Less is known about the process of cellular death in the PPN of animals used as experimental models of PD (Heise et al., 2005).

The results of the present study showed that after injection of the neurotoxin 6-OHDA in rats there are signs of cell death in the PPN ipsilateral to the site of injection. The literature suggests that many experimental models based on cellular damage resulted in cell death by apoptosis or necrosis, with a range of intermediate morphologies (Martin, 2001). It has been pointed out that cell death is actually an apoptosis-necrosis continuum, in which characteristics of both processes coexist and TUNEL immunohistochemistry is unable to differentiate between these forms (Martin, 2001). In this context, a question arises which of the following factors trigger the process of cell death in PPN; either the neurochemical imbalance characterized by a significant increase in the glutamatergic tone or the loss of dopaminergic innervations from the SNc.

The increase in the glutamatergic tone, represented in this study by an increased extracellular concentration of Glu in the PPN, has been associated to a rise in the intracellular calcium concentration that exceeds the limits of the homeostatic mechanisms normally keeping this parameter at a constant value (Dingledine & Bain, 1999; Hirsch et al., 2000). The excitation mediated by an increase in glutamatergic activity (frequently referred to as excitotoxicity) lead

to the activation of several enzymes, such as proteases, phospholipases, and calcium-dependent endonucleases, that degrade the proteins of the neuronal cytoskeleton, the membrane phospholipids, and the cellular DNA, respectively (Bahr *et al.*, 2002).

On the other hand, the excessive release of Glu in absent or minor dopaminergic activity has been associated to the activation of the mitogen-activated protein kinases family (Skapper *et al.*, 2001). These proteins participate in the activation of several subunits of the glutamatergic NMDA receptors and might perpetuate the glutamatergic activity in the PPN cells (Skapper *et al.*, 2001; Nakatsu *et al.*, 2006).

In the pontine neurons from right hemisphere can converge the mechanisms above mentioned and its combination action may trigger the cell death signals observed in this study. The immunohistochemical findings (low amounts of double-labeled TUNEL+ and DAPI+ cells) suggest that cell death, in this case, follows a necrotic course, although the occurrence of apoptotic events can not be excluded.

#### Concluding remarks

Our results confirm that the death of the dopaminergic cells in the SNc is associated to changes in the pattern of release of neurotransmitters such as Glu and GABA, in structures that are anatomically and functionally closely related to the basal ganglia, such as the PPN. Taking together these aspects, both may be triggering factors for mechanisms of cell death in pontine cells, which might contribute, on the long run, to nigral degeneration and establishment of an imbalance between the neurotransmission systems.

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