RESEARCH

Increase in the extracellular concentration of amino acid neurotransmitters and cell death on the pedunculopontine nucleus of hemiparkinsonian rats upon intracerebral injection of 6-hydroxy dopamine

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ABSTRACT

Although the dysfunctions of the pedunculopontine nucleus (PPN) have been linked in the last decade to the physiopathology of Parkinson's disease (PD), the changes in the patterns of neurotransmitter release of this structure remain largely unknown. This study focuses on the changes in the release of glutamate (GLU) and γ-aminobutyric acid (GABA) in the PPN of hemiparkinsonian rats, and on the evaluation of the occurrence of neuronal death in this structure. Three groups of Wistar rats were evaluated: healthy (n = 12); lesioned with 6-hydroxydopamine (6-OHDA) (n = 11), and falsely lesioned (n = 10). In all cases, a microdialysis catheter was implanted in the right NPP, ipsilateral to the injection of 6-OHDA, and the dialysates were analyzed by chromatographic techniques. The neuronal death was followed by TUNEL immunohistochemistry. There were statistically significant increases in the concentration of GLU and GABA for the PPN of hemiparkinsonian rats ($p < 0.001$), and processes of cell death were evidenced in the PPN ipsilateral to the SNc lesion. The higher GLU levels may be related to the hyperactivity of the "indirect pathway" of the basal ganglia (BG), and may trigger neuronal death in the PPN. The increases in the release of GABA may be associated to the hyperactivity of the target nuclei in the BG. These results reinforce the hypothesis of the involvement of the PPN in the physiopathology of PD.

Key Words: pedunculopontine nucleus, glutamate, gaba, cerebral microdialysis, cellular death

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RESUMEN

Aumento de las concentraciones extracelulares de aminoácidos neurotransmisores y muerte celular en el núcleo pedunculopontino de ratas hemiparkinsonianas por inyección intracerebral de 6-hidroxidopamina. En la última década se ha relacionado la disfunción del núcleo pedunculopontino (NPP) con la fisiopatología de la enfermedad de Parkinson (EP); sin embargo, no se conocen los cambios que experimenta la liberación de neurotransmisores en esta estructura. Estudiar los cambios en la liberación de glutamato (GLU) y de ácido γ-aminobutírico (GABA) en el NPP de ratas hemiparkinsonianas, y evaluar la muerte celular en esa estructura, es el objetivo de esta investigación. Se analizaron tres grupos de ratas Wistar: sanas (n = 12); lesionadas con 6-hidroxidopamina (6-OHDA) (n = 11); falsas lesionadas (n = 10). En todas las ratas se implantó una cánula de microdiálisis en el NPP derecho ipsilateral a la inyección de 6-OHDA, y los dializados se analizaron por técnicas cromatográficas. La muerte celular en el NPP de los animales lesionados se evaluó por inmunohistoquímica TUNEL. Hubo un aumento significativo en las concentraciones de GLU y GABA en el NPP de las ratas hemiparkinsonianas ($p < 0.001$). Se evidenció un proceso de muerte celular en el NPP ipsilateral a la lesión del SNc. El incremento en los niveles de GLU puede estar relacionado con la hiperactividad de la «vía indirecta» de los ganglios basales (GB) y a la vez pudiera disparar mecanismos de muerte neuronal en el NPP. El aumento en la liberación de GABA puede asociarse con la hiperactividad de los núcleos diana de los GB. Estos resultados refuerzan la hipótesis de la participación del NPP en la fisiopatología de la EP.

Palabras claves: núcleo pedunculopontino, glutamato, gaba, microdiálisis cerebral, muerte celular

*I*ntroduction

The neurochemical changes occurring at the different nuclei of the basal ganglia as a consequence of the degeneration of the *substantia nigra pars compacta* (SNc) that typically occurs in Parkinson's disease (PD) have been thoroughly studied [1-5]. There are well-established experimental models of parkinsonism by the intracerebral injection of 6-hydroxydopamine (6-OHDA) in rodents [6] and the systemic or intracarotid delivery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropiridine (MPTP) in non-human primates [7, 8] that have been instrumental in the investigation of these changes [1-5]. However, whereas the physiology of the nuclei on the basal ganglia in this setting has been the subject of intense research, this is not true of other closely related nuclei such as those of the pedunculopontine nucleus (PPN) [9-11].

It is well known that the PPN connects to different nuclei on 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)) (Figure 1) [12-14]. The PPN also sends a projection through the medial reticulospinal tract to the interneurons of the spinal chord, and participates in the control of the axial muscles [15].

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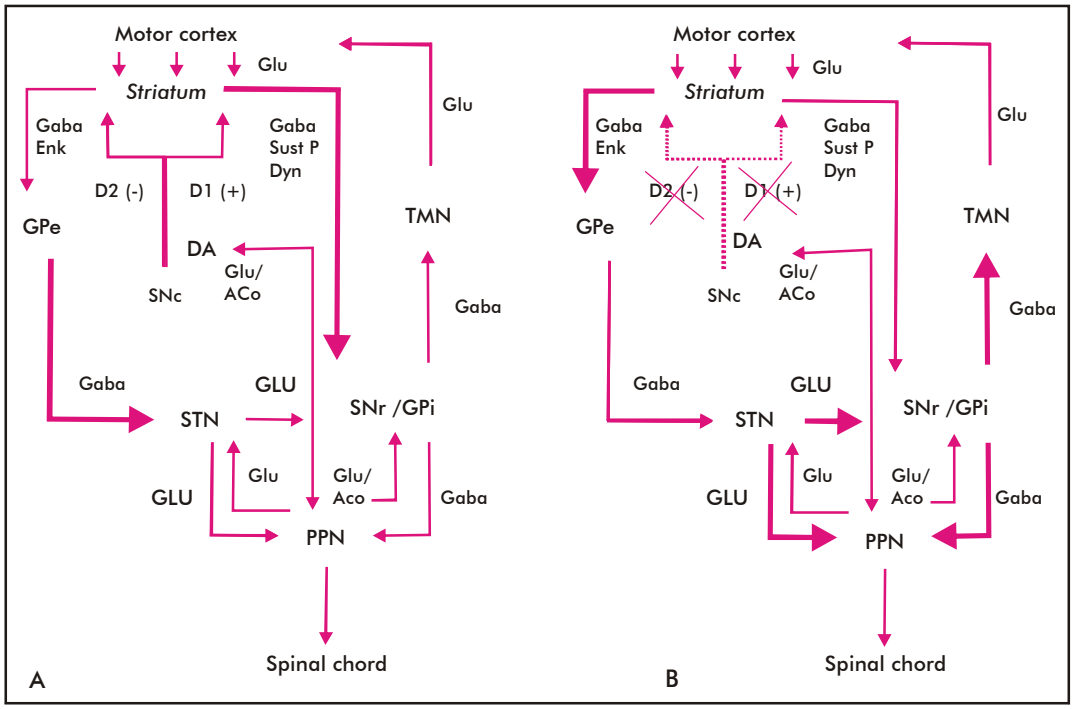

Figure 1. Functional diagram of the motor circuit of the basal ganglia. A. Healthy condition. Dopamine facilitates transmission through the striatonigral «direct pathway» mediated by the dopaminergic D1 receptor and inhibits transmission through the «indirect pathway» mediated by the D2 receptor. B. Parkinsonian status. The effect of dopamine is lost, with an increase in transmission through the «indirect pathway» and a decrease in transmission through the «direct pathway» of the motor circuit.
Legend:
Glu: glutamate, GABA: γ-aminobutyric acid, DA: dopamine, ACo: acetylcholine, Enk: enkephalins, Subst P: Substance P, Dyn: dynorphins, Gpe: external *Globus pallidum*, STN: subthalamic nucleus, GPi: internal *Globus pallidum*, SNr: *substantia nigra pars reticulata*, SNc: *substantia nigra pars compacta*, PPN: pedunculopontine nucleus, TMN: thalamic motor nuclei. Thicker arrows represent facilitation or increase in transmission; thinner lines represent a decrease in transmission. The dashed lines represent the degeneration of the nigrostriatal pathway.

A number of reports published during the last decade agree on considering the PPN as an important structure for the physiopathology of Parkinsonism, based mainly on 3 facts: the location of the PPN on the locomotor mesencephalic area, its involvement in the control of posture and locomotion, and its anatomical and functional relationship to the basal ganglia [10-11].

The physiological changes observed in the PPN for rodent and primate animal models of PD have further emphasized the importance of this structure in processing motor information [11, 16, 17]. An increase in the electric 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 [18]. Other authors describe the reversion of akinesia that characterizes parkinsonian primates by the injection of gabaergic antagonist drugs in the PPN [19]. This last group [19] has also described significant motor improvements for parkinsonian primates subjected to the electrical stimulation of the PPN, and has recently considered the possibility of using this new target for improving the akinesia of parkinsonian patients through deep electric stimulation [20].

The cerebral microdialysis technique has been used to study the release of neurotransmitters in structures of the central nervous system [21-24]. The fact that it offers information on the composition of the extracellular milieu in a particular nucleus *in vivo* has made this methodology an essential tool in neurochemical studies in the last 10 years [25]. However, in spite of the advances in cerebral microdialysis, there are for reports on the patterns of release of aminoacidic neurotransmitters (glutamate (GLU) and γ-amino butyric acid (GABA)) in the PPN of Parkinson animal models [26]. This is explained by a major involvement of other nuclei, such as the SNc, in the motor dysfunctions that characterize PD, which has attracted the attention of most groups focused on the study of dopaminergic neurotransmission. Additionally, although the link between dopaminergic deficiencies and Parkinsonism has long been established, only recently has the PPN come to be recognized as a key structure in the physiopathology of PD.

The paper on the electric activity in pontine cells and its changes in experimental models of Parkinsonism offer very limited information on the patterns of release of neurotransmitters in the PPN, as well as on the effect of the neurotoxic injury of the SNc on the presence of neuronal death processes in this nucleus.

The aim of the present paper is to study the release of GLU and GABA on the PPN of hemiparkinsonian rats, and the evaluation of the occurrence of neuronal

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death on ipsilateral pontine cells after the injection of 6-OHDA.

Materials and methods

Experimental subjects

The study used adult male Wistar rats weighing from 200 to 250 g, from the Center for the Production of Laboratory Animals (CENPALAB, La Habana, Cuba). Three animals were housed per cage throughout the experiment, with a light-darkness cycle of 12-12 hours and water and feed were offered *ad libitum*. The experimental work complied with the *Guidelines for the care, use and reproduction of laboratory animals (…)* [27].

SNc lesion

The rats were anesthetized by the intraperitoneal (i.p.) injection of a chloral hydrate solution (420 mg/kg body weight), and placed on a stereotactic surgery device for rodents (Stoelting, USA); after which they were injected with 3 µL of a solution of 6-OHDA (St. Louis, USA, 8 µg/ 3 µL of 0.9% physiological saline solution + 0.5 mg/mL ascorbic acid) at a rate of 1 µL/min in the right SNc. The procedure used the following stereotactic coordinates (mm), as described in the Atlas from Paxinos and Watson [28]: AP = -0.49, L = 0.17, DV = 0.81.

Rotational activity

One month after the SNc lesion, the rotational activity induced by D-amphetamine (5 mg/kg of body weight, i.p. route) was studied. This variable was evaluated for 90 min., using a sensor-coupled electronic multicounter (LE 3806, PanLab, Barcelona, Spain) that detects the sense of rotation. The study only included animals with at least 7 turns per minute, corresponding to a degree of dopaminergic denervation of 90% or higher (data not shown) [29]. The negative control group (false injured) was formed by animals receiving an injection of 0.9% physiological saline solution (NaCl) with the same volume and stereotactic coordinates.

A total of 3 experimental groups were used: healthy rats (n = 12), rats with SNc injury (n = 11), and rats with a false SNc injury (n = 10).

Microdialysis probes

Concentric-type microdialysis catheters were manufactured as described in the literature [30], using a 1 mm segment from a polyacrylonitrile dialysis membrane with a molecular weight cut-off of 41 000 Da (Hospal Industrie Meyzie, France). The recovery percentage of the microdialysis catheters was determined *in vitro* at room temperature, using standards of known concentration for the amino acids.

In vivo microdialysis

The surgical implantation of the guide cannulae was performed one month after the injection of 6-OHDA on the SNc. Using standard stereotactic techniques, a stainless steel guide cannula was glued to the cranium at the coordinates (mm) corresponding to the right PPN, ipsilateral to the SNc injury: AP = -8.00, L = 1.90, DV = 6.82 (adjusted to the cerebral microdialysis catheter).

The animals were allowed to recover and, three days after the surgery, a microdialysis catheter was introduced through the guide after removing its protective stylet. All the procedures were performed while the animals were fully awake.

The microdialysis probes were connected to an infusion pump (CMA 100; CMA *Microdialysis*, Stockholm, Sweden), and continuously perfused with a solution of artificial cerebrospinal fluid (aCSF) prepared in the laboratory (125 mmol of NaCl, 2.5 mmol of KCl, 0.5 mmol of NaH_2PO_4, 5 mmol of Na_2HPO_4, 1 mmol of MgCl . $6H_2O$, 1.2 mmol of $CaCl_2$, 1.2 mmol of ascorbic acid, pH 7.4 -7.6) at a rate of 2 µL/min. After a stabilization period of 2 h, the samples were manually collected every 20 min.

A total of six dialysates was collected per rat, which were immediately stored at -80 °C for further analysis.

Amino acid analysis

Amino acid concentration in the dialysates was determined by high performance liquid chromatography (HPLC) coupled to fluorometric detection, after derivatization with OPA.

A total of 10 µL of the sample were mixed with 10 µL 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 sample was placed on a shaker for 15 s, and the reaction was stopped by adding 5% acetic acid after 45 s. Twenty microliters of this mixture 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 x 4.6 mm internal diameter, ESA), with a pre-column having a similar stationary phase, using an isocratic chromatography pump (Knauer K1001), and detected with a fluorescence detector set at l_{exc} - 340 nm and l_{exc} = 460 nm (Philips PU 4027). The chromatographic data were recorded using the CHROMATEPC software application, version 4.24 (Philips). The mobile phase was composed of 0.1 M NaH_2PO_4 and 20% methanol. Each sample was analyzed by duplicate.

Morphological study

Tissue sampling: The animals were anesthetized with chloral hydrate (480 mg/kg of body weight, i.p. route), and then perfused through the ascendant aorta. The brains were extracted and fixed in 10% formalin for 24 h. Coronal sections (20 µM) 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 catheters 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, as described in the literature [31].

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

Obtaining and preparing the tissue: The animals with SNc lesions were anesthetized with chloral hydrate (480 mg/kg body weight, i.p. route) and slaughtered by decapitation. The brains were extracted immediately and submerged into 0.9% saline physiological solution at 4 °C, after which they were fixed in a phosphate solution (SBF, 0.1 M, pH 7.4) containing 4% paraformaldehyde and 2% glutaraldehyde for 2 days at 4 °C. After fixing, they were submerged in solutions of increasing sucrose concentration (15%, 20%, 25%, and 30%) for two hours each (24 h for the 30% solution). Coronal sections (10 µM) of the PPN were obtained (Leitz 1720 cryostat, Germany), which were then placed into slides that were stored at 4 °C.

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). A permeabilizing solution was first used (0.1% Triton-X 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 washed three times in phosphate buffer, and then an alkaline phosphatase-conjugated fluoresceinated goat antibody was added (Roche Molecular Biochemical, Germany) and incubated for 30 min at 37 °C. After washing, the substrate was added and incubated for 10 min. at room temperature.

The sections were stained with propidium iodide (Sigma, USA) for 2 min, and, finally, covered with a Vectashield mounting medium containing DAPI at 1.5 µg/mL (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) [32].

Data analysis

The data were tested for normal distribution and homogeneity of variance using the Kolmogorov-Smirnov and the Bartlett tests, respectively. The release of GLU and GABA in the PPN of the experimental animals was compared using a single-classification analysis of variance, followed by Turkey's test. The level for statistical significance was 0.05. The data were processed and analyzed using the statistical software application package Statistica, ver. 6.1.

Results

Release of GLU and GABA in the PPN

The comparison of the release of GLU 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 concentrations of this neurotransmitter in rats with SNc injuries (Figure 2A). Likewise, the comparison of the release of GABA again showed a significant increase in the PPN of rats with SNc injuries ($F_{(2, 31)} = 26.51$, $p < 0.01$) (Figure 2B).

Morphological and immunohistochemical studies

The morphological study confirmed that the cerebral microdialysis device had been correctly placed on the coordinates corresponding to the PPN, located on the distal part of the superior cerebellar peduncle (Figure 3).

The DNA fragmentation study (TUNEL) did not detect TUNEL+ cells in the left PPN, contralateral to the site of the DA injection into the SNc (Figures 4A-4C). 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 (Figures 4D-4F).

There were DAPI+ cells in the left PPN contralateral to the 6-OHDA injection, but none was immunoreactive to TUNEL (Figure 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 cases (Figure 5B).

Discussion

The results of this study evidence the changes in the release of GLU and GABA in the PPN of hemiparkinsonian rats, and are the first description of pontine cell death in this experimental model.

Effect of the SNc lesion on the extracellular concentrations of GLU and GABA on the PPN

Most clinical and experimental studies that try 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 and from there to the spinal cord (Figure 5A) [33-35].

The significant increase of the release of GLU in the PPN of hemiparkinsonian rats agrees with the evidence showing that glutamatergic transmission in the basal ganglia is significantly modified in the parkinsonian status [36]. The death of dopaminergic

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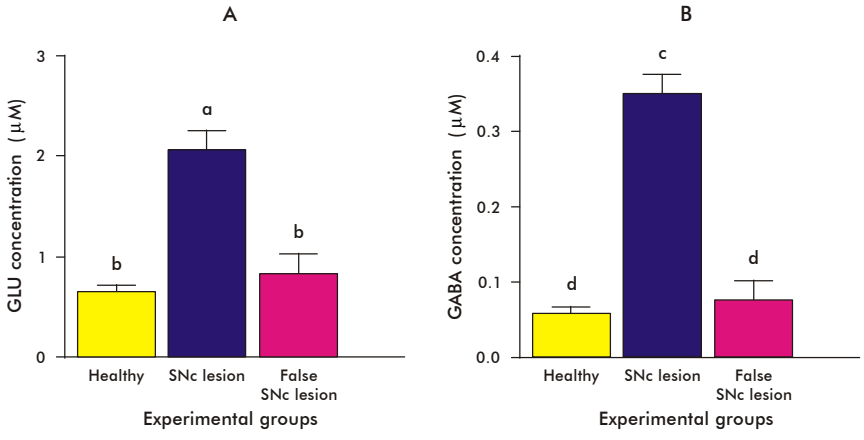

Figure 2. Release of glutamate and GABA in the PPN of healthy, hemiparkinsonian and falsely lesioned rats. A. Inter-group comparison of glutamate release. B. Inter-group comparison of GABA release. The groups were compared using a single-classification analysis of variance, followed by Tukey's test. Differing letters on the bars indicate statistically significant differences: a *vs.* b ($p < 0.001$) and c *vs.* d ($p < 0.01$).

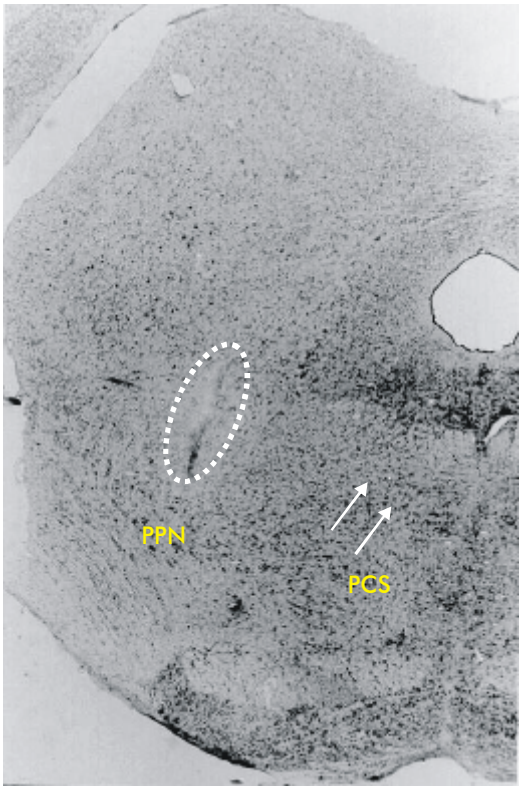

Figure 3. Representative microphotograph of a coronal section stained with cresyl violet, illustrating the site where the cerebral microdialysis catheter is located (5x). The area in the dotted circle is the trace left by the probe in the tissue, at the distal part of the superior cerebellar peduncle.
Legend
PPN: Pedunculopontine nucleus, UCP: superior cerebellar peduncle.

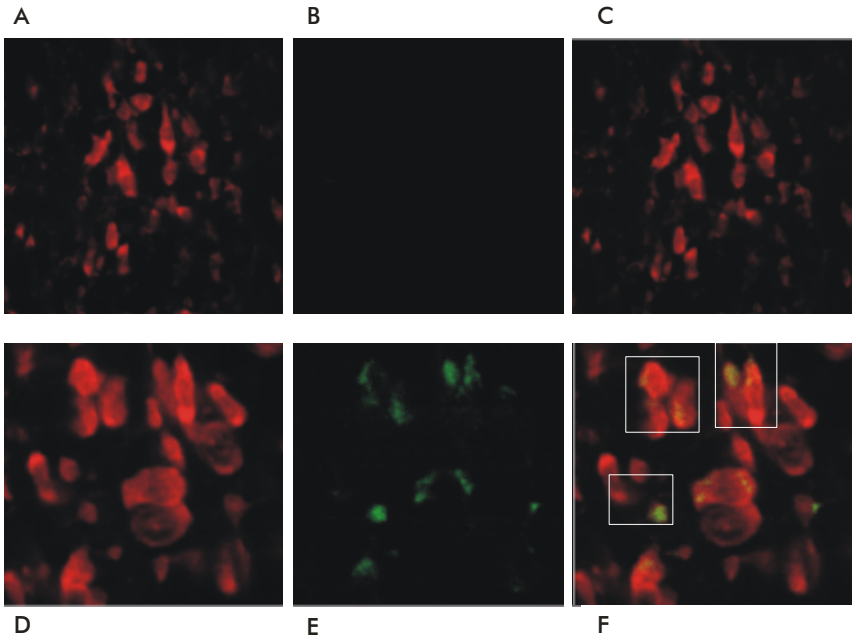

Figure 4. Representative microphotograph, taken with a confocal microscope, of the immunohistochemical study of the cell death process in coronal sections of the PPN of rats with a SNc lesion produced by the injection of 6-OHDA. (A-C 40x). A. Propidium iodide staining in coronal sections of the left PPN, contralateral to the SNc lesion. B. TUNEL-negative cells in the left PPN, contralateral to the SNc lesion. C. Double propidium iodide + TUNEL labeling shows the cell nuclei without evidence of cell death in the left PPN. (D-F 40x2) D. Propidium iodide staining in coronal sections of the right PPN, ipsilateral to the SNc lesion. E. TUNEL-positive cells in coronal sections of the right PPN. F. Double propidium iodide + TUNEL staining in coronal sections of the right PPN. The rectangles show cellular bodies undergoing cell death.

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 [37]. Consequently, there is an increase in neurotransmission through this pathway, which involves the STN (whose cells express GLU as the neurotransmitter) [38] (Figure 1B).

The increase in the release of GLU in the PPN may contribute to perpetuation of the subthalamic hyperactivity, since 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 [34, 39, 40]. The fact that the expression of the mRNA coding for subunit I of cytochrome oxidase is significantly increased in the pontine neurons of parkinsonian rats that project to the STN further substantiates the involvement of the PPN in subthalamic hyperactivity [41].

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, and might, at the same time, have a fundamental role in the stimulation of dopaminergic cells surviving neurotoxic injury. The SNc receives cholinergic and glutamatergic projections from the PPN, and both acetylcholine (ACo) and GLU are capable of modulating the activity of nigral cells [42].

Recent studies have revealed that in parkinsonian rats there is a significant increase on the release of GLU in other structures that, like the PPN, receive glutamatergic afferences from the STN; this is the case of the GP and SNr [43, 44].

The increased release of GABA in the PPN of hemiparkinsonian rats observed in this study may be explained by SNr and Gpi hyperactivity. The glutamatergic neurons of the *pars dissipata* from the PPN of the rats receive a gabaergic projection from these nuclei, whose hyperactivity constitutes a distinctive feature of the changes in the neurotransmission systems during Parkinsonism [11, 45-47].

Although the increase in activity of the pontine cells (fueled by the increased release of GLU of subthalamic origin) may have a positive effect through the stimulation of dopaminergic cells surviving the lesion, this very same phenomenon may perpetuate the hyperactivity of the GP/SNr complex, whose inhibitory gabaergic discharges support some of the characteristic signs of PD such as hypokinesia and bradykinesia [48]. The PPN stimulates the gabaergic cells from the SNr through nicotinic cholinergic receptors, and the dopaminergic cells of the SNc are stimulated through receptors for excitatory amino acids such as glutamate and cholinergic receptors that can be either nicotinic or muscarinic [37]. In this context, a gabaergic feedback to the PPN may contribute to the attenuation of this effect.

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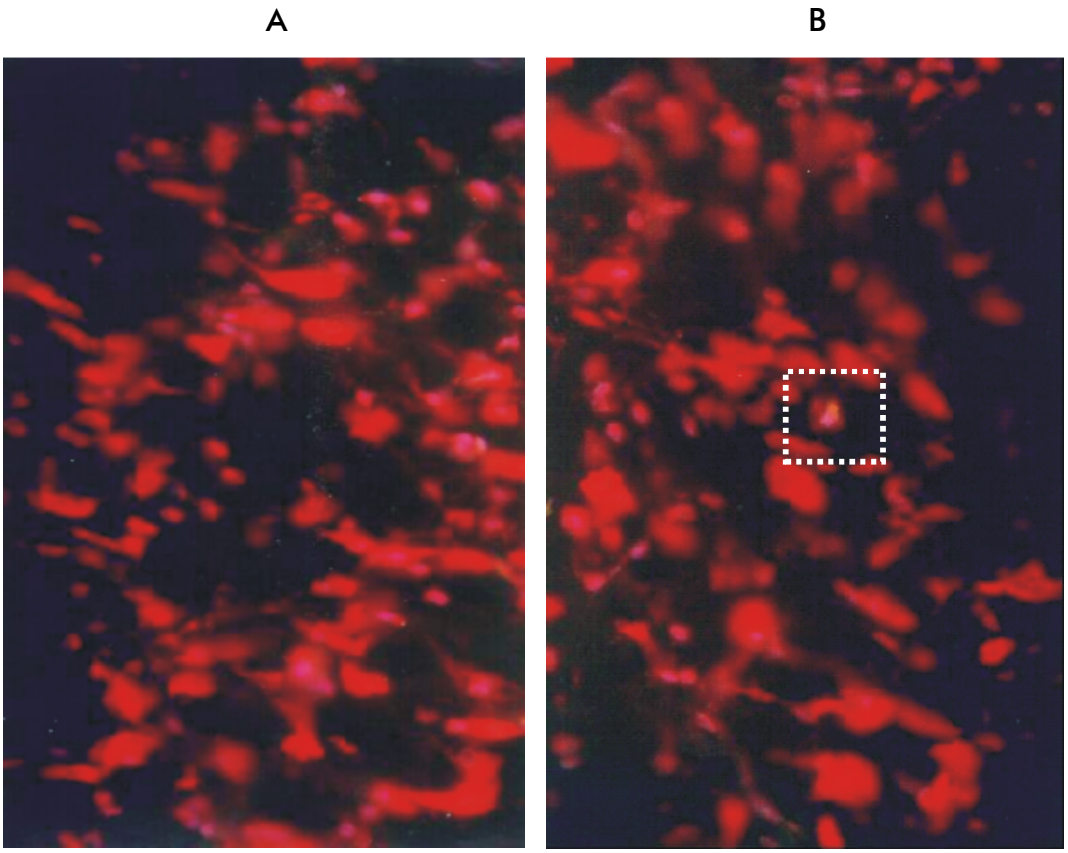

Figure 5. Representative microphotograph (40x) for a coronal section of the PPN, illustrating the results of the TUNEL and DAPI immunohistochemistry study. A. PPN of the non-injured left hemisphere. B. PPN of the right hemisphere, ipsilateral to the SNc lesion, showing a cell with positive labeling for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

Cell death on the PPN of hemiparkinsonian rats

Some studies have documented losses of approximately 40% and even 50% of the cholinergic cells on the lateral part of the PPN, as well as a strong correlation between the death of dopaminergic cells in the SNc and that of presumably cholinergic cells in the PPN of deceased parkinsonian patients [33, 49]. This finding suggests that both cellular populations may have been subjected to the same destructive process, although this hypothesis has not been confirmed thus far [50].

Less is known about the processes of cellular death in the PPN of animals used as experimental models of PD. A recent publication indicated that the morphology, distribution, and number of cholinergic cells in the PPN of non-human primates and hemiparkinsonian rats were no different from those of matched controls, although the presence of cell death in other cellular populations, such as glutamatergic neurons, was not discarded [51].

The results from the present study show that after the injection of neurotoxin 6-OHDA in rats there are signs of cell death in the PPN ipsilateral to the site of injection. However, the TUNEL immunohistochemical technique can not distinguish between glia and neurons, or between cholinergic and glutamatergic cells, although both are present in the PPN. TUNEL is based on the extension of free 3'-OH ends generated by endonucleases with TdT and digoxigenin-labeled dUTP [52].

The literature suggests that many experimental models based on cellular damage produce cell death by apoptosis or necrosis, with a range of intermediate morphologies. The capacity of a cell to enter a programmed cell death may be modulated by damage to neighboring cells, loss of effective heterophagocytosis, or an imposed cellular damage that leads to an energy failure and, ultimately, the interruption of the stages of the apoptotic sequence, thereby promoting the corresponding necrotic cascades [53]. It has been pointed out that cell death is actually an apoptosis-necrosis continuum, with cellular forms in which characteristics from both processes can and in fact do coexist. TUNEL immunohistochemistry is unable to differentiate between such forms [54].

In this context, and considering our findings, the question arises on which of the following factors trigger the processes of cell death in this structure either: the neurochemical imbalance characterized by a significant increase in the glutamatergic tone, the loss of dopaminergic innervation from the SNc, or the modifications in receptor phosphorylation status associated to the changes in the systems of neurotransmitters. Possibly, each one provides important signals that may together trigger neuronal death.

The increase in the glutamatergic tone, represented in this study by an increased release of GLU in the neighborhood of the PPN, has been associated to a rise in the intracellular calcium concentration that goes beyond the limits of the homeostatic mechanisms that

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normally keep this parameter at a constant value (100 nM) [55, 56].

GLU is eliminated from the synaptic cleft and from the extracellular milieu by a Na+-dependent amino acid carrier protein with a high affinity for monocarboxylic acids, located in both pre- and post-synaptic neurons, as well as on glial cells [57, 58]. An efficient capture of GLU contributes to the completion of the synapse, since there are no known enzymes in the synaptic cleft that degrade this amino acid [59].

The excitation mediated by an increase in glutamatergic activity (frequently referred to as excitotoxicity) is regarded as a common factor among neurological disorders such as epilepsy, neurodegenerative disorders, ischemic damage and the consequences of exposure to environmental toxins [57, 60].

There are examples of slow and fast excitotoxic mechanisms in the literature [61]. The fast mechanisms involve an excessive activation of the glutamatergic N-methyl D-aspartate (NMDA) and non-NMDA receptors, leading to a massive rush of sodium and calcium into the cells through the ion channels activated by glutamate itself [62]. The increase in the intracellular concentration of calcium is potentiated, in turn, by the release of calcium from the organelles that normally function as intracellular storages for this ion, such as the endoplasmic reticulum [62, 64]. These conditions lead to the activation of several enzymes such as proteases, phospholipases and calcium-dependent endonucleases, that degrade the proteins of the neuronal cytoskeleton, membrane phospholipids, and the cellular DNA, respectively [65]. A slow excitotoxicity is produced when the cells can not maintain their resting membrane potential, thus relieving the inhibition of the Mg^{2+} ion on the activity of the NMDA receptor under physiological conditions [61]. Under excitotoxic conditions, the physiological concentrations of GLU in the synaptic cleft are high enough to activate the NMDA glutamatergic receptors [66].

Other factors, related to an increased intracellular production of free radicals that ultimately leads to cell death, can be linked to the loss of the dopaminergic tone in pontine cells. The administration of agonist drugs for dopaminergic D1 receptors attenuates the rate of necrotic cell death with an oxidative origin during mouse cortical cell culture [67].

The oxidative stress hypothesis has been used to explain the death of dopaminergic cells from the SNc [68-69]. Although the mechanisms related to an aberrant production of free radicals and to the failure of antioxidative systems in the nigral cells have not been confirmed as the only cause of this neurodegenerative process, there is evidence supporting their involvement as important factors in the signaling pathways that lead to neuronal death [54, 68].

PPN cells under the influence of an increased glutamatergic transmission might become susceptible to oxidative damage, since it is known that an exacerbated glutamatergic activity, secondary to the neurotoxic lesion of the SNc, is associated to excitotoxicity processes that lead to the failure of ATP-dependent ionic pumps and mitochondrial metabolism. Both factors can trigger an increase in intracellular oxidative stress [54].

An excessive release of GLU has been associated to the activation of a secondary group of protein kinases and the phosphorylation of glutamatergic NMDA receptors; some of these enzymes are tyrosine kinase, mitogen-activated protein kinase, and cAMP-dependent protein kinase [70]. Although the substrates of these enzymes are not well known, it has been shown that their inhibition or the inhibition of NMDA receptors can prevent cell death during the culture of hippocampal or cerebellar neurons, or of spinal motoneurons [71, 72].

The pontine neurons in the right hemisphere may be the point of confluence of the mechanisms mentioned above, derived from the increase in glutamatergic neurotransmission and from the loss of the dopaminergic efference from the SNc. The combined action of both mechanisms 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 discarded.

The pontine activity is intact in the left PPN, contralateral to the site of injection of the neurotoxin 6-OHDA. This observation underlines the role of the imbalance between different systems of neurotransmitters that is typical of the parkinsonian state in the elicitation of cell death mechanisms in structures such as the PPN.

These results confirm that the death of dopaminergic cells in the SNc due to the injection of 6-OHDA is associated to changes in the patterns of release of neurotransmitters such as GLU and GABA in structures that are anatomically and functionally closely related to basal ganglia, such as the PPN. These changes, together with the loss of the dopaminergic tone, may be the triggering factors for mechanisms of cell death in pontine cells, which might contribute, on the long run, to nigral degeneration and the establishment of an imbalance between neurotransmission systems.

It would be very interesting to study the molecular events and structural details characterizing the cell death process in the PPN. These studies would confirm or reject the necrotic course hypothesis proposed in this paper.

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