

## Effects of the local injection of 6-OHDA on the density of BDZ and $\mu$ opioid receptors of the substantia nigra pars compacta and pedunclopontine nucleus of rats

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### ABSTRACT

Although there is evidence suggesting a relationship between Parkinsonism and modifications to gabaergic and opioid activity in basal ganglia and related structures, the published results are not conclusive, and the molecular mechanisms underlying these changes remain unknown. Here we study the changes in population density of benzodiazepine (BDZ) and  $\mu$  ( $m$ ) opioid receptors in an animal model of Parkinsonism induced by the intracerebral administration of 6-hydroxydopamine in Wistar rats, using three experimental groups (untreated, treated with 6-OHDA, and treated with saline). One month after inducing lesions on the *substantia nigra pars compacta* (SNc) all rats were slaughtered by decapitation and frozen coronal sections of the samples, representative of the SNc and the pedunclopontine nucleus (PPN), were obtained and studied by autoradiography for BDZ receptors (using 3H-flunitrazepam) or for  $\mu$  opioid receptors (using 3H-DAMGO). The optical density (OD) was measured in SNc and PPN of both hemispheres, using the OD readings of the tritium standards to determine tissue radioactivity values for the accompanying tissue sections and to convert them to fmol/mg protein. The density of BDZ receptors showed statistically significant variations among the experimental groups, being down-regulated both in the SNc ( $p < 0.001$ ) and PPN ( $p < 0.001$ ), ipsilateral to the site of the 6-OHDA injection. On the other hand, whereas the density of  $\mu$  opioid receptors showed statistically significant variations in the PPN ( $p < 0.01$ ), being ipsilaterally down-regulated in the animals receiving 6-OHDA injections, there was no such variation ( $p > 0.05$ ) in the SNc. The results prove that there are changes in the density of BDZ and  $\mu$  opioid receptors in the SNc and the PPN upon intracerebral administration of 6-OHDA, which may constitute one of the steps of the sequence of molecular and neurochemical events underlying the imbalance between "direct" and "indirect" pathways of basal ganglia which is typically seen in Parkinsonism. Additionally, these results underscore the importance of the mesopontine tegmentum in the physiopathology of Parkinson's disease.

Keywords: SNc, NPP, BDZ receptors,  $m$  opioid receptors, 6-OHDA

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### RESUMEN

**Cambios en la densidad de receptores a benzodiazepinas y  $m$  opioides en la *substantia nigra pars compacta* y el núcleo pedunclopontino de ratas, por inyección local de 6-hidroxidopamina.** Existen evidencias de las modificaciones en la actividad gabaérgica y opioide en los ganglios basales y en otras estructuras relacionadas con estos, asociadas al parkinsonismo; pero los resultados informados no son concluyentes y aún no se conocen las bases moleculares que subyacen en estos cambios. Estudiar los cambios en la densidad de receptores benzodiazepínicos (BDZ) y  $m$  ( $m$ ) opioides en el modelo de enfermedad de Parkinson inducido por la administración intracerebral de 6-hidroxidopamina (6-OHDA), en ratas. Se organizaron tres grupos de ratas Wistar: sanas, lesionadas con 6-hidroxidopamina y falsas lesionadas. Un mes después de la lesión de la *substantia nigra pars compacta* (SNc) se sacrificaron todas las ratas por decapitación. Se obtuvieron cortes coronales representativos de SNc y del núcleo pedunclopontino (NPP), en los cuales se practicó la técnica de autorradiografía para receptores BDZ (con el empleo de <sup>3</sup>H-flunitrazepam) y para receptores  $m$  opioides (con el empleo de <sup>3</sup>H-DAMGO). Se realizó la lectura de la densidad óptica (DO) en SNc y NPP de ambos hemisferios, la cual se convirtió en fmol/mg de tejido sobre la base de los valores obtenidos en los estándares de tritio. La densidad de receptores BDZ mostró diferencias significativas, desde el punto de vista estadístico, entre los grupos experimentales. Esta variable disminuyó tanto en la SNc ( $p < 0.001$ ) como en el NPP ( $p < 0.001$ ) ipsilateral a la inyección de 6-OHDA. La densidad de receptores  $m$  opioides en la SNc no exhibió diferencias significativas ( $p > 0.05$ ) entre los grupos experimentales. Esta variable mostró una disminución estadísticamente significativa en el NPP ipsilateral a la inyección de 6-OHDA ( $p < 0.01$ ). De acuerdo con los resultados, existen cambios en la densidad de receptores BDZ y  $m$  opioides en la SNc y el NPP asociados con la administración intracerebral de 6-OHDA. Estos cambios pudieran representar un eslabón en la cadena de eventos moleculares y neuroquímicos que caracterizan el desbalance entre las dos vías de proyección, las cuales conducen la información motora, desde la corteza motora pasando por los núcleos que conforman los ganglios basales, el tálamo y el regreso a la corteza motora en condiciones de parkinsonismo.

Palabras claves: SNc, NPP, receptores BDZ, receptores  $m$  opioides, 6-OHDA

## Introduction

Most neurochemical studies carried out on experimental models of Parkinson's disease (PD) have focused on the dopaminergic system [1, 2]. The study of other neurotransmitters, such as amino acids and opioid peptides, has largely depended on experimental designs centered on the determination of the effect of dopaminergic drugs on these molecules [3-6]. Additionally, although the populations of dopaminergic receptors have been extensively studied in models of Parkinsonism, less is known about the behavior of receptors for other neurotransmitters in PD.

The GABA<sub>A</sub> receptors are part of a macromolecular complex known as the benzodiazepine (BDZ) receptor, which includes a selective chloride channel and binding sites for different molecules, including benzodiazepines [7, 8]. Benzodiazepine drugs act by improving gabaergic transmissions through an increase in the opening frequency of the chloride channel and, consequently, a decrease in neuronal excitability [7, 9, 10].

A number of evidences suggest that the chronic stimulation of dopaminergic receptors results in an increased regulation of the GABA<sub>A</sub> / BDZ complex [11], and this mechanism has been postulated to be involved in the dyskinesias induced by L-DOPA in the experimental primate models of Parkinsonism, but not in humans [11]. Similarly, dopamine is thought to modulate the activity of the GABA<sub>A</sub> / BDZ receptor complex, mainly in the *striatum* [9].

On the other hand, there are reports in the literature on changes in the peptidergic neurotransmission of basal ganglia related to nigral degeneration [12]. Many neuropeptides localize the circuits of basal ganglia [13-15]. The "direct pathway" of the motor circuits co-expresses, together with  $\gamma$ -aminobutyric acid (GABA), peptides such as substance P and dynorphins, whereas the "indirect pathway" co-expresses GABA and enkephalins [16, 17]. From the point of view of their anatomical localization, the *mu* ( $\mu$ ), *delta* ( $\delta$ ) and *kappa* ( $\kappa$ ) opioid receptors are found in the *substantia nigra pars compacta* (SNc), the *Globus pallidus* (GP), the *striatum* and the cortex [16, 18]. The opioid antagonist known as (-) naloxone is used as an anti-parkinsonian drug, mainly for the treatment of the dyskinesias induced by L-DOPA [19, 20]. Similarly, studies on the neuroprotective role of (-) naloxone have revealed that this drug inhibits the production of superoxide anion free radicals in microglia, and this effect might attenuate or slow down the progress of neurodegenerative processes [21].

Currently, the role of the pedunculopontine nucleus (PPN) on the physiopathology of PD is the subject of some debate [22-25]. From a neurochemical point of view, the PPN is regarded as a heterogeneous structure [26]. This nucleus sends a cholinergic projection, together with another, presumably glutamatergic projection, to the SNc and the subthalamic nucleus (STN) [24, 25]. Simultaneously, the PPN receives dopaminergic, glutamatergic and gabaergic afferences from the SNc [4, 27], the NST [27-29] and the efferent structures of the basal ganglia, *substantia nigra pars reticulata* (SNr) and internal segment of the GP [29], respectively. The pontine cells express glutamatergic receptors for N-methyl D-aspartate (NMDA) and  $\alpha$ -amino-3-hydroxy-5-

methylisoxazole-4-propionic acid (AMPA) [30], cholinergic, muscarinic and nicotinic receptors, [31], GABA<sub>A</sub> gabaergic receptors [32, 33], and receptors to different neurotrophins such as fibroblast-derived growth factor [26].

The purpose of this paper is to study the changes in density of  $\mu$  opioid and BDZ receptors in a rat model of hemi-parkinsonism by the intracerebral injection of 6-hydroxydopamine (6-OHDA).

## Materials and methods

The animals used were adult male Wistar rats, with a weight range of 200 to 250 grams, obtained from the Center for the Production of Laboratory Animals (CENPALAB, La Habana, Cuba). Three animals were kept per cage throughout the experiments, with a cycle of 12 hours of light alternating with 12 hours of darkness, and water and food *ad libitum*. The experimental work respected and followed the Practical Guidelines for the use of laboratory animals.

### SNc lesions

The rats were anesthetized by the intraperitoneal (i.p.) administration of chloral hydrate at 420 mg/kg, after which they were placed in a stereotaxic surgery device for rodents (David Kopf Instruments, U.S.A.). They received a 3  $\mu$ L injection of a solution of 6-OHDA (8  $\mu$ g/3  $\mu$ L of 0.9% physiological saline solution and 0.5 mg/mL ascorbic acid), at a rate of 1  $\mu$ L/min, on the right SNc, using the following stereotactic coordinates, according to the atlas of Paxinos and Watson [34]: AP = -4.4 mm; L = 1.2 mm; V = 7.8 mm.

One month after the SNc lesion, the rotatory activity induced by D-amphetamine (5 mg/kg, i.p.) was analyzed. This variable was studied for 90 min., using an electronic multiscaler (LE 3806, PanLab, Barcelona, Spain), coupled to sensors to detect the direction of rotation (LE 902, PanLab, Barcelona, Spain). The study only included animals displaying more than 7 turns per minute, which corresponds to a dopaminergic denervation equal to or higher than 90% [35, 36]. A control group of «mock-injured» animals was produced by administering physiological saline solution (0.9% NaCl) under the same conditions.

The animals were distributed in three experimental groups: healthy rats (n = 9), rats with SNc lesions (n = 7) and rats with mock SNc lesions (n = 5).

Upon concluding the study of rotatory activity, the rats were anesthetized by the i.p. administration of a higher dose of chloral hydrate (480 mg/kg, i.p.) and slaughtered by decapitation. Brains were extracted and, after freezing in dry ice, they were stored at -80 °C until further processing.

For the autoradiography studies, a series of six coronal sections (20  $\mu$ m) were obtained using the coordinates of the SNc and the PPN. The SNc first appeared on the anteroposterior coordinate -4.3 and disappeared on coordinate -6.8; and the PPN first appeared on the anteroposterior coordinate -7.3, disappearing on coordinate -8.8.

### Autoradiography for BDZ and $\mu$ opioid receptors

For the autoradiography experiments for the detection of BDZ receptors, the cerebral sections were incubated

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for 45 min at 4 °C in a solution containing 2.08 nM of 3H-flunitrazepam (exogenous agonist for the site of BDZ action in the GABA<sub>A</sub> complex) in Tris-HCl 170 mM, pH 7.6 buffer, in the absence or presence of 1  $\mu$ M diazepam. Receptor binding in the presence of diazepam was considered as unspecific [38].

The autoradiographies for  $\mu$  opioid receptors were performed by prewashing the brain sections in a solution of Tris 50 mM buffer for 30 min. at room temperature, and then incubating for 60 min. at 25°C in a solution containing 2 nM [<sup>3</sup>H]Tyr-D-Ala-(Nme)Phe-Gly-ol] (3H-DAMGO) (exogenous agonist for  $\mu$  opioid receptors) in Tris HCl 50 mM buffer, in the absence or presence of 2  $\mu$ M naloxone (competitive antagonist for opioid receptors). Receptor binding in the presence of this compound was regarded as unspecific [39].

For both receptor populations, the incubations were finished by two successive washings in Tris HCl 50 mM pH 7.5 (1 min. each) at 4 °C. Finally, the sections were rinsed in distilled water for 2 s at 4°C and dried with cold air.

### Exposure and development of the sections

The sections were placed in lead cartridges for autoradiography together with tritium standards, and exposed to tritium-sensitive film (Kodak MR-2). The cartridges were kept away from light, at room temperature, for two weeks in the case of the BDZ receptors and for 10 weeks for the  $\mu$  opioid receptors. The films were development using the Kodak (D 11) developer and high-speed fixer. The optical density (OD) of the autoradiograms was measured using an image analysis software application (JAVA, Jandel Video Analysis Software). The OD readings from the standards were used to determine radioactivity values for the tissue in the adjacent sections. For each area studied, a minimum of 10 OD readings were taken, in at least 5 sections, and the mean was computed. Readings were taken from both hemispheres for each structure under study. The OD values were converted to femtomoles per milligram of tissue, using the values obtained from the tritium standards.

### Data processing

Compliance of the data to a normal distribution was checked with the Kolmogorov-Smirnov test. Receptor density of the different experimental groups was compared using a single classification analysis of variance, followed by a separate Tukey test for each structure and receptor. For groups in which the density of receptors of the structures was compared between the left and right brain hemispheres, a Student's t-test was used. All statistical tests used a minimal value of 0.05 for statistical significance.

## Results

### BDZ receptor density in the SNc

The comparison of the BDZ receptor density between both brain hemispheres revealed statistically significant differences for the group of rats with SNc lesions ( $t = 5.50, p < 0.01$ ), with a decreased value for this variable on the right SNc, ipsilateral to the site of injection of 6-OHDA. The remaining groups did not have

statistically significant differences for this variable between both hemispheres (figures 1A and 2A).

When comparing the experimental groups, there was a statistically significant decrease of this variable in the group of rats with SNc injuries (figures 1B and 2A).

### BDZ receptor density in the PPN

The comparison of the BDZ receptor density between both brain hemispheres revealed a statistically significant decrease of this variable in the right PPN of the rats with SNc lesions ( $t = 5.26, p < 0.01$ ). There were no statistically significant differences between the brain hemispheres of the remaining experimental groups (figures 1C and 2B).

The comparison between experimental groups for BDZ receptor density in the PPN revealed statistically significant differences ( $F_{(2, 18)} 8.54, p < 0.001$ ). These differences were caused by a statistically important decrease of this variable in the group of rats with SNc injuries, compared to the remaining control groups (figures 1D and 2B).

### Density of $\mu$ opioid receptors

There were no statistically significant differences ( $p > 0.05$ ) for the density of  $\mu$  opioid receptors in the SNc, whether comparing brain hemispheres or experimental groups (figures 3A and 4A).

However, the same analysis performed in the PPN showed a statistically significant decrease in this value when comparing both brain hemispheres, located in the right PPN, ipsilateral to the site of the 6-OHDA injection ( $t = 3.00, p < 0.05$ ) (Figure 3B). In turn, when comparing the experimental groups it showed a statistically significant decrease in the group of rats

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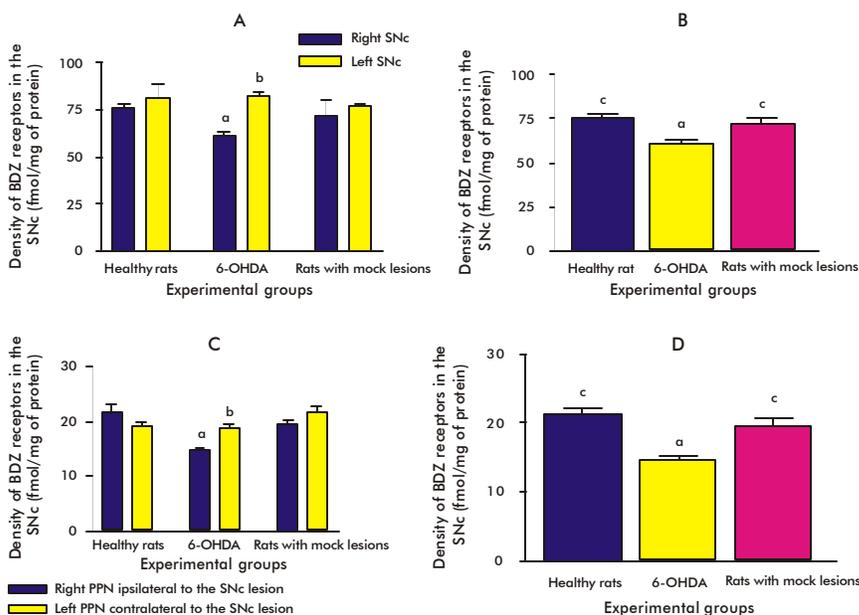


Figure. 1 Effect of the intracerebral injection of 6-hydroxydopamine (6-OHDA) on the density of benzodiazepinic receptors in the substantia nigra pars compacta (SNc) and pedunculopontine nucleus (PPN). A and C) Comparison between hemispheres (Student's t-test); B and D) Comparison among groups (single classification analysis of variance and Tukey test). Legend: The presence of different letters atop the bars indicates statistically significant differences: a vs. b ( $p < 0.05$ ) and a vs. c ( $p < 0.001$ ).

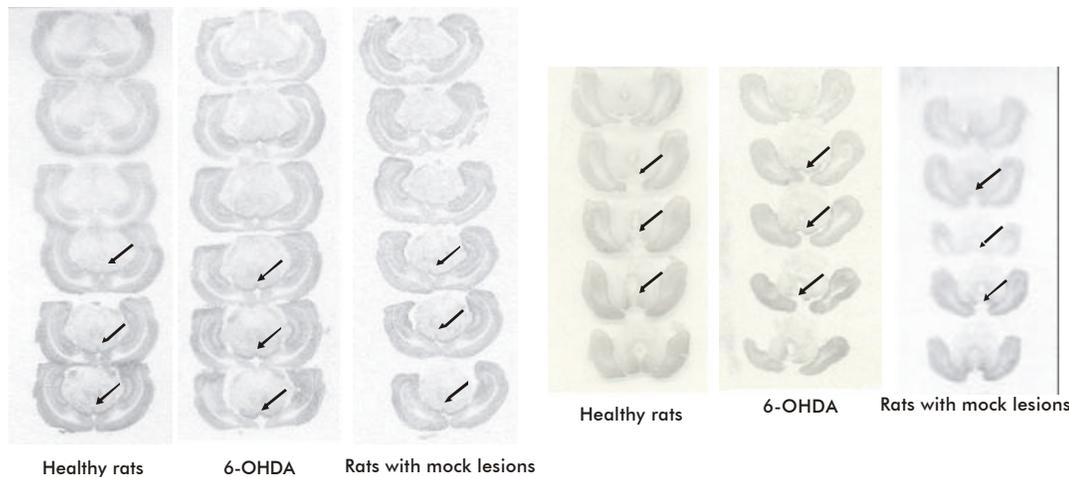


Figure. 2 Autoradiograms for BDZ receptors in sequential coronal sections of: A) *Substantia nigra pars compacta* B) Pedunculopontine Nucleus, on each of the experimental groups.

Legend: The arrows point to the zone chosen for the optical density readings in the right hemisphere for each structure been under study. BDZ: benzodiazepinic receptors.

with SNc injuries, in contrast with the control groups ( $F_{(2, 14)} = 6.02, p < 0.01$ ) (figures 3C and 4B).

### Discussion

The gabaergic and peptidergic neurotransmission systems have been extensively studied in experimental models of the addiction to psychotropic drugs and other substances such as alcohol and opiates, as well as in models of epilepsy [20, 40-44]. When these systems have been studied in experimental models of Parkinsonism, the analyses have involved a wide range of *in vitro* and *in vivo* experimental techniques, such as histological studies [43], *in situ* hybridization for measuring mRNA expression levels [44] and pharmacological manipulations using brain microdialysis [47], among others.

#### BDZ receptor density

Although there are reports on the activity of the gabaergic  $GABA_A$  and  $GABA_B$  receptors in the context of Parkinsonism, there is very little information available on the changes of the BDZ site of the  $GABA_A$  receptor in experimental models for this disorder.

The present study shows the presence of changes in the populations of BDZ gabaergic receptors which are associated to nigral degeneration. These changes suggest that there is a significant modification of gabaergic neurotransmission in the 6-OHDA model.

The decrease in the values of this variable in the SNc might be explained by the neurotoxicity of 6-OHDA. The nigral cells receive a gabaergic projection from the external segment of the GP [13, 14]. Since it has been described that the  $GABA_A$  gabaergic receptor has a mainly post-synaptic localization [7], the loss of nigral cells should have a sensible effect on the presence of these receptor populations.

The decrease in density of the BDZ receptors in the PPN ipsilateral to the 6-OHDA lesions suggests that there are changes in gabaergic activity in the PPN under conditions of Parkinsonism. Although the PPN is located anatomically outside the basal ganglia, it is

however highly connected to them through reciprocal connections involving several nuclei [22]. The PPN is a point of convergence for projections of varying nature and origin, including glutamatergic, gabaergic and dopaminergic projections from the NST, SNr and/or Gpi, and the SNc, respectively [26-29].

According to the currently accepted functional model for basal ganglia [16, 48], during the parkinsonian status there is an increase in gabaergic activity in the output

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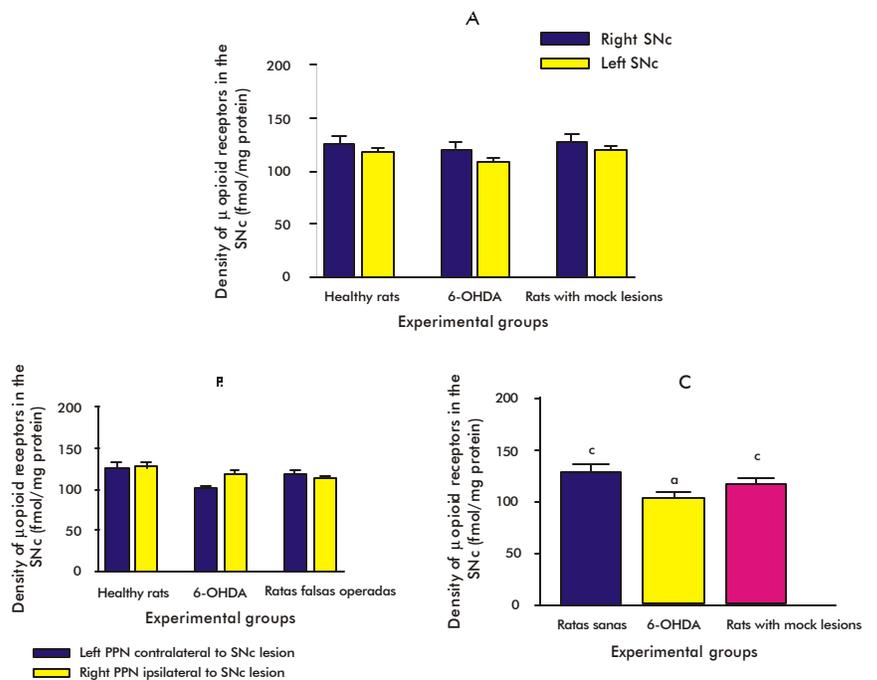


Figure 3. Effect of the intracerebral injection of 6-hydroxydopamine (6-OHDA) on the density of  $\mu$  opioid receptors in the *substantia nigra pars compacta* (SNc) and the pedunculopontine nucleus (PPN). A and B) Comparison between hemispheres (Student's t-test); C) Comparison between groups (single classification analysis of variance and Tukey test). The different letters atop the bars indicate statistically significant differences: a vs. b ( $p < 0.05$ ) and a vs. c ( $p < 0.01$ ).

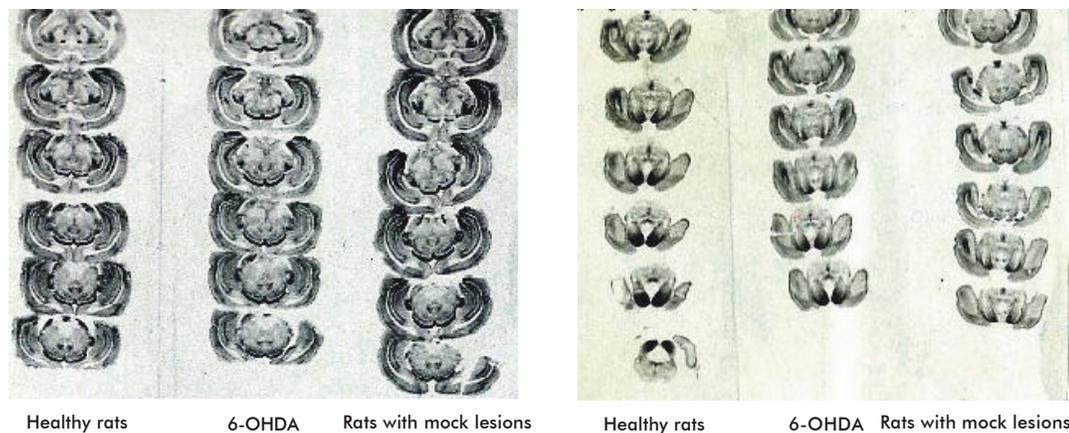


Figure 4. Autoradiograms for  $\mu$  opioid receptors in coronal sections of: A) *Substantia nigra pars compacta* (SNc); B) *Pedunculopontine nucleus* (NPP), for each one of the experimental groups. Legend: The triangles and small rhombi indicate the zone chosen for the optical density readings in each structure.

nuclei of the basal ganglia, as a result of the hyperactivity of the indirect pathway of the motor circuit [48, 49]. It is known that there is a significant increase in the release of GABA in the PPN of hemiparkinsonian rats [49]. Additionally, the decrease in the density of BDZ receptors in the PPN ipsilateral to the 6-OHDA lesions further supports the hypothesis that a compensatory mechanism for adaptation to the increase in gabaergic activity is established, which would be an expression of the mechanisms of synaptic plasticity at this level [50].

Other authors have pointed out that the microinjection of agonist drugs for GABA<sub>A</sub> receptors, such as bicuculine in the PPN, attenuates the akinesia and other parkinsonian symptoms in models of Parkinsonism in non-human primates based on the systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [51]. Bicuculine is a GABA<sub>A</sub> antagonist that decreases the frequency and average time for the opening of the chloride channel, which constitutes an integral component of the GABA<sub>A</sub> receptor macromolecular complex [7]. This drug competes with GABA for one or several receptor binding sites, and it has been postulated that one of these sites may be the BDZ site of the GABA<sub>A</sub> receptor [7].

Other studies deal with the role of the BDZ receptors in the *striatum*, showing an increase of the density of these receptor populations in the putamen of brains from deceased parkinsonian patients [52]. This finding underscores the relationship between the degeneration of the nigrostriatal dopaminergic route and the state of the populations of gabaergic receptors [53].

The changes in density of the BDZ receptors in the PPN represent a link in the chain of molecular and neurochemical events that characterize the imbalance between the two projection routes for the passage of motor information from the cortex, passing through the nuclei in the basal ganglia and the thalamus, and back to the motor cortex, which is typical of Parkinsonism.

#### Density of $\mu$ opioid receptors

This study failed to find any modifications in the density of  $\mu$  opioid receptors in the SNc which could

be attributed to a parkinsonian status. This may be explained by the existence of a compensatory mechanism in the dopaminergic cells surviving the neurotoxic damage caused by the injection of 6-OHDA. These cells receive different excitatory projections which modify their rate of synthesis and storage of neurotransmitters, as well as the phosphorylation status of their receptors [28, 36, 47]. In general, opioid receptors have this type of activity [15, 18], with the net result of a tendency toward normality, as observed in this investigation.

The results show that there was a discrete decrease in the density of  $\mu$  opioid receptors in the PPN ipsilateral to the 6-OHDA injection. This is especially significant, considering the fact that no peptidergic afferences reaching the PPN have been described, although, on the other hand, the PPN has an important representation of opioid receptors [26].

It is known that the interactions between opioid peptides and their cognate receptors does not always follow the classical synaptic pathway of the release from a pre-synaptic terminal and binding to receptors in post-synaptic terminals [15, 54]. It is also known that peptides can follow alternative transmission routes, such as diffusion to receptors located in structures which do not necessarily receive the corresponding afferent innervations [54]. These are some of the reasons why the peptides are classified as neuromodulators, rather than classical neurotransmitters [15].

The changes in density of the  $\mu$  receptors in the PPN may have their origin in changes in the diffusion of some of the endogenous peptides, such as  $\beta$ -endorphins, which constitute the best ligand for this type of receptors [55]. However, taking into account that the ligand:receptor ratio for opioid peptides is not 1:1, it is possible that the changes in  $\mu$  receptor density may also be due to other peptides [56].

The interaction of endogenous opioid peptides (enkephalins, cephalins, dynorphins and  $\beta$ -endorphins) with their respective receptors modulates a number of intracellular activities: mitochondrial respiration, ionic channel activity and synthesis of mediators of the immune response, among others [57, 21]. It has been pointed out that these peptides, alone, do not trigger

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changes in membrane potential of the post-synaptic neuron, but rather modulate the capacity of other neurotransmitters to modify neuronal excitability, thereby justifying their classification as neuromodulators [58].

There are significant changes in peptidergic activity during Parkinsonism [12]. The studies using binding assays have revealed marked regional differences regarding the number of neuropeptide receptors found in the brain of deceased parkinsonian patients, as compared to healthy persons [12].

The interactions between the different receptors are often established through the molecules responsible for intracellular signaling, such as protein G [59, 60]. Several types and subtypes of protein G-coupled receptors form heterodimeric complexes with this protein that then modulate the activity of other receptors of the family of ionic channels or specific for kinase-type enzymes [59].

These interactions do take place between gabaergic and opioid receptors, and have been studied in models of epilepsy [61, 62].

Although finding a possible relation between the changes in the populations of BDZ receptors and those of  $\mu$  opioid receptors in the PPN of hemiparkinsonian rats is not the objective of this study, it is interesting to note that the modifications in both receptors, in both cases, result in a decrease in their density.

It has only been ten years since PPN was first considered as a key factor for a thorough understanding of the physiopathology of PD. The changes in this structure regarding the populations of  $\mu$  opioid and BDZ receptors in the model of 6-OHDA confirm the dysfunctional nature of these neurotransmission systems during PD, providing evidence that underscore the importance of the mesopontine segment in the function of the basal ganglia.

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