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*Review article*

# Study of the Neuropeptide Function in Parkinson's Disease Using the 6-Hydroxydopamine Model of Experimental Hemiparkinsonism

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**Abstract:** Parkinson's disease, one of the most common neurodegenerative diseases, characterized by unilateral brain dopamine damage in its initial stages, remains unknown in many respects. It is especially necessary to improve the early diagnosis and, in order to improve the treatment, to go thoroughly into the knowledge of its pathophysiology. To do this, it is essential to perform studies in appropriate animal models of the disease. One of those is generated by the unilateral intracerebral administration of the neurotoxic 6-hydroxydopamine that produces clear asymmetrical cerebral dopamine depletion. Currently the neuronal coexistence of several neurotransmitters is obvious. Particularly interesting is the coexistence of dopamine with various neuropeptides. If the neuronal content of dopamine is asymmetrically altered in the early stages of the Parkinson's disease, the coexisting neuropeptides may also be asymmetrically altered. Therefore, their study is important to appropriately understand the pathogenesis of the Parkinson's disease. The function of the neuropeptides can be studied through their metabolism by neuropeptidases whose activity reflects the functional status of their endogenous substrates as well as the one of the peptides resulting from their hydrolysis. Here we review the 6-hydroxydopamine model of experimental hemiparkinsonism as an

appropriate model to study the initial asymmetric stages of the disease. In particular, we analyze the consequences of unilateral brain dopamine depletions on the functionality of brain neuropeptides through the study of the activity of cerebral neuropeptidases.

**Keywords:** Parkinson's disease; neuropeptides; neuropeptidases; dopamine; brain asymmetry

## 1. Parkinson's Disease

Parkinson's disease (PD) like Alzheimer's dementia is considered the most common neurodegenerative disorder of aging brain. Clinically, the tetrad of tremor at rest, slowness of voluntary movements, rigidity, and postural instability characterize PD. However, patients with PD exhibit other symptoms such as autonomic and cognitive disorders beyond motor consequences [1]. PD is a neurodegenerative disease that results mainly from the death of the dopaminergic neurons in the pars compacta of the substantia nigra (SN), whose axons project to the striatum (ST). At the first stages, the degenerative process develops unilaterally becoming progressively bilateral over the years [1]. The first clinical signs and symptoms appear only after a loss of 50%-70% of the dopaminergic neurons. Unfortunately, the diagnosis of these major neurodegenerative disorders remains insufficient, being mainly based on clinical criteria and limited laboratory investigations. Whereas its pathogenesis begins to be understood as the result of a better knowledge of multiple various deleterious factors, the true etiology of PD remains largely mysterious and, until now, very little is known about why and how this neurodegenerative process begins and progresses. Most observations on its pathogenesis come from studies performed in experimental models essentially produced by neurotoxins, the 6-hydroxydopamine (6-OHDA) being one of the most widely employed [2, 3]. This neurotoxin destroys the dopaminergic neurons of the nigrostriatal system causing depletion of dopamine (DA), the main feature of the disease. However, other neurotransmitters, such as some neuropeptides like oxytocin, enkephalins or angiotensins, may be also involved in the pathogenesis of PD [1].

## 2. Neuropeptides and Coexistence of Neuropeptides and Classical Neurotransmitters

As opiate narcotics exhibit a marked degree of stereospecificity, Goldstein *et al.*[4] argued that the active isomers should bind stereospecifically to receptors. Based on this proposal, Pert *et al.* [5] and Simon *et al.* [6] identified and characterized specific receptors for those active narcotic isomers.

Obviously, these definite receptors, although having high affinity for morphine derivatives, had to exist to bind the endogenous opiates. The discovery of these substances did not take long before two small peptides called Leu- and Met-enkephalin were soon discovered having high affinity for the receptors discovered by Hughes *et al.* [7]. These findings were at the beginning of a real revolution in the field of neuroscience. Numerous brain peptides (neuropeptides) grouped in families [8] were added to a small number of classical neurotransmitters to investigate the brain function. Brain research became more complex, but certainly more interesting and stimulating. Neuropeptides are defined strictly as "*small proteinaceous substances produced and released by neurons through the regulated secretory route and acting on neural substrates*" [9]. However, if the discovery of neuropeptides was a milestone in brain research, another crucial step, even more interesting, was the finding that different types of neurotransmitters, such as classical neurotransmitters and neuropeptides, could coexist in the same neuron. Thus, in the 1980s, several works [10-12] among many others showed that the so-called Dale's principle [13] was not correct since it was shown that in the same neuron different types of neuropeptides, classical neurotransmitters as well as other neurotransmitter substances [8] could coexist. Particularly, dopaminergic neurons of mesencephalon and hypothalamus contain DA and neurotensin (NT) [14, 15]. Most dopaminergic neurons of the SN express cholecystokinin (CCK) [16] and neurons of the ventrotegmental area (VTA) contain NT as well as CCK [17]. Hypothalamic neurons containing DA also have other peptides such as galanin and/or opiates [14]. These significant findings immediately drew attention to the subcellular storage of such substances in the neuron. It is now widely accepted that large synaptic vesicles store neuropeptides whereas the small ones accumulate classical neurotransmitters such as DA [18].

Therefore, if different types of neurotransmitters coexist in a neuron, its damage will affect the functions that such various neurotransmitters could exert as well as the interactions they exert with other non-lesioned neurons. In consequence, the treatments aimed at compensating the neuronal deficit should consider the different neurotransmitters involved. It is therefore unquestionable that in the neurological disorders in which DA is altered, the functions of coexisting neuropeptides will also be altered. PD is perhaps the most important neurological disorder that primarily involves a degeneration of the brain dopaminergic system. PD patients not only develop the classic motor alterations (also present in animal models) such as tremor at rest, slowness and poverty of voluntary movements, rigidity and postural instability but also autonomic alterations including cardiovascular symptoms, gastrointestinal and urogenital disorders as well as cognitive alterations such as depression or memory impairment [1]. The cause of such alterations will indubitably be not only in the dopaminergic degeneration but also in the deficit of coexisting neuropeptides.

Using the experimental Parkinson's model of animals treated with 6-OHDA, several studies

have analyzed the consequences of dopaminergic neuronal degeneration on the neuropeptides they contain. A brief description of this animal model may be useful in the context of the present review to understand the consequences of the neuropeptide dysfunction.

## 3. Oxidative Stress and 6-Hydroxydopamine

The 6-OHDA and several other synthesized analogs were introduced as catecholaminergic neurotoxins and they have been widely used for both *in vitro* and *in vivo* investigations of neurodegenerative diseases involving dopaminergic alterations. 6-OHDA shares some structural similarities with DA and norepinephrine, exhibiting a high affinity for their plasmatic membrane transporters. Consequently, 6-OHDA can be introduced into both dopaminergic and noradrenergic neurons to cause damage in the peripheral and central nervous system pathways of both monoamines. Therefore, in order to develop a high quality model of PD, with a specific lesion of the nigrostriatal dopaminergic pathway, it is essential to carefully select the mode of administration of 6-OHDA [19]. Regarding its mode of action, it is well accepted that 6-OHDA destroys catecholaminergic neurons by a combined effect of reactive oxygen species and quinones [20]. It was indeed demonstrated that 6-OHDA, once dissolved in an aerobic and alkaline milieu, readily oxidizes, yielding hydrogen peroxide and para-quinone [21].

Like other parkinsonian neurotoxins, 6-OHDA could be administrated by systemic injection. However, contrary to other ways, this route of administration does not produce the desired nigrostriatal lesion but only cause a chemical sympathectomy by damaging the peripheral nervous system [19]. Indeed, 6-OHDA poorly crosses the blood-brain barrier and therefore does not accumulate within the brain parenchyma to reach significant neurotoxic concentrations after systemic injections.

Several sites of injection have been used to cause damage of the central dopaminergic pathways [19]. Intraventricular and intracisternal administration of 6-OHDA produce a bilateral lesion, after few hours, usually with poor recovery of the affected neurons [19]. However, the damage is so severe that animals often die primarily due to the occurrence of marked aphagia and adipsia [22]. Therefore, 6-OHDA must be injected directly into the brain by means of stereotaxic techniques. Accordingly, a much more useful model to induce 6-OHDA damage is the unilateral intracerebral injection, particularly intrastriatal, to successfully destroy a precise catecholaminergic pathway of the brain [23]. To specifically damage the nigrostriatal dopaminergic pathway, the 6-OHDA is injected stereotaxically into the SN, the medial forebrain bundle (that comprises the nigrostriatal tract) or the ST [19, 24]. When 6-OHDA is injected into the SN or the medial forebrain bundle, the cell dies

within the first 24 h [25] and the maximal reduction of striatal DA level is reached within 3-4 d after lesion. In most studies, residual striatal DA content is less than 20% of controls. Interestingly, despite the dramatic loss of dopaminergic neurons in the SN after a medial forebrain bundle injection of a high dose of 6-OHDA, levels of extracellular DA are still close to normal [26]. This can perhaps be explained by a somatodendritic release of DA from the few remaining active neurons of the SN. When injected into the SN, 6-OHDA produces a more exacerbated retrograde degeneration of the nigrostriatal system which take place between one and three weeks after treatment [27]. In addition to the lesion of the dopaminergic system, gliosis is also a major characteristic of the 6-OHDA model [28] and there are many data indicative that the glial response, especially of microglia, exacerbates the degeneration of the dopaminergic neurons [21].

Regarding behavioral motor abnormalities, it was reported that animals surviving to bilateral 6-OHDA lesions exhibit motor abnormalities that could be partially corrected by drugs stimulating the dopaminergic receptors [29]. In contrast, unilateral injections of 6-OHDA cause a typical asymmetric circling motor behavior whose magnitude in rodents depends on the degree of nigrostriatal lesion [27, 30]. This specific behavioral abnormality is most prominent after administration of drugs that stimulate dopaminergic receptors, such as apomorphine (rotation away from the lesion), or drugs that stimulate the release of dopamine, such as amphetamine (rotation toward the lesion), due to physiologic imbalance between the lesioned and the non-lesioned ST. Quantification of this turning behavior has been extensively used to assess the antiparkinsonian potency of new drugs [31], and to study the motor fluctuations in the chronic treatment with levodopa [32].

In conclusion, the unilateral 6-OHDA rat model of experimental hemiparkinson has been and continues to be one of the most widely used experimental models of PD when it comes to analyze its physiopathological basis and consequences, the identification of key neurotransmitter pathways governing the function of the basal ganglia as well as the preclinical testing of new symptomatic therapies, neuroprotective strategies and transplantation approaches [2] (Figure 1). The use of this model is especially interesting because it reflects the initial stages of PD in which there is unilateral dopaminergic damage [1]. Therefore, its study can provide valuable data for the early diagnosis of the disease. Moreover, it can provide data on the behavior of neuropeptidases and, consequently, on their endogenous substrates (neuropeptides) in the early stages of the disease.

As shown in Figure 1, after anesthetizing the animal and placing it on a stereotaxic instrument, an injection of the neurotoxin 6-OHDA is administered into the left or right striatum to produce a left or right experimental hemiparkinsonism [33], according to the stereotaxic coordinates obtained from the atlas of Paxinos and Watson [34]. The neurotoxin has affinity for dopaminergic transporters

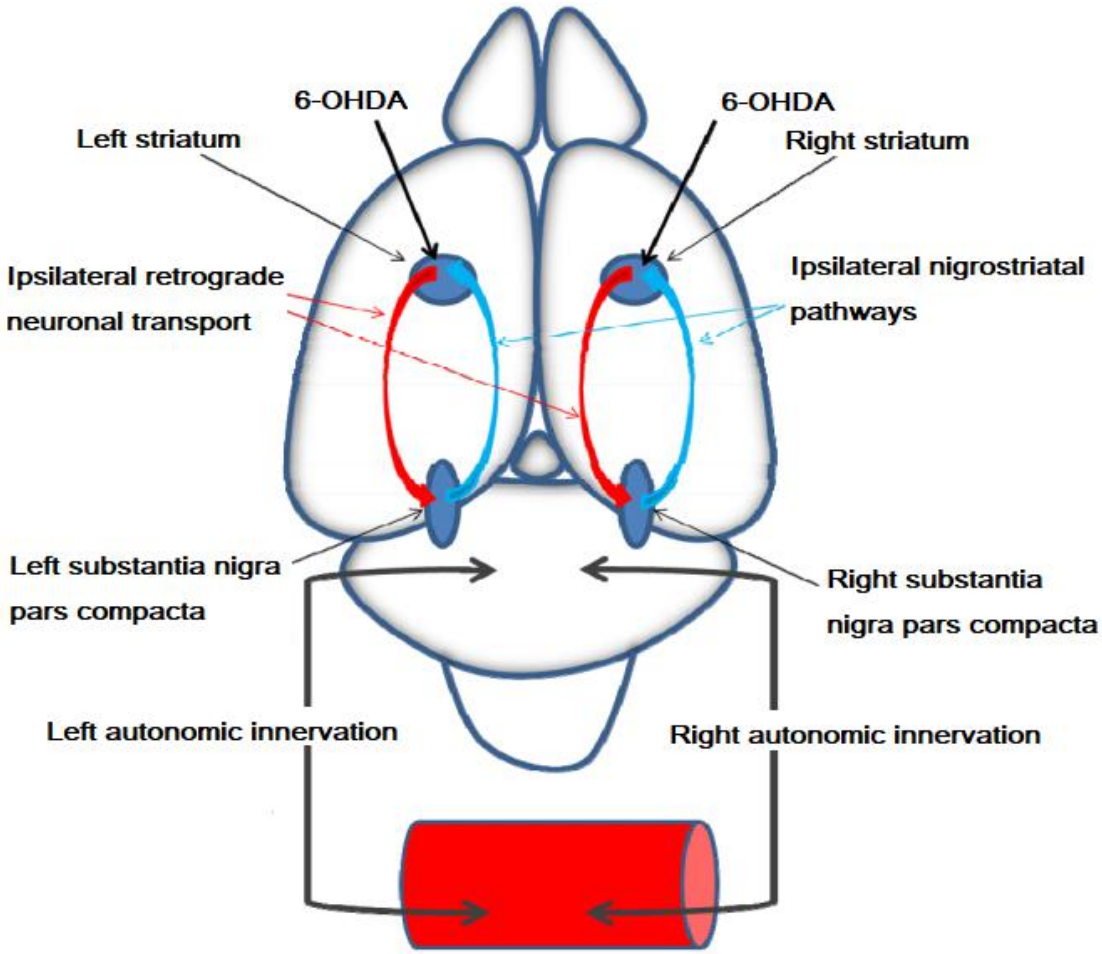


**Figure 1. Animal model of experimental hemiparkinsonism induced by unilateral administration of 6-hydroxydopamine.** 6-OHDA: 6-hydroxydopamine.

located in axonal DA terminals of nigral afferent to the ST. Thus the 6-OHDA is internalized and transported to the soma of neurons of SN. The 6-OHDA has effective influence on the mitochondrial respiratory chain and finally the neurons of SN die by apoptosis in a progressive way [35, 36]. To improve specificity and solely perform lesions of the nigrostriatal pathway, the injection is made into the ST. Since this dopaminergic pathway is unilateral without bilateral crossover, it is essential that only the injured hemisphere is DA depleted, leading to a left or right experimental hemiparkinsonism [23]. The bilateral innervation of peripheral tissues is also indicated.

## 4. Neuropeptides in 6-Hydroxydopamine Lesioned Rats

Luthman *et al.* [37] administered intracerebroventricularly (ICV) 6-OHDA in neonatal Sprague-Dawley rats. After two months, while no alteration of norepinephrine was observed, a drastic reduction of DA was demonstrated in ST and limbic regions. A marked increase of

serotonin was demonstrated only in ST. In addition substance P in ST, accumbens and VTA, CCK in accumbens and NT levels in VTA were reduced. The authors speculate that behavioral disturbances following DA alterations might be due to concomitant modifications in neuropeptides and other neurotransmitters. On the other hand, 6-OHDA lesions of the SN induced the expression of enkephalin, neurotensin, and substance P immunoreactivity in neurons of the globus pallidus [38].

An increase of acetylcholine was observed in ST after depletion of DA from nigro-striatal dopaminergic neurons with 6-OHDA [39]. Using *in vivo* microdialysis, CCK induced an increased release of DA, dynorphin and aspartate levels in ST and SN [40]. After the ICV administration of 6-OHDA, the levels of CCK in the frontal cortex (FC), ST, hippocampus, SN, and nucleus accumbens increased transitorily on day 1. On day 7, the levels fell progressively bellow the basal ones in FC, ST and SN [41]. The co-release of DA and CCK was studied in nigrostriatal neurons of the cat [42].

After 6-OHDA lesions of the SN, CCK levels were not affected in the caudate nucleus but decreased in the SN. While the activation of dopaminergic neurons induced by the nigral application of alpha-methyl-para-tyrosine stimulated the release of CCK and DA in the caudate nucleus, the opposite effects were seen in the SN. DA stimulated CCK release when it was administered into the caudate nucleus. The local application of CCK into the SN increased the firing rate of dopaminergic neurons and stimulated the release of newly synthesized DA from dendrites and nerve terminals [42].

The study of the functional role of neuropeptides can be carried out by analyzing their synthesis, by determining their own levels or by investigating the receptors to which they specifically bind [43]. But their function can also be investigated through the study of their metabolism through the action of proteolytic enzymes that will generate inactive metabolites or new peptides with actions differing from those of their substrates [44].

Aminopeptidases, the most abundant proteolytic enzymes in the brain [45], play an essential role in the inactivation and biotransformation of brain neuropeptides their study being a valuable tool for the analysis of their functional role. How these enzymes act synaptically, inactivating or biotransforming their neuropeptidergic substrates is still under discussion [46-50] (Figure 2). In order to better understand the behavior of neuropeptides in PD, the following section discusses the influence of unilateral hemispheric lesions with 6-OHDA on certain brain aminopeptidases (neuropeptidases) responsible for the hydrolysis of some neuropeptides such as angiotensins, enkephalins, oxytocin or vasopressin.

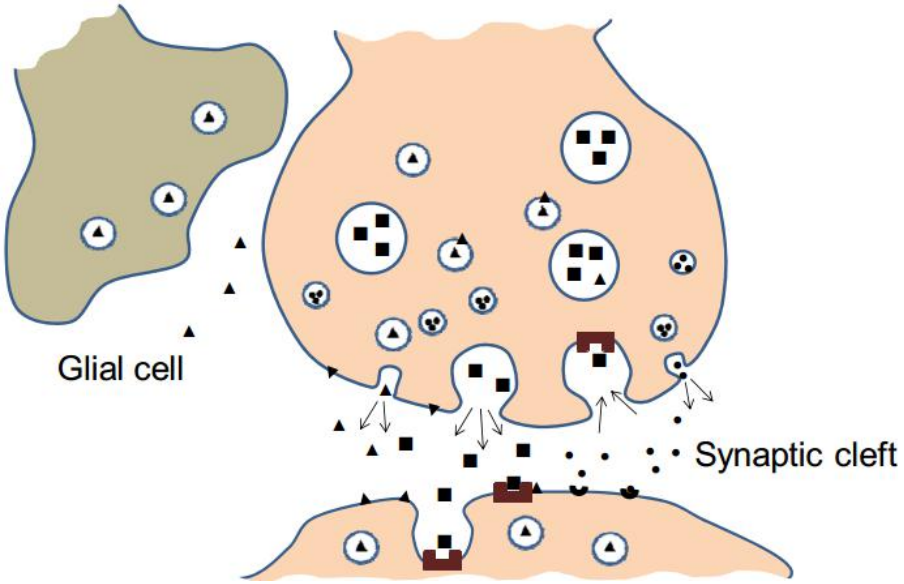


**Figure 2. Hypothetic model of neuropeptide metabolism at the synaptic level.** It is accepted that neuropeptides (■) are stored in large vesicles and classical neurotransmitters such as dopamine (•) in small ones [18]. Neuropeptidases (▲) could be stored in independent vesicles or co-stored in the same vesicles as neuropeptides. Following the release of the vesicular content by calcium-dependent mechanisms and the pre- or post-synaptic neuropeptide-receptor interaction, the neuropeptide may bemetabolized by membrane-bound (pre-or post-synaptic) neuropeptidases, associated or not to receptors, or by soluble neuropeptidases from neural and / or glial origin or, after a potential internalization of the neuropeptide-receptor complex, by fusion with vesicles of enzymatic content. The enzymatic activity and specificity would depend on the type of enzyme (soluble or membrane bound), the cerebral location, the concentration of the susceptible neuropeptide or may depend on the status of the surrounding biochemical milieu [46-50].

## 5. Neuropeptidase Activities in 6-Hydroxydopamine Lesioned Rats

As previously indicated, the study of neuropeptidase activities reflects the functional status of their endogenous neuropeptidergic substrates [44]. PD is an entity that initially develops unilaterally, progressing with years to a bilateral process [51]. If initially, it is an asymmetric alteration, the investigation that is carried out on it should contain primarily this asymmetric aspect. Therefore, the experimental hemiparkinsonism is a very appropriate model to analyze the PD in its initial stages in which not only appear altered motor symptoms, consequence of the unilateral nigrostriatal lesion, but also other asymmetric non-motor autonomic or cognitive alterations [1, 51] that may also involve

other brain regions. In order to search possible interactions between DA and some neuropeptides involved in cognitive and autonomic functions, the study of neuropeptidase activities is a good approach that offers a reflective thought on the function of such neuropeptides. In this regard, an interesting work of Okada and Kato [52] in 1985, in which they surgically disconnected the regions of the nigrostriatal system, suggested a projection of neuropeptidases between ST and SN thus involving directly these enzymes in the function of that system.

More recently, based on the known cognitive alterations observed in the early stages of PD, consequence of the initial unilateral hemispheric damage, the behavior of oxytocinase activity (neuropeptidase that regulates the anxiolytic oxytocin function) was studied in the medial prefrontal cortex (mPFC), a region of the mesocortical dopaminergic system involved in cognitive functions as well as exhibiting asymmetric behavior. A left and right hemiparkinsonism by injections of 6-OHDA into the left or right ST was induced in rats in which the oxytocinase activity was measured in the left and right mPFC. Results demonstrated a marked bilateral change in 6-OHDA lesioned animals in comparison with the bilateral distribution observed in controls: the lateralized distribution of oxytocinase in mPFC of lesioned animals depended on whether the left or right was the hemisphere depleted of DA [53]. On the other hand, Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) are extensively used models for blood pressure (BP) studies but also are validated approaches for mood disorders research. While WKY rats present low motor activity and high anxiety, SHR have high locomotor activity and low anxiety [54]. Unilateral brain depletions of DA by inducing left or right experimental hemiparkinsonism with 6-OHDA injections in both strains were performed. Results demonstrated a marked discrepancy in behavior and lateralization between WKY and SHR but also a striking difference between left or right lesioned animals: the bilateral behavior of neuropeptidases in left DA depleted animals was different to the lateralization observed after right DA depletion [55].

Regarding the autonomic consequences of the dopaminergic alteration in PD and taking into account that asymmetries in the neuroendocrine system [56] may extend from brain regions to the periphery through an asymmetrical autonomic innervation of tissues, the central asymmetric damage of dopaminergic neurons may be also reflected asymmetrically [57-63]. Obviously, aminopeptidases such as oxytocinase, vasopressinase, enkephalinases and angiotensinases modified their levels in plasma depending on whether the depleted hemisphere was the left or right one [57]. In addition, it is well known that nitric oxide (NO) is released to plasma by sympathetic activation [58] and a similar effect was also postulated for aminopeptidases such as the one that metabolizes angiotensin II (aminopeptidase A, AP A) [57]. Angiotensin II, NO and DA interact in the control of BP [59, 60] and AP A and NO are increased in SHR in which the sympathetic system is activated [61]. Based on

these data, it was speculated that the behavior of AP A and NO could differentially reflect in plasma the central asymmetry induced by unilateral depletions of DA [62].

The results demonstrated an inverse relationship between NO and AP A in WKY and SHR this response differing if the injured hemisphere was the left or the right one. Whereas the inverse relationship was observed in right lesioned WKY, it was in the left ones in SHR. Since NO is known to be released to plasma by the autonomic innervation of the vessels and as it was also been suggested for AP A [57], the changes observed depending on left or right brain DA-depletion may reflect asymmetries in the autonomic innervation of the vessels [62].

Finally, in line with these results, a dramatic progressive increase of BP was observed from pre-lesion values until the end of the study in sham-left and left-lesioned WKY and SHR but no differences throughout the study were observed in the right sham or lesioned animals. These results represent direct experimental evidence of an asymmetrical cardiovascular response to unilateral brain lesions, suggesting that left injury may have a worst prognosis [63]. These data are important because they reflect peripherally biochemical and functional changes dependent on the side on which the degenerative process begins. Therefore, they may be susceptible to be used in the early diagnosis of the disease. In fact, studies in patients with PD have led to the proposal of the analysis of plasma aminopeptidases as potential biomarkers of the disease [64, 65].

## 6. Conclusions

The experimental hemiparkinsonism, induced by unilateral hemispheric injections of 6-OHDA, has demonstrated to be an useful model to analyze the central and peripheral consequences of the unilateral dopaminergic damage, on brain neuropeptide and peripheral peptidergic functions as occurs in the early stages of PD. The available data support the assumption that DA interacts asymmetrically with neuropeptides in brain regions involved in cognitive functions and that alterations in these functions due to dopaminergic damage may not only be caused by DA deficit but also by alterations in some neuropeptides as well as in changes in the lateralized distribution of these substances: DA, oxytocin, enkephalin and their corresponding neuropeptidase activities. The data also demonstrate that central asymmetries, induced by left or right DA-depletions, are differentially reflected (probably by asymmetries in the autonomic nervous system) in the periphery by changes in plasmatic NO and aminopeptidase activities and by changes in BP.

## Conflict of Interest

The authors declare no conflicts of interest.

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