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ORIGINAL PAPER

The Hydrolysis of Striatal Adenine- and Guanine-Based Purines in a 6-Hydroxydopamine Rat Model of Parkinson's Disease

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Abstract Parkinson's disease (PD) is characterized by a progressive neurodegeneration in the substantia nigra and a striatal dopamine decrease. Striatal extracellular adenosine and ATP modulate the dopaminergic neurotransmission whereas guanosine has a protective role in the brain. Therefore, the regulation of their levels by enzymatic activity may be relevant to the clinical feature of PD. Here it was evaluated the extracellular nucleotide hydrolysis from striatal slices 4 weeks after a unilateral infusion with 6-OHDA into the medial forebrain bundle. This infusion increased ADP, AMP, and GTP hydrolysis by 15, 25, and 41%, respectively, and decreased GDP hydrolysis by 60%. There was no change in NTPDases1, 2, 3, 5, 6, and 5′-nucleotidase transcription. Dopamine depletion changes nucleotide hydrolysis and, therefore, alters the regulation of striatal nucleotide levels. These changes observed in 6-OHDA-lesioned animals may contribute to the symptoms observed in the model and provide evidence to indicate that extracellular purine hydrolysis is a key factor in understanding PD, giving hints for new therapies.

Keywords Adenosine · Guanosine · Parkinson's · 6-OHDA · NTPDases · 5′-nucleotidase

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Introduction

Parkinson's disease (PD) is characterized by a progressive neurodegeneration in the *substantia nigra* with a subsequent reduction in the striatal dopamine content. Its cause is not well understood. Current therapies do not stop the neurodegenerative process and have several side effects [1]. Administration of 6-hydroxydopamine (6-OHDA) into the rat medial forebrain bundle (MFB) leads to a depletion of dopamine in the basal ganglia [2, 3]. This depletion is, however, not restricted to dopamine in animal models of PD: for instance, the nucleosides adenosine [4] and guanosine [5] also decline in their extracellular striatal levels, but in a lesser degree, with possible consequences in the purinergic signalling and in the expression of the motor symptoms observed.

Endogenous adenosine and ATP modulate the dopaminergic neurotransmission in the basal ganglia [6, 7]. P2

receptor agonists and adenosine A_{2A} receptor antagonists reduce the neurotoxic effects on dopaminergic neurons and improve motor activity in animal models of PD. Therefore, ATP and adenosine may have protective and deleterious effects, respectively, on dopaminergic neurons [8–10].

Guanosine is known by its anti-apoptotic, anti-inflamatory, and neurotrophic effects, and also to its ability to prevent glutamate excitotoxicity by stimulating astrocytic glutamate uptake [11–25]. In animal models of PD, the oral administration of guanosine prevents neuronal death or, at least, reduces motor symptoms [24, 26]. Therefore, PD is a condition in which guanosine levels are likely to be relevant, even implicated, in its pathogenesis.

Since adenine- and guanine-based purine levels may be implicated in PD, understanding how the mechanisms controlling their levels are affected in animal models may be a valuable strategy for understanding the mechanisms underlying this disease or for developing new therapies. Among the biochemical agents involved in the regulation of the extracellular levels of these purines, there are the enzymes that belong to the NTPDase (nucleoside triphosphate diphosphohydrolase) family and the 5′-nucleotidase, which mediate the hydrolysis of triphosphate and diphosphate nucleotides to their respective nucleosides, such as adenosine and guanosine [27, 28]. NTPDase1 hydrolyzes ATP and ADP about equally well whereas NTPDase3 and NTPDase8 reveal a preference for ATP over ADP as substrate. NTPDase2 stands out for its high preference for nucleoside triphosphates and, therefore, has previously also been classified as an ecto-ATPase [29]. NTPDases 4, 5, and 6 exhibit intracellular localization and have a marked preference for nucleoside 5′-diphosphates, especially for UDP and GDP [30]. More importantly, guanosine protects neurons in several models of neurodegenerative processes when it is generated by the activity of the enzymes above-mentioned, as revised elsewhere [22]. Therefore, studying the hydrolysis of adenine- and guanine-based purines is a highly relevant issue in animal models of PD and probably in PD itself. Thus, the aim of this study was to evaluate, 4 weeks after lesioning the *substantia nigra* with a unilateral infusion of 6-OHDA into the rat MFB, (1) the level of adenine- and guanine-based purine hydrolysis and (2) the NTPDase and 5′-nucleotidase mRNA levels from striatal slices ipsilateral to the infusion site.

Experimental Procedure

Chemicals

Nucleotides, Malachite Green Base, Coomassie Brilliant Blue G, Trizma base, and 6-OHDA were purchased from Sigma (St. Louis, MO, USA). The other reagents were of analytical grade.

Subjects

Adult male Wistar rats (290–350 g, 3–4 months old) were obtained from the Animal House Unit of the Department of Biochemistry of the Federal University of Rio Grande do Sul and kept on a 12-h light/dark cycle (lights on from 7 a.m. to 7 p.m.) in a temperature-controlled (22°C) colony room. The animals were housed 5 by cage with access to water and standard lab chow ad libitum. They were handled in accordance with the governmental and Brazilian Experimental Biology Societies Federation guidelines.

6-OHDA Treatment

The animals were anaesthetised with 40 mg/kg of sodium thiopental and placed in a stereotaxic apparatus. After that, they received two unilateral infusions of vehicle (0.2% ascorbic acid-saline, Sham) or 6-OHDA (11.0 μg) into the right MFB (1st infusion: 0.5 μL/min, 2.5 μl, AP: −4.4 mm, LL: −1.8 mm, DV: −8.8 mm from bregma; 2nd infusion: 3.0 μl, AP: −4.0 mm, LL: −1.6 mm, DV: −9.0 mm; incisor bar: −3.3 mm). An injection needle attached to a 10-μl microsyringe (Hamilton, 701 N) and an infusion pump (Insight, Brazil) were used. The needle was kept in the brain for 4 min after each infusion to allow drug diffusion. Rats received post-operative care until awake and were returned to their home cages. A second control group was used in which the animals were not submitted to the surgical procedure (Control). Two weeks after the surgical procedure, each animal was challenged with 3 mg/kg of amphetamine i.p. (Sigma, USA) and, 40 min later, it was placed on an 80 cm-diameter circular arena for 20 min. The number of ipsilateral and contralateral rotations was registered.

Two weeks after amphetamine challenging, the rats were individually sacrificed by decapitation and the biochemical analyses were carried out. Different cohorts of animals were used either for the hydrolysis assays, immunohistochemistry, or the RT–PCR. In the 6-OHDA group, only animals that presented rotational behavior were used.

Striatal Slices

The brains were rapidly removed to the incubation medium (bicarbonate-buffered salt solution with 115 mM NaCl, 3.0 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM glucose, 2.0 mM $CaCl_2$, pH 7.4), which was gassed with a 95% O_2 and 5% CO_2 mixture. Then, they were longitudinally cut, the striatum ipsilateral to the lesion dissected,

and coronal 400-μm thick slices were obtained with a McIlwain chopper.

Assay for Nucleotide Hydrolysis

Two slices per tube from the striatum ipsilateral to the infusion site (approx. 0.16 mg protein) were preincubated for 10 min at 37°C with 500 μL of the incubation medium and gassed directly with 95% O_2 and 5% CO_2. Then, the incubation was started by adding one of the adenine- or guanine-based nucleotides at 2.0 mM at 37°C, being stopped 10 min later by adding 100 μl of 10% trichloroacetic acid. The amount of nonenzymatic Pi released from nucleotides into the assay medium without slices and of Pi released from slices without nucleotide was subtracted from total Pi released during incubation. All assays were performed in duplicate or triplicate. Pi was measured according to the literature [31] and the enzymatic activity is expressed as nmol of Pi/min/mg. Previously, it was established that striatal purine hydrolyses are linear in the incubation period and protein concentration ranges used in the present study.

Semi-Quantitative RT–PCR

Total RNA from the striatum ipsilateral to the infusion site was isolated with TRIzol™ Reagent (Invitrogen) and the cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen) from 3 μg of total RNA in a total volume of 20 μl with an oligo (dT) primer. cDNA reactions were performed for 50 min at 42°C and stopped by boiling for 5 min. Two microliters of cDNA were used as PCR template with primers specific for 5′-nucleotidase, NTPDases1, 2, 3, 5, 6, and β-actin (0.4 μM) in a total volume of 25 μl. dNTPs (200 μM) and 1 U Taq polymerase (Invitrogen) were included in the supplied reaction buffer.

The PCR cycling conditions, the amplification products, and the set of primers are described elsewhere [32]. Seven microliters of the PCR reaction were analyzed on a 1%-agarose gel containing gel Red. The Low DNA Mass Ladder (Invitrogen) was used as a molecular marker and normalization was performed employing β-actin as a constitutive gene. The relative abundance of each mRNA versus β-actin was determined by densitometry using ImageJ 1.37 for Windows.

Protein Determination

Protein was determined by the Coomassie Blue method using bovine serum albumin as standard [33].

Tyrosine Hydroxylase Immunohistochemistry

Three rats were selected for tyrosine hydroxylase (TH) immunohistochemistry. They were anesthetized (ketamine + xilazine mix) and transcardially perfused with 0.9%-NaCl solution (150 mL) followed by a 4%-paraformaldehyde solution (about 150 mL) before the removal of their brain. The brains were further post-fixed overnight in a 4%-paraformaldehyde phosphate buffer saline (PBS) solution (pH 7.4) and then cryoprotected in a 30%-sucrose PBS solution until they were completely submerged. Coronal slices (45 μm) were obtained using a −20°C cryostat (Leica Microsystems GmbH). Midbrain slices were washed five times for 5 min each in PBS at room temperature and incubated with a rabbit anti–TH primary antibody (AB 152, Chemicon/Millipore, USA, 1:750) at room temperature for 18 h. After that, they were washed five times for 5 min each in PBS and incubated with the secondary antibody (AlexaFluor 568 A11036, Molecular Probes, USA, 1:400) at room temperature for 60 min. After another wash, sections were mounted and received an antifading solution (Fluorsave, Calbiochem, USA) and coverslipped. Antibodies were diluted in a pH 7.4 PBS. Slices were selected according to their coronal section and observed under a fluorescence microscope. The images were analyzed using the Scion Image for Windows program by pairing the amount of nigral TH-positive neurons in the SNpc from both hemispheres, where the value of the contralateral hemisphere to the 6-OHDA administration site was normalized to 100%. Duplicates were obtained from each rat.

Statistical Analysis

Data are shown as mean ± SD unless indicated otherwise. The statistical analysis was performed using the one-way ANOVA test followed by the Dunnett post-hoc test. $P < 0.05$ indicated statistical difference.

Results

The percents of nonenzymatic Pi released from nucleotides into the assay medium without slices and of Pi released from slices belonging to any group without nucleotide were less than 0.1% and of approximately 5%, respectively.

The effects of the unilateral infusion of 6-OHDA into the MFB on ATP, ADP, and AMP hydrolysis from striatal slices are shown in Fig. 1. There was no difference among groups in ATP hydrolysis (ANOVA, $F(2,12) = 0.079$; $P = 0.925$). However, there was a difference in ADP hydrolysis (ANOVA, $F(2,13) = 4.991$, $P = 0.025$), since it was increased in the animals lesioned with 6-OHDA

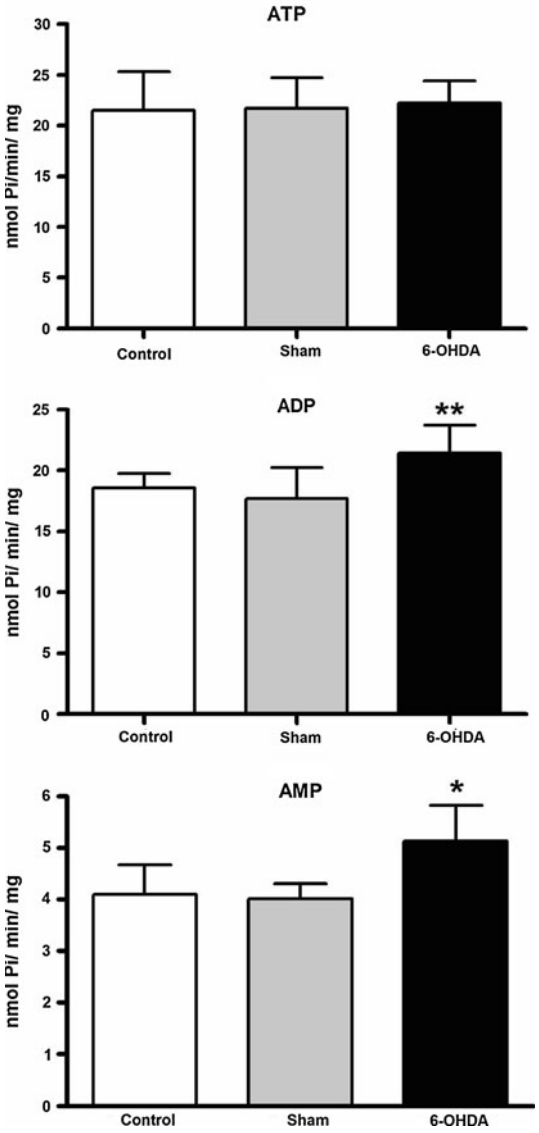

Fig. 1 ATP, ADP, and AMP hydrolysis from striatum of saline (Ctrl), Sham and 6-OHDA-rats. *Bars* represent mean ± SD of at least three animals. *Simple* and *double asterisk* indicates a difference from both control groups ($P < 0.05$ and 0.01, respectively)

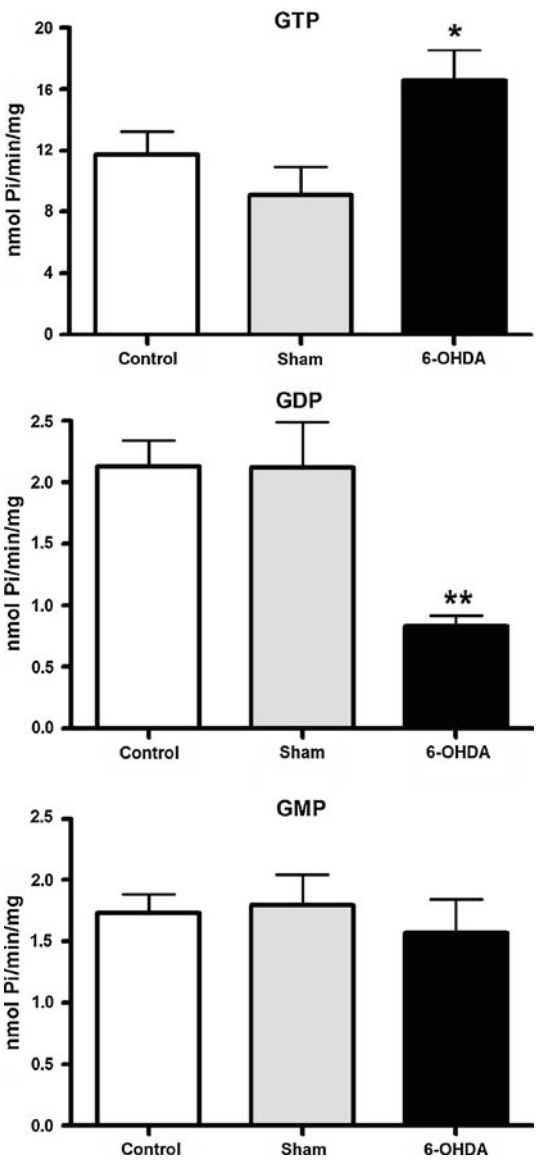

Fig. 2 GTP, GDP, and GMP hydrolysis from striatum of saline (Ctrl), Sham and 6-OHDA-rats. *Bars* represent mean ± SD of at least three animals. *Simple* and *double asterisk* indicates a difference from both control groups ($P < 0.05$ and 0.01, respectively)

relative to control and sham-operated animals ($P = 0.024$ and 0.049, respectively). In addition, there was a difference in AMP hydrolysis (ANOVA, $F(2,10) = 1.400$, $P = 0.029$), since it was increased in the animals lesioned with 6-OHDA relative to control and sham-operated animals ($P = 0.050$ and 0.045, respectively).

The effects of the unilateral infusion of 6-OHDA into the MFB on GTP, GDP, and GMP hydrolysis from striatal slices are shown in Fig. 2. The animals submitted to 6-OHDA treatment presented a 41%-increase in GTP hydrolysis relative to control and sham-operated animals (ANOVA, $F(2,16) = 3.957$, $P = 0.040$; $P = 0.036$ and 0.048, respectively). In contrast, GDP hydrolysis was decreased by 60% in the 6-OHDA group relative to control and sham-operated animals (ANOVA, $F(2,12) = 8.93$, $P = 0.004$ and 0.006, respectively). The infusion of 6-OHDA did not change GMP hydrolysis (ANOVA, $F(2,12) = 0.185$, $P = 0.833$).

The changes promoted by 6-OHDA infusion on nucleotide hydrolysis could be a consequence of transcriptional control. The expression patterns of NTPDase1, 2, 3, 4, 5, 6, and 5′-nucleotidase from naïve (Control), Sham, and 6-OHDA rats were represented. There was no difference among groups in the transcript mRNA striatal levels of the enzymes 5′-nucleotidase and NTPDases1, 2, 3, and 5 (ANOVA, $F(2,8) < 1.916$, $P > 0.22$), and of NTPDase 6 (ANOVA, $F(2,8) = 3.517$, $P = 0.098$) (Fig. 3).

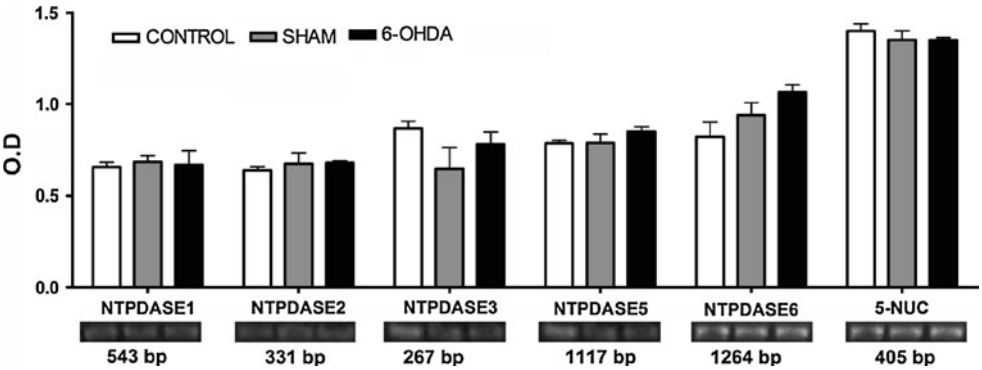

Fig. 3 Representative semi-quantitative RT–PCR mRNA for striatal ectonucleotidases from saline (Ctrl), Sham and 6-OHDA rats. The NTPDases to β-actin mRNA ratio was the same for all samples. *Bars* represent mean ± SE of three independent experiments with entirely consistent results

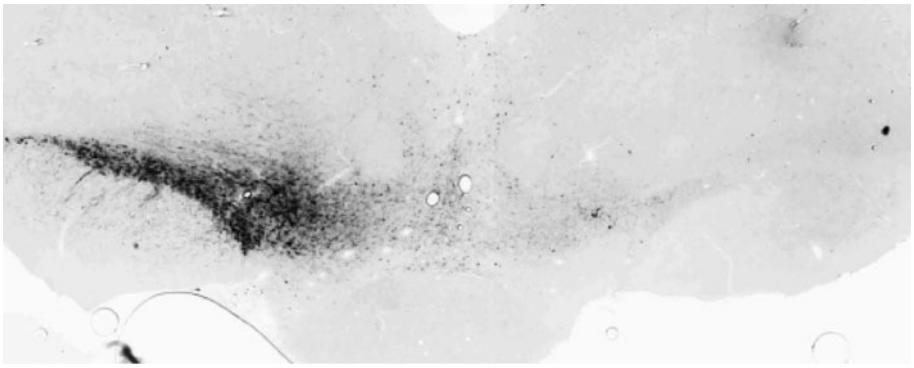

Fig. 4 Immunohistochemistry showing the decrease of the TH content in the substantia nigra ipsilateral to the MFB infused with 6-OHDA (*right side*)

TH immunohistochemistry revealed a reduction between 98.3 and 99.1% in the levels of mesencephalic TH in the three rats analyzed, which indicates an extensive lesion of the nigrostriatal pathway (Fig. 4).

Discussion

The present results show that the infusion of 6-OHDA into the rat MFB increases ADP, AMP, and GTP hydrolysis whereas it decreases GDP hydrolysis in biochemical assays using slices from the striatum ipsilateral to the infusion site. In addition, no change was found in the transcription of the 5′-nucleotidase and of NTPDase1, 2, 3, 5, and 6, which are enzymes that control the rate, amount, and timing of nucleotide degradation and formation, promoting the regulation of nucleotide-mediated signaling in purinoceptors [34, 35]. To our knowledge, this is the first time that alterations in adenine- and guanine-based purine hydrolysis cascades are shown in the 6-OHDA model of PD. First, this discussion will focus on how these alterations may affect the extracellular levels of adenine-based purines and their consequences in synaptic and systems level. Then, guanine-based purines are discussed concerning the present results.

There is compelling evidence to indicate that adenosine is implicated in PD. Adenosine A_{2A} and D_2 receptors are coexpressed in GABAergic striatopallidal spiny neurons [36, 37]. In these cells, adenosine increases and dopamine decreases the production of cAMP [37, 38]. Therefore, these neurotransmitters have antagonistic effects and changes in the balance of their levels may cause the system to malfunction; then, since the dopaminergic activity is highly decreased, any attempt to restore the balance between both neurotransmitters should be helpful. This may explain the fact that adenosine A_{2A} receptor antagonists enhance the motor activity in animal models of PD and are candidates for PD therapy [39, 40].

In rats, adenosine decreases by 35% when measured 15 days after a 6-OHDA infusion into the MFB [4]. Then, this decrease is far less than that of dopamine, which might indicate that adenosine levels should be even lower to counterbalance the effect of dopamine depletion. Intriguingly, adenosine even increases in rats treated with MPTP [41], which indicates that the mechanism of action and the consequences of acute toxin infusion are quite different in PD animal models regarding the purinergic system. Despite of this difference in adenosine levels, the imbalance between adenosine and dopamine favors the former in both models, and considering this factor only, adenosine production should be decreased in order to reestablish the balance. Therefore, one crucial point to this issue is how the extracellular adenosine levels are affected by the enzymes that hydrolyze the adenine-based nucleotides. Since it was found an increase in the hydrolysis of two nucleotides, ADP and AMP, it is evident that the direction

of both changes itself only intensifies the imbalance, regardless whether it is little or high, or whether these changes are or not due to a homeostatic control of adenine-based purine levels.

Ecto-5′-nucleotidase has different immunological isoforms in nerve terminals [42] and it is the rate-limiting step that regulates the output of the pathway [35]. Adenosine formation is tightly controlled by a feed-forward inhibition of ecto-5′-nucleotidase by ATP and/or ADP [43, 44]. When ATP release is low, there is a low and constant formation of adenosine, which is insufficient to activate A2A receptors [45]. As ATP release is high, extracellular AMP accumulates and adenosine is produced in an 'explosive' mode after ATP and ADP levels decrease below the ecto-5′-nucleotidase inhibition threshold [43]. Thus, it is expected that A2A receptor activation occurs only at high frequencies of stimulation and ATP release. This matches the observation that tonic A1 receptor inhibition and tonic A2A receptor facilitation predominate at low and high concentrations of adenosine, respectively [46, 47]. In fact, ecto-5′-nucleotidase blockade abolishes A2A, but not A1, receptor-mediated tonic modulation [48]. Then, as a consequence of the ADP hydrolysis increase, ADP levels decreases and this may relief ecto-5′-nucleotidase from inhibition in 6-OHDA-lesioned rats. Combining this effect with the increase in AMP hydrolysis, it is likely that both increases contribute to adenosine formation in high transient amounts and facilitate A2A receptor activation. Adding the possibility that 6-OHDA infusion promotes an increase of the level of mRNA for A2A receptors [4], the sum of these individually small changes would contribute to high transients of adenosine formation and, in turn, to trigger A2A receptor-mediated facilitation. All of this favors the imbalance between adenosine and dopamine levels, contributing to the evolution of motor and non-motor symptoms promoted by the 6-OHDA infusion. Therefore, targeting these enzymes with specific inhibitors in order to reestablish neurotransmitter balance is likely to be an extremely attractive strategy for PD therapy. Unfortunately, specific inhibitors or stimulators for each NTPDase remain to be discovered.

Interestingly, there was no significant change in ATP hydrolysis in slices from 6-OHDA-lesioned animals. However, the involvement of extracellular ATP in the neurochemical changes induced by the 6-OHDA infusion must not be ruled out. On the contrary, there is evidence to indicate that ATP induces neurotoxicity in vivo in the rat striatum [49] and that the P2X7 receptor antagonist A-438079 partially prevents the depletion of striatal dopamine stores but not the dopamine cell loss induced by 6-OHDA [50]. Therefore, any increase in extracellular ATP hydrolysis would be beneficial if considering this aspect only. Since 6-OHDA causes mitochondria to malfunction and depletes intracellular ATP levels [51, 52], biochemical events that reestablish the balance between neuronal firing and ATP synthesis [53, 54] might also affect ATP hydrolysis in our assays, a concept that demands further investigation.

Here, it was shown that GDP hydrolysis relatively decreases more than GTP hydrolysis increases in 6-OHDA-lesioned rats. These alterations raise several questions, among them: what are their effects on guanine-based purine levels, on the viability of remaining neurons and on the motor symptons in the model? How may this knowledge contribute in finding new therapies for PD? What are the mechanisms underlying the differential change in GTP and GDP hydrolysis?

There is growing evidence to indicate that guanosine has a neuroprotective role in neurodegenerative processes due to anti-apoptotic, anti-inflamatory and neurotrophic effects, and also to its ability to prevent glutamate excitotoxicity by stimulating astrocytic glutamate uptake [11–25, 55]. More importantly, it has already been shown that, in two different models of PD, the administration of guanosine can promote neuroprotection or, at least, reduce motor symptoms [24, 26]. In addition, guanosine increases dopamine turnover in PC12 cells at certain concentrations, which indicates that this purine may positively regulate the dopaminergic transmission [56]. GDP hydrolysis is much greater than that of GMP, which would favor, in normal conditions, the accumulation of GMP and, consequently, of guanosine [22]. Then, GDP hydrolysis should not have decreased in 6-OHDA-lesioned rats, since a decrease in extracellular guanosine production due to this factor would be expected. In consonance with this, there is evidence to indicate that striatal guanosine levels decreases by 41% after dopamine depletion due to a 5-day reserpine treatment [5]. Thus, it is likely that the decrease of GDP hydrolysis contributes to diminish the viability of the remaining nigral neurons in the 6-OHDA model. Since exogenous guanosine is beneficial in this model [24, 26], targeting GDP hydrolysis with specific enzyme positive modulators in order to promote neuroprotection is likely to be a very attractive strategy for PD therapy in the future, even in the case guanosine level were not altered in a patient.

It cannot be ruled out the possibility in which alterations in the level of guanine-based purines other than guanosine are relevant. The present results also point to an accumulation of GDP, since its production is increased and its hydrolysis is decreased. If this were the case, this excess of GDP would, in turn, leak to the cerebrospinal fluid, for example, instead of being substrate of hydrolysis for guanosine production in the striatum. A further study may clarify this. However, it is worth to point out that GDP hydrolysis in the cerebrospinal fluid is much higher than that of ADP when these nucleotides are at very high

concentrations, a condition that occurs at neural insults, such as seizures and ischemia [28]. In addition, the present study does contribute in the understanding of the 6-OHDA model regarding the fact that the decrease in GDP hydrolysis does decrease guanosine levels and may diminish the viability of the remaining dopamine neurons. Then, it is possible that the changes in activity of the enzymes responsible for hydrolyzing both adenine- and guanine-based purines are very relevant even without considering the putative changes in purine levels in PD patients for the reasons mentioned above.

NTPDases have different affinities for each purine as substrate [57] and significant changes in adenine- and guanine-based purine hydrolytic activity were observed after 6-OHDA treatment. Since it was found no alteration in mRNA levels of the enzymes tested, it is likely that alteration in NTPDase and 5′-nucleotidase activity rather than expression is involved in the hydrolytic activity changes observed in the present study.

Finally, it is essential to point out that the 6-OHDA model does not have the time course of years over which PD develops. Therefore, it remains to be elucidated in further studies whether alterations in the ectonucleotidase levels or even in adenine- and guanine-based purine levels may correspond to those alterations that occur in PD patients' brains. Despite of this, the suggestion of focusing the ectonucleotidases with specific modulators as a clinical strategy seems to be promising even if purine levels do not change in PD patients' striata.

In summary, the present results point to several changes in striatal adenine- and guanine-based purine hydrolysis in the 6-OHDA model. These changes might contribute to the clinical feature seen in PD, since adenosine and dopamine have opposite physiological roles in striatopallidal synapses, and extracellular guanosine prevents neurotoxicity in the striatum. Therefore, the present results reinforce the hypothesis that changes in purinergic systems are involved in PD, embracing the activity of ectoenzymes in this issue.

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