Combined administration of an A_{2A} receptor antagonist and a 5-$HT_{1A/1B}$ receptor agonist prevents dyskinetic movements and neuroinflammatory effects in a rat model of Parkinson's disease

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Abstract

Several lines of evidence have strongly implicated neuroinflammation in Parkinson's disease (PD) progression and L-dopa-induced dyskinesia. The present study investigated whether early subchronic pretreatment with the serotonin 5-HT1A/1B receptor agonist eltoprazine plus the adenosine A2A receptor antagonist preladenant counteracted L-dopa-induced abnormal involuntary movements (AIMs, index of dyskinesia), and neuroinflammation, in unilateral 6-hydroxydopamine(6-OHDA)-lesion rat model of PD. The immunoreactivity of glial-fibrillary-acidic-protein(GFAP), and the colocalization of ionized calcium-binding-adaptor-molecule-1(IBA-1), with interleukin (IL)-1β, tumor-necrosis-factor-α(TNF-α) and IL-10 were evaluated in denervated caudate-putamen (CPu) and substantia nigra pars-compacta (SNc). The combined subchronic pretreatment with L-dopa plus eltoprazine and preladenant reduced AIMs induced by acute L-dopa challenge in these rats and decreased GFAP and IBA-1 immunoreactivity induced by the drug in both CPu and SNc, with reduction in IL-1β in IBA-1-positive cells in both CPu and SNc, and in TNF-α in IBA-1-positive cells in SNc. Moreover, a significant increase in IL-10 in IBA-1-positive cells was observed in SNc. Evaluation of immediate-early-gene zif-268 (index of neuronal activation) after L-dopa challenge, showed an increase in its expression in denervated CPu of rats pretreated with L-dopa or L-dopa plus preladenant compared with vehicle, whereas rats pretreated with eltoprazine, with or without preladenant, had lower zif-268-expression. Finally, tyrosine hydroxylase and dopamine transporter examined to evaluate neurodegeneration, showed a significant equal decrease in all experimental groups. The present findings suggest that combination of L-dopa with eltoprazine and preladenant may be promising therapeutic strategy for delaying the onset of dyskinesia, preserving L-dopa efficacy and reducing neuroinflammation markers in nigrostriatal system of 6-OHDA-lesioned rats.

Keywords: astroglia, abnormal involuntary movements, dyskinesia, GFAP, IBA-1, microglia, nigrostriatal system, neuroinflammation, zif-268

1. Introduction

The most effective therapy for Parkinson's disease (PD) remains dopamine replacement with the dopamine precursor L-3,4-dihydroxyphenylalanine (L-dopa) (in combination with a peripheral decarboxylase inhibitor) (Olanow et al., 2009). Although, L-dopa is of substantial benefit as it alleviates the main motor symptoms in parkinsonian patients, it loses effectiveness over time, since the long-term therapy is associated with the development of motor complications, including dyskinesia (Obeso et al., 2004; Olanow et al., 2009). Dyskinesia affects about 40% of chronically treated patients and consists of choreic and/or dystonic involuntary movements, which could become a major source of disability (Jankovic 2005; Nutt et al., 2010; Obeso et al., 2004). Dyskinesia has largely studied evaluating abnormal involuntary movements (AIMs) induced in PD rodent models by subchronic administration of L-dopa (Cenci and Crossman, 2018; Chen et al., 2020; Lundblad et al., 2002; Olanow, et al 2020).

Among the different non-dopaminergic systems explored in preclinical and clinical studies, the serotonergic system has been shown to modulate the expression of dyskinesia and motor PD-like symptoms (Bezard et al., 2013; Carta and Bezard, 2011; Cenci, 2014; Del Bel et al., 2016; Goetz et al., 2007; Pisanu et al., 2018; Svenningsson et al., 2015). This system seems also to play a critical role in neuroinflammation associated to PD (Barcia et al., 2011; Lee et al., 2019; Tansey and Golderg, 2010) and, more recently, in neuroinflammation associated to dyskinesia (Carta et al., 2017; Del Bel et al., 2016; Julien et al., 2006). In particular, the role of the pro-inflammatory cytokines, interleukin (IL)-1β, tumor necrosis factor-alpha (TNF-α), and IL-6 and anti-inflammatory cytokines, such as IL-10 and growth factors, appears to be important for neuroinflammation related to PD and/or dyskinesia (Boi et al., 2019; Hirsch and Hunot 2009; Tronci et al., 2017; Wolf et al., 2017). Interestingly, a recent study in rats demonstrated that chronic pulsatile L-dopa treatment induced neuroinflammatory responses in the dorsal caudate-putamen (CPu), with a strong increase in TNF-α production by microglia, while continuous delivery of the L-dopa did not produce any inflammatory and dyskinetic response (Mulas et al, 2016). Recent studies indicate, in addition, a possible role of serotonin receptors (specifically 5-HT1A and 5-HT1B) in the modulation of gliosis correlated with PD and dyskinesia (Farajdokht et al., 2020; Garrett et al., 2013; Miyazaky et al., 2013; Pisanu et al., 2018). Moreover, IL-1β and TNF-α, increased the activity and expression of the serotonin reuptake transporter (SERT) in mouse midbrain and striatal synaptosomes, which caused a decrease in extracellular levels of serotonin (Wu et al., 2015; Zhu et al., 2006, Carta and Bezard, 2011). Indeed, several findings demonstrated that L-dopa reduced striatal serotonin tissue concentrations in PD rodents (Carta et al., 2007), and the severity of dyskinetic movement induced by L-dopa is inversely correlated with striatal serotonin concentrations (Gil et al., 2011). A direct involvement of SERT on dyskinesia has been confirmed by several findings showing that its inhibition with serotonin reuptake inhibitors (SSRIs) reduced dyskinesia, without affecting the therapeutic efficacy of L-dopa, in PD animal models (Bezard et al., 2006; Bishop et al., 2012; Chung et al., 2010; Conti et al., 2014; Farajdokht et al., 2020; Fidalgo et al., 2015). In addition, serotonin 5-HT1A receptors are expressed by astrocytes (Whitaker-Azmitia et al., 1993), and the activation of these receptors seems to enhance their proliferation, together with an upregulation of antioxidative molecules (Miyazaki et al., 2013). Similarly to serotonin, adenosine A2A receptors are also deeply involved in PD pathology having a critical role in neuroinflammatory and neuroprotective processes through modulation of microglial and astroglial activity and potentiating the motor stimulation induced by L-dopa (Armentero et al.,

2011; Carta et al., 2009; Chen et al., 2001; Gyoneva et al., 2014; Illes et al., 2020; Jenner, 2014; Kalda et al., 2006; LeWitt et al., 2008; Pinna et al., 2010, Pinna and Morelli, 2014; Simões et al., 2012). Interestingly, beside to localization of A2A receptors post-synaptically in striatopallidal neurons or pre-synaptically in nerve terminals (Rosin et al. 1998; Rebola et al. 2005), A2A receptors are also expressed by microglial and astroglial cells (Fiebich et al. 1996; Saura et al. 2005). A2A receptors play an important role in the activation of microglia, and A2A antagonists may limit the progression of a fully activated microglia phenotype (Armentero et al., 2011; Gyoneva et al., 2014; Orr et al., 2009; Yu et al., 2008). Moreover, antagonism of A2A receptors located in astrocytes seems to decrease toxic astrogliosis and neurodegeneration, suggesting an important role of these receptors in reducing neuroinflammation on PD models (Armentero et al., 2011; Brambilla et al., 2003; Ke et al., 2009; Minghetti et al., 2007).

To study the basis of a pharmacological therapy that could alleviate dyskinesia without worsening PD disability, previous studies from our laboratory have investigated the efficacy of a combined treatment with the serotonin 5-HT1A/1B agonist eltoprazine and the adenosine A2A receptor antagonist preladenant on motor impairment and dyskinetic movements in both the unilaterally 6-hydroxydopamine (6-OHDA)-lesioned rat and the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated primate models of PD (Ko et al., 2017; Pinna et al., 2016). Results demonstrated that acute and chronic combined administration of a subthreshold dose of L-dopa plus eltoprazine and preladenant significantly inhibited or reduced dyskinetic-like behavior, without impairing motor activity (Pinna et al., 2016, 2018) and produced a lower expression of the immediate-early gene zif- 268, an index of long-term changes (Pinna et al., 2016; 2018). This finding suggests that this combination of drugs may not only be able to contrast the development of dyskinetic movements during chronic treatment, but also to influence changes in gene expression associated with dyskinesia induced by L-dopa (Carta et al., 2008; Muñoz et al., 2008; Pinna et al., 2016; 2018).

Starting from these findings, the present study aimed to evaluate whether the antidyskinetic effect of the early subchronic combined administration of L-dopa plus eltoprazine and preladenant could be correlated with a reduction in the neuroinflammation process and dyskinetic effects induced by a challenge with L-dopa in these suchronically treated 6-OHDA-lesioned rats. To this end, four days after a subchronic pretreatment with L-dopa plus eltoprazine and preladenant, unilaterally 6-OHDA- lesioned rats were evaluated for astrogliosis and microgliosis induced by a L-dopa challenge, by means of glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule-1 (IBA- 1) immunoreactivity, in the CPu and substantia nigra pars compacta (SNc). Moreover, IBA-1 colocalization with the pro-inflammatory IL-1β, TNF-α and the anti-inflammatory IL-10 were evaluated together with the early gene zif-268 and markers of dopaminergic degeneration, tyrosine hydroxylase (TH) and dopamine transporter (DAT).

2. Materials and Methods

2.1 Animals

Male Sprague–Dawley rats (Charles River, Calco, Italy) were housed in groups of 4–6 in standard polycarbonate cages with sawdust bedding and maintained on a 12-hour-light/dark cycle (lights on at 8:00 am). Food and water were freely available. All experiments were conducted in accordance with the guidelines for animal experimentation of the EU directives (2010/63/EU; L.276; 22/09/2010) and with the guidelines approved by the Ethical Committee of the University of Cagliari. Experiments were designed to minimize animal discomfort to the least extent possible and to reduce the number of animals used.

2.2. Unilateral 6-OHDA lesion

Rats (275–300 g) were anesthetized and infused unilaterally into the left medial forebrain bundle (A: −2.2, L: +1.5 from bregma, V: −7.9 from dura, according to the atlas of Pellegrino et al. (1979) with 6-OHDA (8 μg/4 μl saline containing 0.05% ascorbic acid) or vehicle (saline containing 0.05% ascorbic acid), through a stainless steel needle, at a rate of 1 μl/min. All rats were pretreated with desipramine (10 mg/kg i.p.) 30 min before 6-OHDA injection to prevent damage to noradrenergic neurons (Fuzzati-Armentero et al., 2015).

2.3. Drugs and subchronic treatment

Preladenant was purchased from Sequoia-Research-Products (UK), suspended in 0.4% methylcellulose and administered orally. Eltoprazine was purchased from Sequoia-Research-Products (UK), dissolved in saline, and injected subcutaneously. The 0.4% methylcellulose suspension served as vehicle control for preladenant. 6-OHDA, L-dopa–methyl ester hydrochloride, desipramine hydrochloride, and benserazide were purchased from Sigma-Aldrich (Italy). L-dopa–methyl-ester and benserazide were injected subcutaneously. Preladenant and eltoprazine were administered 60 minutes before L-dopa or simultaneously, respectively.

Three weeks after the unilateral 6-OHDA lesion, rats were allocated to one of four groups, balanced on the basis of their deficit on the performance of right paw in the adjusting step test after the lesion (Chang et al., 1999; Pinna et al., 2007; 2010; 2016), and treated for 14 days as follow: 1) L-dopa/benserazide (4 mg/kg, n=11) alone; 2) L-dopa/benserazide plus eltoprazine (0.6 mg/kg, n= 10); 3) L-dopa/benserazide plus preladenant (0.3 mg/kg, n=10); 4) L-dopa/benserazide plus eltoprazine plus preladenant (n=11); or 5) vehicle (n=10). Sham-operated rats were treated with vehicle (n=8).

Immediately after the drug administration, rats were tested for AIMs and rotational behaviors (Pinna et al., 2016). During treatment, AIMs and rotational behavior were evaluated three times each, whereas motor activity was evaluated one time (Fig. 1A).

Four days after treatment discontinuation, rats were challenged with L-dopa/benserazide (4 mg/kg) or vehicle and their AIMs were evaluated.

2.4. Assessment of rotational behavior

Rotational behavior was assessed in hemispherical bowls (50 cm diameter), with sawdust on the floor, in which each rat was connected to an automated rotameter system (Panlab s.l., Barcelona, Spain) capable of detecting the number of full (360°) rotations in both directions (ipsilateral and contralateral to the lesioned hemisphere) (Pinna et al., 2016). Rats were placed in the bowls 30 min before drug administration in order to acclimatize and extinguish any spontaneous rotational behavior, and both contralateral and ipsilateral rotations were measured every 10 min for 120 min after drug injection. Total number of contralateral and ipsilateral rotations (mean ± SEM) in 2 h testing-period were calculated.

2.5. Abnormal Involuntary Movements

AIMs were evaluated according to the rat dyskinesia scale as previously described (Lundblad et al., 2002; Pinna et al., 2016) by experimenters' blind to the treatments. Briefly, AIMs were classified into four subtypes, according to their topographic distribution, as forelimb, orolingual, axial, and locomotive dyskinesia (displayed as contralateral rotation). The severity of each AIM subtype was

assessed using scores from 0 to 4 (1: occasional, i.e. present less than 50% of the time; 2: frequent, i.e. present more than 50% of the time; 3: continuous, but interrupted by strong sensory stimuli; 4: continuous, not interrupted by strong sensory stimuli).

2.6. Evaluation of motor activity

Motor activity: was assessed using an activity meter equipped with infrared photocell emitters–detectors (Pinna et al., 2016). Locomotion and non-finalized movements (total motor activity) were evaluated immediately after L-dopa administration. Motor activity counts were evaluated every 10 min for 120 min.

2.7. Immunohistochemistry

2.7.1. Tissue preparation

Two hours after the challenge with L-dopa/benserazide or vehicle, rats were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). Brains were postfixed overnight in the same solution (4°C) and coronally cut on a vibratome (40 μm) 2 days later. For each rat, three coronal sections were collected from 1.70 mm to 0.20 mm (CPu) and from −4.80 mm to −5.80 mm (SNc) relative to bregma, according to the rat brain atlas of Paxinos and Watson (1998).

2.7.2. Immunoreaction protocols

Free-floating sections were rinsed in 0.1 M PB, blocked in a solution containing 3% normal donkey/goat serum (Jackson ImmunoResearch Europe, Newmarket, UK and ABC, Vector, Peterborough, UK, respectively) and 0.3% Triton X-100 in 0.1 M PB at room temperature for 2 h, and incubated at 4°C in the same solution with the primary antibody for 2 nights at 4 °C. After the incubation with the primary antibody was completed, sections were rinsed three times in 0.1 M PB and incubated with the secondary antibody in 0.1 M PB at room temperature for 2 h. Table 1 summarizes the features and dilutions of the primary antibodies used in this study. After the incubation with the secondary antibody was completed, sections were rinsed and immediately mounted onto glass slides coated with gelatin in Mowiol mounting medium. To allow visualization of cell nuclei, sections were incubated for 10 min with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; 1:10,000, Sigma-Aldrich, Milan, Italy) and then mounted onto glass slides (Sirabella et al., 2018). Omission of either the primary or secondary antibodies served as negative control and yielded no labeling (data not shown).

2.7.3. Image acquisition

Images of single wavelength were obtained with an epifluorescence microscope (Axio Scope A1, Zeiss, Oberkochen, Germany) connected with a digital camera (1.4 M Pixels, Infinity 3-1, Lumenera, Nepean, Canada). In each of the three brain sections immunostained for GFAP and IBA-1, two portions from the CPu (dorsolateral and ventromedial) and the whole SNc, left and right, were acquired using a 20× objective. For the colocalization of IBA-1 with IL-1β, TNF-α and IL-10, the dorsolateral and ventromedial CPu and the whole SNc, left and right, were acquired using a 40× objective from the same epifluorescence microscope cited above (Costa et al., 2019). For zif-268, DAT and TH, the dorsolateral and ventromedial CPu was acquired using a 20× objective from the same epifluorescence microscope cited above.

2.7.4. Image analysis for GFAP, IBA-1, IBA-1 and IL-1β, TNF-α or IL-10, DAT and TH

The ImageJ software (US National Institutes of Health, Bethesda, MD, USA) was used to determine quantitatively the number of GFAP- and IBA-1- and TH-positive cells with the Multi-points plug-in of the ImageJ software (US National Institutes of Health). Briefly, this plug-in creates a point selection to count the number of GFAP-, IBA-1- or TH-positive cells (Costa et al., 2020). The number of cells labeled with the nuclear marker DAPI was obtained separately for each level of the CPu and SNc. The quantitative analysis of colocalization of IBA-1 and IL-1β, TNF-α or IL-10 was conducted using the ImageJ plug-in JACoP (Just Another Colocalization Plugin) (Bolte and Cordelières, 2006). A correlation of signal intensity was calculated as a Pearson's correlation coefficient (Rr). Rr is a quantitative measurement that estimates the degree of overlap between fluorescence signals obtained in two channels. The density of immunoreactive fibers positive for DAT or TH in the CPu was used to determine quantitatively using the ImageJ software (Costa et al., 2019). Sections were captured in black and white 8-bit monochrome and the density of fibers was determined in fixed regions using a threshold level that was kept constant across all images. The pixels were converted into square micrometers by employing a suited calibration procedure, in order to represent the area occupied by a specific immunoreaction product in square micrometers. The final values were expressed as a percentage of the respective vehicle group. For each level of the CPu and SNc, the value obtained was first normalized with respect to vehicle, and then the values from different levels were averaged. No significant differences in the density of immunoreactive cells, fibers or neurons were found among the three coronal sections of a given area in the same rat. All rats included in this study showed an almost complete lesion with over 85% dopaminergic cell loss in the lesioned side. Rats that did not reach this lesion level were discarded.

2.8. Data analysis and statistics

Statistical analysis was performed with Statistica for Windows (StatSoft, Tulsa, OK, USA) or Prism (GraphPad, La Jolla, CA, USA). Statistical significance was assessed by one-way and/or two-way analysis of variance (ANOVA), followed by Newman–Keuls or Tukey post-hoc test. All data were evaluated for normality and Levene's test was applied to check for homoscedasticity of data before ANOVA analysis.

The results of AIMs were confirmed with Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons test.

Pearson analysis was applied to evaluate whether in unilaterally 6-OHDA-lesioned rats pretreated with L-dopa and with the combination of L-dopa plus eltoprazine and preladenant, a correlation existed between the total AIMs and the levels of markers of astrogliosis and microgliosis (GFAP and IBA-1 positive cells).

For this correlation analysis, data of total AIMs expressed by rats pretreated with L-dopa and with the combined administration of L-dopa plus eltoprazine plus preladenant were pooled together and correlated with the respective value of GFAP and IBA-1 positive cells in both the CPu and SNc. Some of the rats treated with L-dopa (2 animals) or L-dopa plus eltoprazine and preladenant (1 animal) were not included in the immunohistochemical analysis because of a loss of data. Significance was set at $p < 0.05$ and the results are expressed as mean ± SEM for every analysis performed.

The values of immunoreactivity presented in results and figures are referred to the denervated CPu and SNc corresponding to the 6-OHDA-lesioned side of the unilateral 6-OHDA-lesioned rats compared with the sham-operated rats.

3. Results

3.1. Behavioral studies

3.1.1. Total AIMs induced by subchronic treatments

Evaluation of the total AIMs during the 14th day of subchronic pretreatments showed a similar prevention of AIMs in unilateral 6-OHDA-lesioned rats treated with L-dopa (4 mg/kg) plus eltoprazine (0.6 mg/kg) and preladenant (0.3 mg/kg) or with L-dopa plus eltoprazine compared with L-dopa alone, and/or L-dopa plus preladenant ($F_{3,38} = 102.8$, $p < 0.0001$; Tukey's post-hoc test: $p < 0.0001$ L-dopa + elt + prel vs L-dopa; $p < 0.0001$ L-dopa + elt + prel vs L-dopa + prel; $p < 0.0001$ L-dopa + eltoprazine vs L-dopa) (Fig. 1B), in line to what reported by Pinna and collaborators (2016). Furthermore, two-way ANOVA showed a significance of treatments (prel: $F_{1,38} = 9.702$, $p < 0.005$; elt: $F_{1,38} = 301.82$, $p < 0.0001$), but a non-significant interaction (prel*elt: $F_{1,38} = 1.409$, $p = 0.24$), showing that regarding AIMs expression the two drugs (eltoprazine plus preladenant) act independently in two different neurotransmitter systems rather than interact between them. However, the Tukey's post-hoc test confirmed the statistical significance obtained by one-way ANOVA described above (Fig. 1B).

The results of one-way ANOVA were confirmed with Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons test. Moreover, these effects were maintained throughout the whole period-treatment (Table 1).

3.1.2. Rotational behavior and motor activity induced by subchronic treatments

In unilateral 6-OHDA-lesioned rats, subchronic administration with eltoprazine produced a reduction of L-dopa-induced rotational behavior (*$p < 0.05$ vs L-dopa) (Tab. 1); by contrast, chronic administration of preladenant (0.3 mg/kg) significantly potentiated contralateral rotations induced by L-dopa (4 mg/kg) (*$p < 0.05$, **$p < 0.005$ vs L-dopa) (Table 1). Subchronic treatment with the combination of L-dopa (4 mg/kg) plus eltoprazine (0.6 mg/kg) plus preladenant (0.3 mg/kg) did not significantly reduce L-dopa-induced contralateral rotations compared to L-dopa alone during the whole period of treatment (Table 1), with a significant increase of L-dopa-induced contralateral rotations as compared to eltoprazine without preladenant (^$p < 0.05$ L-dopa + elt + prel vs L-dopa plus elt) (Table 1). Consistent with rotational behavior evaluation, the assessment of general motor activity, showed that in unilateral 6-OHDA-lesioned rats, L-dopa plus eltoprazine and preladenant did not reduce motor activity compared with L-dopa (Table 1). In contrast, preladenant plus L-dopa significantly increased the total activity compared with L-dopa ($p<0.05$ vs L-dopa; Table 1), while eltoprazine significantly reduced L-dopa-induced total activity ($p<0.05$ vs L-dopa; Table 1). Moreover, it is important to underline that dDuring the evaluation of rotational and motor behavior, rats treated with the association of drugs showed a fluid motor behavior, indeed animals move and explore without any stereotyped movements or AIMs (Pinna et al., 2016). It is important to note that evaluation of rotational behavior and motor activity, demonstrated that the combined administration of L-dopa (4 mg/kg) plus eltoprazine (0.6 mg/kg) and preladenant (0.3 mg/kg), preserves motor behavior, while AIMs are decresed.

3.2.1. Acute L-dopa challenge after subchronic treatments

Acute challenge with L-dopa four days after the termination of the subchronic pretreatments with L- dopa (4 mg/kg) plus eltoprazine (0.6 mg/kg) and preladenant (0.3 mg/kg), produced a significant lower number of AIMs compared with L-dopa-pretreated rats ($p < 0.005$ L-dopa + elt + prel vs L-dopa) (Fig. 1C). Similarly, the acute challenge with L-dopa in rats pretreated with L-dopa plus

eltoprazine induced a significant lower number of AIMs compared with L-dopa ($p < 0.0001$ L-dopa + elt vs L-dopa) (Fig. 1C). In contrast, the acute challenge with L-dopa did not show any reduction in AIMs in rats pretreated with L-dopa plus preladenant ($p = 0.99$ L-dopa vs L-dopa + prel) compared with those pretreated with L-dopa alone (Fig. 1C). Furthermore, two-way ANOVA showed significance of one treatment (let: $F1,38 = 43.16$, $p < 0.0001$), but non-significance for other treatment (Prel: $F1,38 = 0.197$, $p = 0.66$), and a non- significant interaction ($F1,38 = 0.491$, $p = 0.49$). However, the Tukey's post-hoc test confirmed the statistical significance of one-way ANOVA described above (Fig. 1B,1C).

The results of ANOVA were confirmed with Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons test.

3.2. Immunohistochemical studies

As described in figure 1A, four days after the last drug pretreatment (two weeks) with: vehicle; L-dopa/benserazide (4 mg/kg); L-dopa/benserazide in combination with eltoprazine (0.6 mg/kg) and/or preladenant (0.3 mg/kg); all rats received a challenge with L-dopa/benserazide or vehicle and then were sacrificed for immunohistochemical studies.

The values of immunoreactivity presented in the results and figures refer to the denervated CPu and SNc of unilateral 6-OHDA-lesioned rats compared with sham-operated rats.

3.2.1. Immunoreactivity for GFAP

One-way ANOVA of GFAP immunoreactivity revealed a significant effect of treatment both in the CPu ($F5,51 = 66.35$, $p \leq 0.0001$) and in the SNc ($F5,50 = 16.55$, $p \leq 0.0001$).

In the CPu, sham-operated rats displayed astroglial cells with highly branched morphology, tiny processes, and a small body (Fig. 2). The 6-OHDA lesion in the vehicle-pretreated rats produced an increased number of GFAP-positive cells compared with sham-operated rats (Fig. 2). Pretreatment with L-dopa elicited a further elevation in astroglia compared with vehicle-pretreated 6-OHDA-lesioned rats (Fig. 2). Interestingly, pretreatment with L-dopa plus eltoprazine, L-dopa plus preladenant, and L-dopa plus eltoprazine and preladenant elicited a lower increase in astroglia compared with L-dopa-pretreated 6-OHDA-lesioned rats (Fig. 2).

In the SNc, sham-operated rats displayed fewer astroglial cells and the morphology was similar to that of the CPu (Fig. 2). In contrast, the 6-OHDA lesion increased the number of GFAP-positive cells in all the experimental groups compared with sham-operated rats (Fig. 2). Moreover, pretreatment with L-dopa plus preladenant and with L-dopa plus eltoprazine and preladenant to 6-OHDA-lesioned rats elicited a lower increase in astroglia compared with L-dopa- and L-dopa plus eltoprazine- pretreated rats (Fig. 2).

3.2.2. Immunoreactivity for IBA-1

One-way ANOVA of IBA-1 immunoreactivity revealed a significant effect of treatment both in the CPu ($F5,51 = 17.54$, $p \leq 0.0001$) and in the SNc ($F5,51 = 10.50$, $p \leq 0.0001$).

In the CPu, sham-operated rats displayed fewer microglial cells with a small body (Fig. 3), whereas, the 6-OHDA lesion in vehicle-pretreated rats did not significantly increase the number of IBA-1-positive cells compared with sham-operated rats (Fig. 3). Pretreatment with L-dopa elicited an increase in microglia compared with sham-operated and vehicle-pretreated 6-OHDA-lesioned rats

(Fig. 3). Interestingly, pretreatments with L-dopa plus eltoprazine, L-dopa plus preladenant, and L-dopa plus eltoprazine and preladenant elicited a lower increase in microglia compared with L-dopa-pretreated 6-OHDA-lesioned rats (Fig. 3).

In the SNc, sham-operated rats displayed fewer microglial cells and the morphology was similar to that of the CPu (Fig. 3), whereas pretreatment with L-dopa increased the number of IBA-1-positive cells compared with sham-operated and vehicle-pretreated 6-OHDA-lesioned rats (Fig. 3). Interestingly, pretreatment with L-dopa plus eltoprazine, L-dopa plus preladenant, and L-dopa plus eltoprazine and preladenant elicited a lower increase in microglia compared with L-dopa-pretreated 6-OHDA-lesioned rats. Moreover, pretreatment with L-dopa plus preladenant and L-dopa plus eltoprazine and preladenant elicited a lower increase in microglia compared with L-dopa plus eltoprazine-pretreated 6-OHDA-lesioned rats (Fig. 3).

3.2.3. Double immunoreactivity for IBA-1 and IL-1β, TNF-α, IL-10

In the CPu, one-way ANOVA revealed a significant effect of treatment for the double immunoreactivity of IBA-1 and IL-1β, but not for IBA-1 and IL-10 (IL-1β: $F_{5,50} = 5.27$, $p < 0.0001$; IL-10: $F_{5,49} = 1.63$, $p > 0.05$). In the SNc, one-way ANOVA revealed a significant effect of treatment for the double immunoreactivity of IBA-1 and IL-1β, IL-10 and TNF-α (IL-1β: $F_{5,44} = 4416$, $p < 0.005$; IL-10: $F_{5,41} = 2.13$, $p < 0.05$; TNF-α: $F_{5,36} = 4.78$, $p < 0.01$).

In the CPu, pretreatment with vehicle or L-dopa of 6-OHDA-lesioned rats elicited an increase in the colocalization of IBA-1 and IL-1β, in the 6-OHDA-lesioned side compared with sham-operated rats (Table 2). Interestingly, pretreatment with L-dopa plus eltoprazine, L-dopa plus preladenant, and L-dopa plus eltoprazine and preladenant elicited a decrease in the colocalization of IBA-1 and IL-1β compared with vehicle- and L-dopa-pretreated rats (Table 2). No significant differences were observed in the colocalization analysis of IBA-1 and IL-10 (Table 3).

In the SNc, the 6-OHDA lesion in vehicle-pretreated rats elicited an increase in the colocalization of IBA-1 and IL-1β compared with sham-operated rats (Table 2). Moreover, pretreatments with L-dopa plus eltoprazine, L-dopa plus preladenant, and the combination of L-dopa plus eltoprazine and preladenant elicited a decrease in the colocalization of IBA-1 and IL-1β compared with vehicle-pretreated 6-OHDA-lesioned rats (Table 2).

Moreover, in the SNc, a decrease in the colocalization of IBA-1 and TNF-α was also observed after pretreatments with L-dopa plus eltoprazine, L-dopa plus preladenant and with L-dopa plus eltoprazine and preladenant to 6-OHDA-lesioned rats compared with vehicle- and L-dopa-pretreated 6-OHDA- lesioned rats (Table 4).

Finally, pretreatment with L-dopa plus preladenant and with L-dopa plus eltoprazine and preladenant elicited an increase in the colocalization of IBA-1 and IL-10 compared with sham-operated rats and vehicle-pretreated 6-OHDA-lesioned rats (Table 3).

3.2.4. Immunoreactivity of TH and DAT

In order to evaluate whether the drug treatments could influence the degree of lesion of dopaminergic neurons, quantitative image analysis of TH and DAT immunoreactivity in the CPu and SNc was performed.

Analysis in the CPu revealed a significant decrease in TH-immunoreactive fibers after the 6-OHDA lesion in all the experimental groups compared with sham-operated rats ($F_{5,50} = 169.06$, $p < 0.0001$) (Table 5). Moreover, terminal damage was confirmed by the significant decrease in DAT-

immunoreactive fibers in the 6-OHDA-lesioned side in all the experimental groups compared with sham-operated rats (Table 6). In the SNc, as for the CPu, the denervated side of the 6-OHDA-lesioned rats showed a decreased number of TH-positive neurons compared with sham-operated rats in all the experimental groups ($F_{5,47} = 172.86$, $p < 0.0001$) (Table 5). Tukey's post-hoc test confirmed that all unilateral 6-OHDA-lesioned experimental treated groups did not differ in extension of lesion for both TH density in CPu and TH neurons in SNc (Table 5).

3.2.5. Immunoreactivity for zif-268

After the acute challenge with L-dopa, expression of zif-268 was increased in the CPu of L-dopa- and L-dopa plus preladenant-pretreated rats compared with vehicle-pretreated rats. In contrast, rats pretreated with L-dopa and eltoprazine (with or without preladenant) had a lower zif-268 activation, after acute challenge with L-dopa (Fig. 4).

One-way ANOVA of zif-268 immunoreactivity in the CPu revealed a significant effect of treatment ($F_{5,48} = 8$, $p < 0.001$). In particular, high levels of zif-268 were found after the 6-OHDA lesion in L- dopa and L-dopa plus preladenant-pretreated rats compared with sham-operated and vehicle-pretreated 6-OHDA-lesioned rats (Fig. 4). On the other side, pretreatment with both L-dopa plus eltoprazine or L-dopa plus eltoprazine and preladenant showed a lower increase in zif-268 compared with rats pretreated with L-dopa alone or L-dopa plus preladenant (Fig. 4).

3.3. Correlations between AIMs and neuroinflammatory markers (IBA-1 and GFAP in CPu and SNc) in unilaterally 6-OHDA-lesioned rats pretreated with L-dopa and L-dopa plus eltoprazine and preladenant.

The analysis of Pearson's correlation coefficient was performed in unilaterally 6-OHDA-lesioned rats, pretreated with L-dopa and with the combined administration of L-dopa plus eltoprazine plus preladenant; this Pearson's analysis revealed that a positive correlation existed between total AIMs and GFAP and IBA-1 positive cells in both the CPu and SNc, considering total AIMs evaluated in both the 14th day of pretreatment or after L-dopa challenge (Table 7).

4. Discussion

The present study demonstrates that the pretreatment with eltoprazine plus preladenant and L-dopa reduces dyskinetic movements and the activation of GFAP- and IBA-1-positive cells in the CPu and SNc, compared with rats pretreated with L-dopa alone. Likewise, pretreatment with L-dopa plus preladenant reduced the activation of astroglia and microglia in the CPu and SNc, whereas L-dopa plus eltoprazine reduced astrogliosis in the CPu and microgliosis in the CPu and SNc compared with pretreatment with L-dopa alone.

All the above-mentioned changes were associated to a lower increase in IL-1β and TNF-α in IBA-1- positive cells in the CPu and/or SNc, whereas, in the SNc, a significant increase of IL-10 in IBA-1- positive cells was observed. These results show the new interesting finding that the combined drug pretreatments mentioned above, reduce neuroinflammation and the production of pro-inflammatory cytokines associated to an increase in the production of anti-inflammatory cytokines induced in these subchronically treated rats by a challenge with L-dopa. All these changes were associated with a reduction of AIMs and zif-268 induced by L-dopa. Moreover, the pretreatments

did not seem to have any effect on neurodegeneration of dopaminergic neurons and terminals, since both TH and DAT levels in the denervated side of unilateral 6-OHDA-lesioned rats were similar in all experimental group suggesting that the preladenant and eltoprazine combination reduced the neuroinflammation correlated with dyskinesia but did not influence the already established degeneration of dopaminergic neurons.

In the present study the early combined pretreatment with eltoprazine and preladenant, besides preventing the onset of AIMs induced by L-dopa in unilateral 6-OHDA-lesioned rats, is also able to reduce the expression of AIMs after discontinuation of treatment when L-dopa is administered acutely. Specifically, after acute challenge with L-dopa, 6-OHDA-lesioned rats pretreated with eltoprazine plus preladenant and L-dopa displayed a lower number of AIMs compared with those pretreated with L- dopa alone. These findings confirm the previous findings of Pinna et al., 2016, which demonstrated that the combination of eltoprazine and preladenant prevented and reduced Ldopa-induced AIMs, without impairing the efficacy of L-dopa in relieving motor symptoms (Ko et al., 2017; Pinna et al., 2016). At the same time, the present results increase the importance of those findings showing that this drug combination not only reduces AIMs during treatment, but also delays the onset of AIMs and contrasts their expression after acute administration of L-dopa.

Beside the anti-dyskinetic effect of eltoprazine, it is important to notice, that the role of A2A antagonist in the drug combination is fundamental in order to preserve the therapeutic efficacy of L-dopa, which is partially lost by the coaministration of L-dopa with eltoprazine (Bezard et al., 2013), as

demonstrated by findings of rotational behavior and motor activity (Pinna et al., 2016). Moreover, fluidity of movements during the whole subchronic pretreatment was preserved, as demonstrated by contralateral/ipsilateral rotations displayed by unilateral 6-OHDA-lesioned rats; indeed, contralateral rotational behavior was also qualitatively different from stereotyped contralateral rotation characterized by the typical bent position observed after L-dopa (Pinna et al., 2016).

In this study, the doses of drugs have been chosen on the basis of our previous publication by Pinna et al., 2016, in which we performed a dose finding study in order to obtain the optimal doses for this combination drugs.

The present study was focused on neuroinflammation induced by a L-dopa challenge in these subchronically pretreated rats, which is considered to be a very important aspect of PD neuropathology, as confirmed by recent preclinical findings (Bishop, 2019; Del Bel et al., 2016; Pisanu et al., 2018; Tansey and Goldberg, 2010). Indeed, microgliosis, astrogliosis and the production of pro-inflammatory cytokines by microglia seem to be involved in the development and expression of L-dopa-induced dyskinesia (Bishop, 2019; Carta et al., 2017; Del Bel et al., 2016, Pisanu et al., 2018).

The mechanism of the anti-neuroinflammatory activity of the combined treatment of eltoprazine and preladenant on L-dopa-induced neuroinflammation, could involve a concomitant and additional action of serotonergic 5-HT1A/1B and adenosine A2A receptors. In particular, the activation of serotonergic 5- HT1A/1B autoreceptors, dampening the peak of dopamine concentration after L-dopa administration, normalizes dopamine release, and through this mechanism reduces the induction of dyskinetic movements and the associated astrogliosis and microgliosis. Consistent with this suggestion, Mulas and collaborators (2016) demonstrated that intermittent dyskinetic L-dopa treatment exacerbated microgliosis and increased microglial TNF-α, while non-dyskinetic L-dopa treatment characterized by a continuous subcutaneous infusion, was devoid of pro-inflammatory effects in the unilateral 6-OHDA- lesioned rat. Moreover, several antidepressant drugs acting on

serotonergic transmission showed an antidyskinetic efficacy (Conti et al., 2014; 2016; Farajdokht et al., 2020). Among them, the SSRIs fluoxetine and paroxetine revealed an anti-inflammatory activity that was attributed to the inhibition of microglial activation and the reduction in various pro-inflammatory factors (Chung et al., 2010; Farajdokht et al., 2020; Zhang et al., 2012). Furthermore, Miyazaki and collaborators (2013) demonstrated that repeated stimulation of astroglial 5-HT1A receptors by the 8-hydroxy-2-(di-n- propylamino)tetralin (8-OH-DPAT) agonist promotes astrocyte proliferation but not activation, and upregulates antioxidative molecules both in vitro and in vivo. Therefore, treatment with 8-OH-DPAT protected dopaminergic neurons against oxidative stress through serotonin 5-HT1A receptors on astrocytes demonstrating an involvement of astroglia in the anti-inflammatory and neuroprotective mechanism of 5-HT1A agonists (Miyazaki et al., 2013). Regarding the mechanism involving adenosine A2A receptors, several findings demonstrated that blockade of the A2A receptors produces neuroprotective and anti-inflammatory effects in PD animal models (Armentero et al., 2011; Carta et al., 2009; Chen et al., 2001; Kalda et al., 2006; Pinna et al., 2010). A2A receptors play an important role in the activation of microglia, and A2A antagonists may limit the development of a fully activated microglia phenotype (Armentero et al., 2011; Gyoneva et al., 2014; Orr et al., 2009; Yu et al., 2008). It has recently been found that pretreatment of slices from MPTP-injected mice with the A2A receptor antagonist preladenant plays an important role in restoring the ability of activated microglia to respond to tissue damage (Gyoneva et al., 2014). Moreover, an in vitro study suggested that a possible mechanism underlying the striking ability of A2A receptor antagonists to limit neuronal damage caused by a variety of brain insults, including glutamate-induced neurotoxicity and neuroinflammation, involves the blockade of the release of pro-inflammatory cytokines, such as IL-1β (Simões et al., 2012). A2A antagonists down-regulated GFAP immunoreactivity in primary astrocytes and rodent models of neurodegeneration (Brambilla et al., 2003; Ke et al., 2009; Minghetti et al., 2007), and consistent with these data, A2A receptor knockout mice exhibit reduced astroglial development (Bura et al., 2008; Carta et al., 2009). Thus, the blockade of A2A receptors in astrocytes appears to reduce toxic astrogliosis and neurodegeneration, suggesting an important role of these receptors in reducing neuroinflammation on PD models. Specifically, astrocytes play an important role by modulating glutamate activity, through A2A receptors (Cunha, 2005). Whereas, chronic neuroinflammation is associated with the inhibition of glutamate uptake and enhanced release of glutamate by astrocytes, a phenomenon further amplified by activated microglia (Bezzi et al., 2001; Rothwell et al., 1997).

Moreover, both 5-HT1A agonists and A2A antagonists may play an important role in neurodegeneration and/or astrogliosis, by reducing the glutamate release and subsequent excitotoxicity on traumatic insult or neurodegenerative disease, including PD (Armentero et al., 2011; Bezard et al., 2006; Chen and Pedata, 2008; Li et al., 2001).

Altogether, the above-described mechanisms might prevent and delay the onset of dyskinesia by modulating microgliosis and astrogliosis induced by 6-OHDA lesion and treatment with L-dopa. However, we should evidence that the combined administration of L-dopa plus preladenant without eltoplazine, reduced astrogliosis and microgliosis but was not able to reduce or prevent AIMs. Thus, the reduction of neuroinflammation might not be the only mechanism involved in the reduction of dyskinesia.

Besides the anti-inflammatory factors that participates to the prevention of L-dopa-induced dyskinesia by this combination of drugs, it is important to underline that two complementary mechanisms may take place to this antidyskinetic effect; one presynaptic mechanism mediated by serotonergic neurons (Carta e Bezard, 2011) that stabilizes dopamine release from serotonin terminals and the other postsynaptic related to A2A receptors that increases dopamine D2 receptor responses allowing the utilization of low non-dyskinetic dosage of L-dopa (Morelli et al., 2007). It should be mentioned that a study by Lukasiewicz and co-workers (2007), which demonstrated a

direct A2A/5-HT1A receptor-receptor interaction in co-transfected cells lacks evidence in vivo. Another important finding of this study is that after challenge with L-dopa, unilateral 6-OHDA-lesioned rats pretreated with eltoprazine plus preladenant and L-dopa displayed a lower number of AIMs compared with those pretreated with L-dopa alone. Interestingly, this finding was associated with changes in the early gene zif-268, an index of long-term neuroplastic changes (Carta et al., 2008; Muñoz et al., 2008; Pinna et al., 2016; Simola et al., 2013). In agreement with previous studies, the early gene zif-268 was increased in L-dopa- and L-dopa plus preladenant-pretreated rats that displayed dyskinetic movements, whereas rats pretreated with eltoprazine plus L-dopa, with or without preladenant, had a lower zif-268 induction after the challenge with L-dopa compared with L-dopa-pretreated rats. Therefore, the subchronic pretreatment with eltoprazine, with or without preladenant, besides preventing and delaying AIMs induced by a challenge with L-dopa in these rats, reduced modifications in gene expression that are associated with dyskinesia (Pinna et al., 2016). Consistent with this finding, it has been reported that other serotonin 5-HTA/1B receptor agonists reduced the induction of other early genes, such as Fos-B, in the CPu in unilateral 6-OHDA-lesioned rats chronically treated with L-dopa (Muñoz et al., 2008). In conclusion, the present findings show that a challenge with L-dopa induces a reduced neuroinflammatory response in 6-OHDA-lesioned rats pretreated with an association of A2A antagonists and 5-HT1A/1B agonists, suggesting that this drug association may be a promising therapeutic strategy for preventing both neuroinflammation and dyskinesia.

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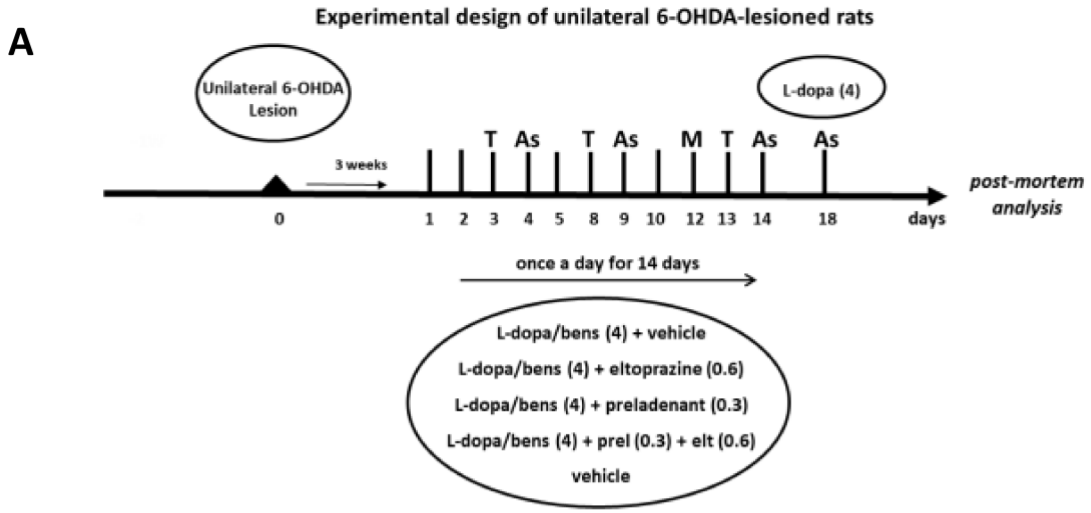
Figure Legends



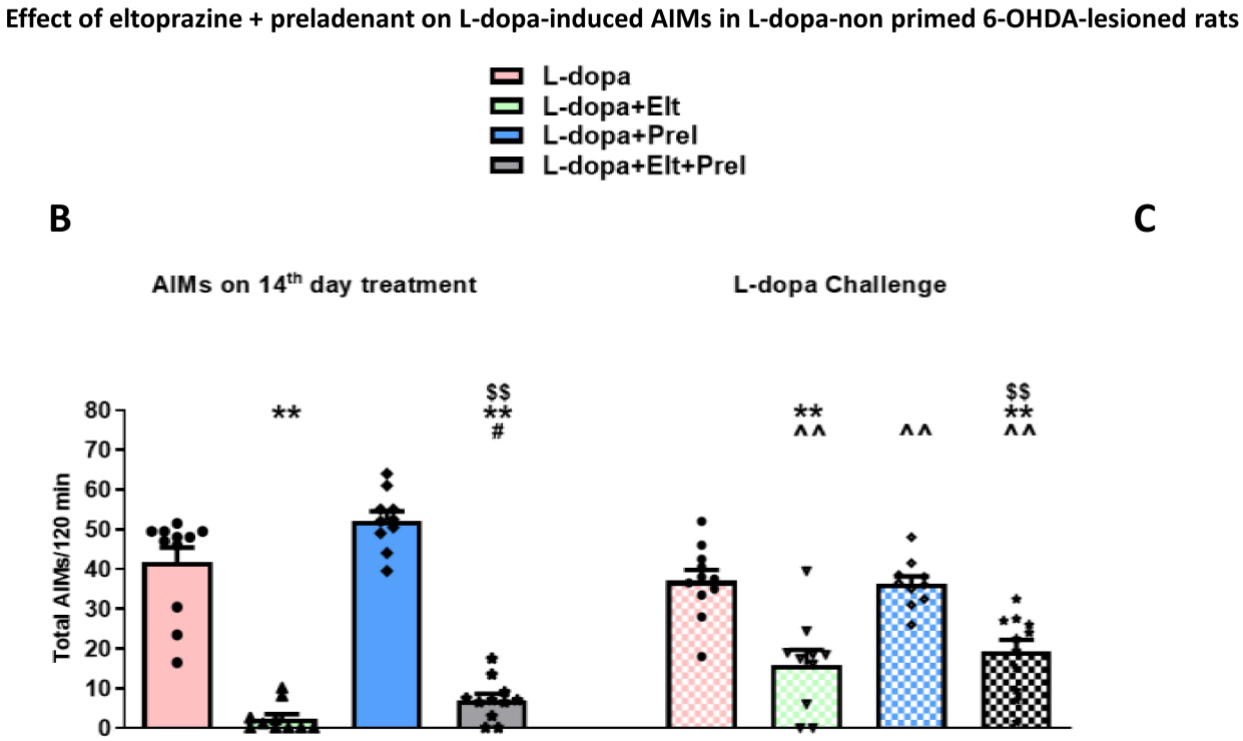
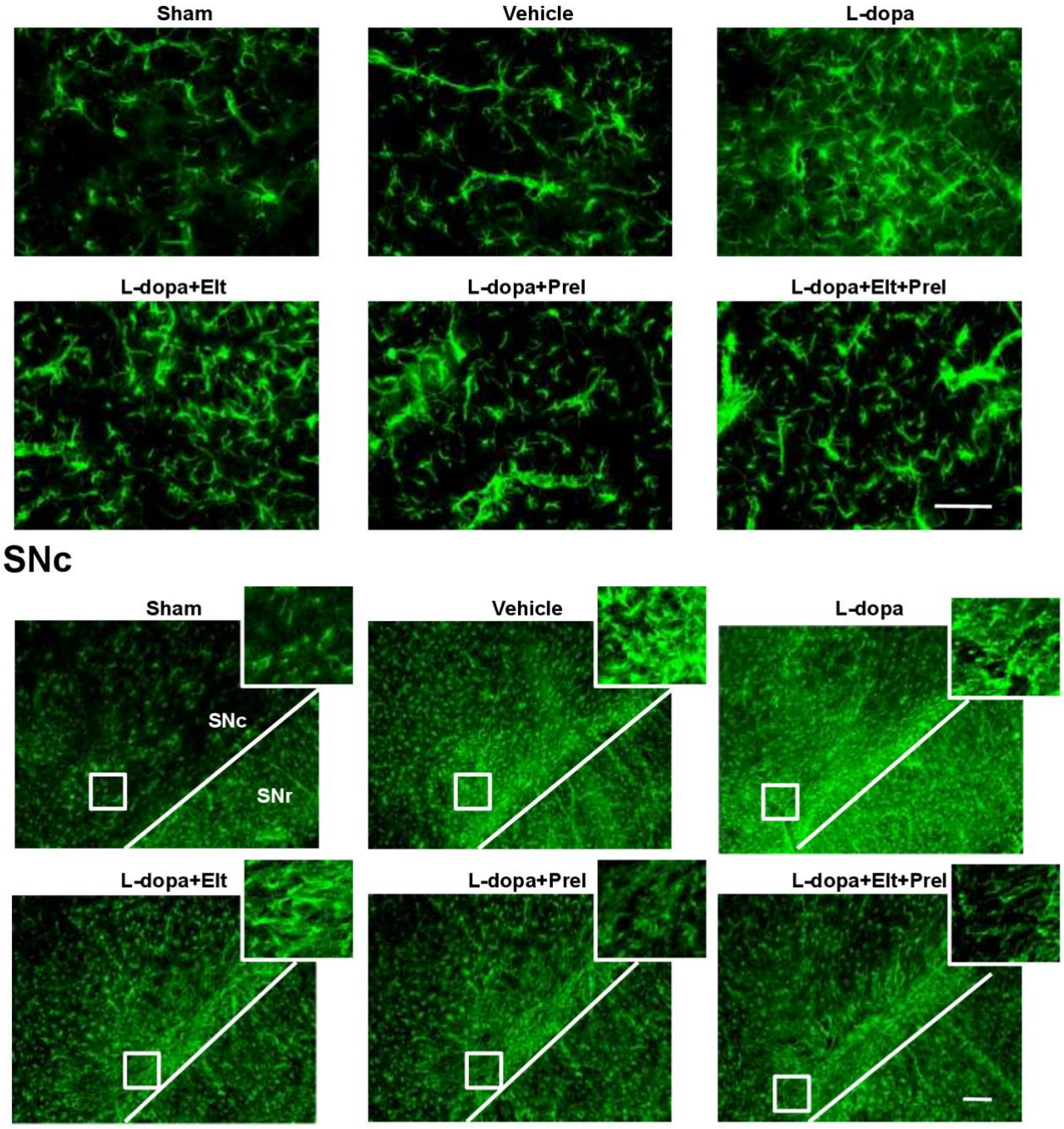


Fig. 1. Experimental design (A) and effect of subchronic pretreatment of eltoprazine (Elt) plus preladenant (Prel) on L-dopa-induced abnormal involuntary movements (AIMs), during 14th day of treatment (B) and during acute challenge with L-dopa (C) in unilateral 6-OHDA-lesioned rats.

Three weeks after lesion, rats were administered for two weeks as follow: L-dopa (4 mg/kg) plus benserazide (4 mg/kg) alone and/or with eltoprazine (0.6mg/kg) and/or with preladenant (0.3mg/kg) or vehicle, and their AIMs(As) and Turns (T) were evaluated three times each and motor activity (M) one time, during treatment. Four days after the treatment discontinuation, rats were challenged with L-dopa/benserazide (4 mg/kg) or vehicle and their AIMs were evaluated. Two hours after L-dopa challenge, rats were sacrificed for post-mortem immunohistochemical analysis (A).

Mean ± SEM of total AIMs scores measured in 120 min (B-C). Statistical significance was determined by one-way ANOVA followed by the Tukey's post hoc test. The results of ANOVA were confirmed with Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons test. ** $p < 0.005$ vs L-dopa; $$ $p < 0.005$ vs L-dopa+Prel; # $p < 0.05$ vs L-dopa+Elt. ^^ $p < 0.005$, ^ $p < 0.05$ vs the same group during the 14th day of treatment.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SNc
Sham
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SNr
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L-dopa+Elt+Prel

Fig. 2. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on glial fibrillary acidic protein (GFAP) immunoreactivity in the caudate-putamen (CPu) and substantia nigra pars compacta (SNc).

Representative sections of the CPu (upper photograms) and SNc (lower photograms) immunostained for GFAP and histograms of the number of GFAP-positive cells in CPu (on the left) and SNc (on the right). Experimental groups: sham-operated rats (CPu: n=8; SNc: n=7); vehicle-pretreated 6-OHDA- lesioned rats (CPu: n=10; SNc: n=10); L-dopa-pretreated 6-OHDA-lesioned rats (CPu: n=9; SNc: n=9); L-dopa plus eltoprazine-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus preladenant-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus eltoprazine and preladenant-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10). Values are expressed as mean ± SEM. $$$p < 0.001, $ p < 0.05 vs sham-operated rats; *** p < 0.001 vs vehicle-pretreated rats; ^^^ p < 0.001, ^^ p < 0.005 vs L-dopa-pretreated rats; & p < 0.05 vs L-dopa plus eltoprazine- pretreated rats. Scale bar: 50 μm.

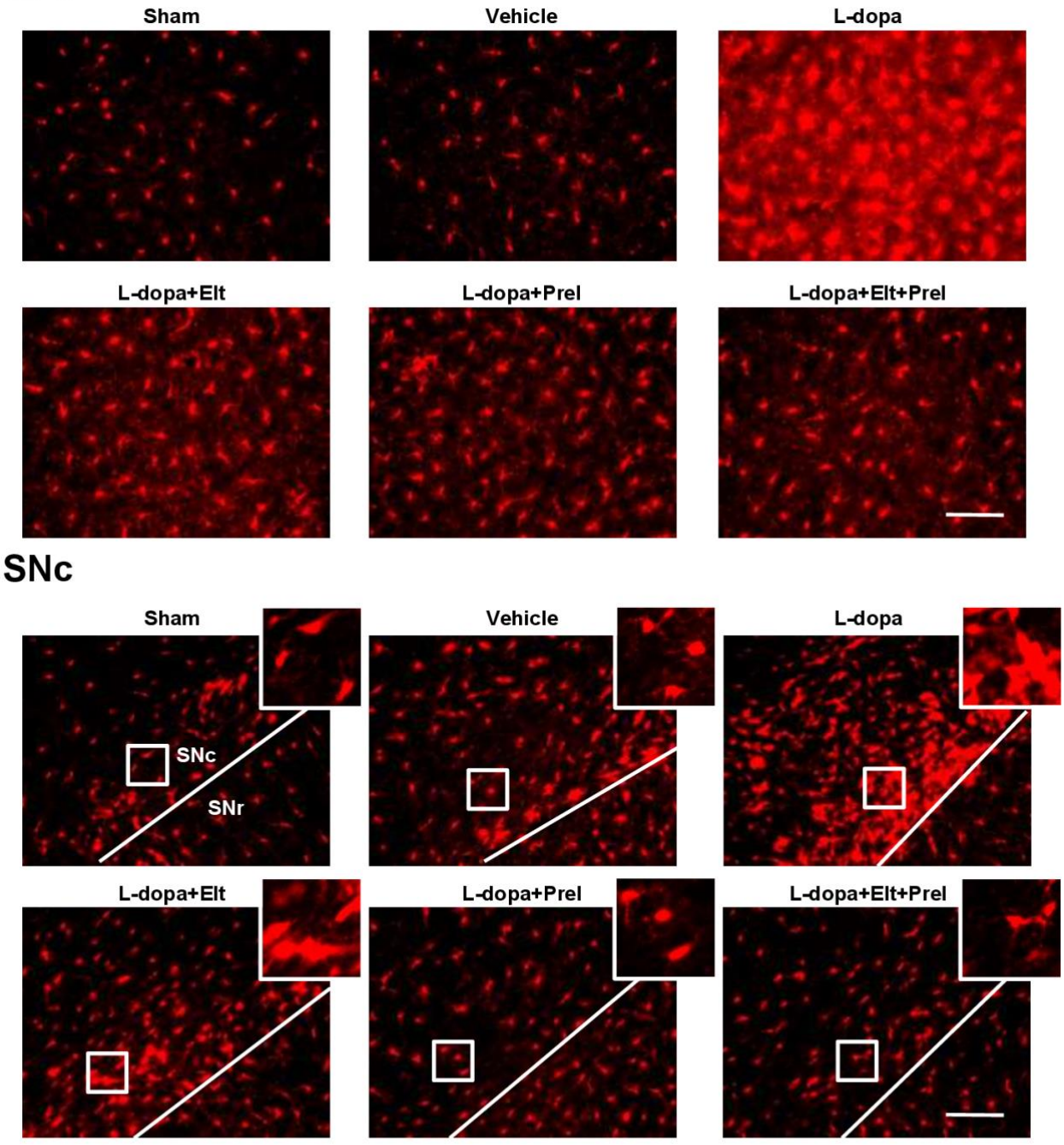
CPu
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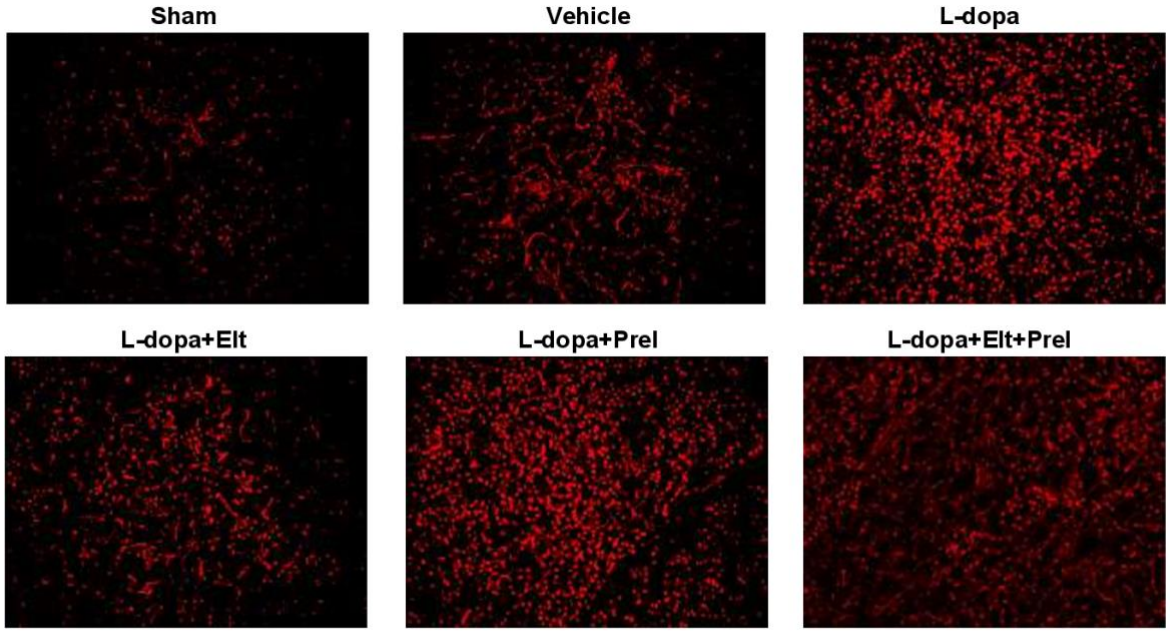
Fig. 3. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on ionized calcium binding adaptor molecule-1 (IBA-1) immunoreactivity in the caudate-putamen (CPu) and substantia nigra pars compacta (SNc).

Representative sections of the CPu (upper photograms) and SNc (lower photograms) immunostained for IBA-1 and histograms of the number of IBA-1-positive cells in CPu (on the left) and SNc (on the right). Experimental groups: sham-operated rats (CPu: n=8; SNc: n=7); vehicle-pretreated 6-OHDA- lesioned rats (CPu: n=10; SNc: n=10); L-dopa-pretreated 6-OHDA-lesioned rats (CPu: n=9; SNc: n=9); L-dopa plus eltoprazine-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus preladenant-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus eltoprazine and preladenant-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10). Values are expressed as mean ± SEM. $$$ $p < 0.001$, $$ $p < 0.005$ vs sham-operated rats; *** $p < 0.001$, ** $p < 0.005$, * $p < 0.05$ vs vehicle-pretreated rats; ^^^ $p < 0.001$ vs L-dopa-pretreated rats; & $p < 0.05$ vs L-dopa plus eltoprazine-pretreated rats. Scale bar: 50 μm.

CPu


Sham
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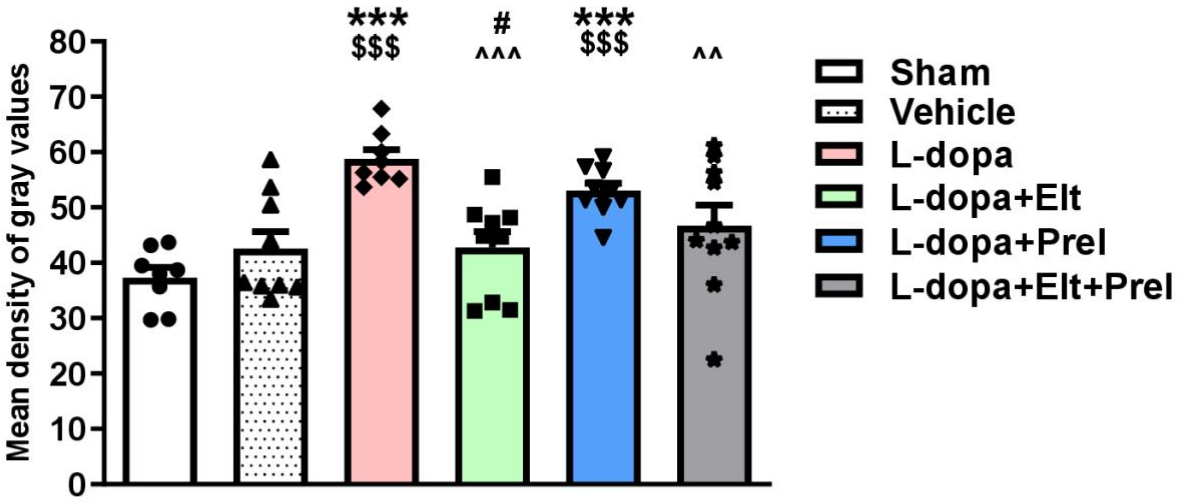
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L-dopa+Elt
L-dopa+Prel
L-dopa+Elt+Prel

Fig. 4. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on zif-268 immunoreactivity in the caudate-putamen (CPu).

Representative sections and histograms of the CPu immunostained for zif-268. Experimental groups: sham-operated rats (CPu: n=8; SNc: n=7); vehicle-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa-pretreated 6-OHDA-lesioned rats (CPu: n=9; SNc: n=9); L-dopa plus eltoprazine-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus preladenant-pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10); L-dopa plus eltoprazine and preladenant- pretreated 6-OHDA-lesioned rats (CPu: n=10; SNc: n=10).

Values are expressed as mean ± SEM. $$$ $p < 0.001$ vs sham-operated rats; *** $p < 0.001$ vs vehicle- pretreated rats; ^^^ $p< 0.001$, ^^ $p < 0.005$ vs L-dopa-pretreated rats; # $p < 0.05$ vs L-dopa plus preladenant-pretreated rats. Scale bar: 50 μm.

Table 1. Effects of combined administration of L-dopa plus eltoprazine and preladenant on AIMs score, rotational behaviors and motor activity counts

Treatments and **dose**			**Total AIMs score**			**Total motor activity**
L-dopa (4)	**Elt (0.6)**	**Prel (0.3)**	**Day 4**	**Day 9**	**Day 14**	**Day 12**
+	-	-	38.9 ± 2.3	41.56 ± 2.73	41.77 ± 2.97	6452 ± 522
+	+	-	0.3 ± 0.3**	0.6 ± 0.4**	2.33 ± 1.29**	4003 ± 693*
+	-	+	44.3 ± 3.2	49.94 ± 3.78	52.25 ± 2.3*	11398 ± 1789*
+	+	+	2.08 ± 0.9**##	4.41 ± 2.01**##	7.05 ± 1.26**##^	9013 ± 1272^^

Treatments and **dose**			**Contralateral / Ipsilateral total rotations**					
			Day 3		**Day 8**		**Day 13**	
L-dopa (4)	**Elt (0.6)**	**Prel (0.3)**	contralateral	ipsilateral	contralateral	ipsilateral	contralateral	ipsilateral
+	-	-	224 ± 43	109 ±25	343 ± 75	114 ±17	296 ± 56	110 ±20
+	+	-	31 ± 11**	59 ± 23	47 ± 16**	64 ± 23	68 ± 25**	79 ± 29
+	-	+	842 ± 236*	97 ± 21	1284 ± 222**	67 ± 11*	1486 ± 238**	73 ± 13
+	+	+	130 ± 36#^	131 ± 29	241 ± 59##^	165 ± 28##^	235± 49##^	203 ± 39*#^

Number ± SEM of total AIMs (axial, limb and orolingual) and number ± SEM of total contralateral and ipsilateral rotations after administration of L-dopa (4 mg/kg sc) alone and/or in combination with eltoprazine (Elt)(0.6 mg/kg sc) and/or preladenant (Prel)(0.3 mg/kg po) in already dyskinetic rats.

Number ± SEM of total counts of motor activity, after administration of L-dopa (4 mg/kg) alone and/or in combination with eltoprazine (0.6 mg/kg) and/or preladenant (0.3 mg/kg) in 6-OHDA-lesioned rats.

Statistical significance was determined by one-way ANOVA followed by Newman-Keuls post-hoc test.

**p<0.005, *p<0.05 vs L-dopa;

##p<0.005, #p<0.05 L-dopa+Elt+Prel vs L-dopa+Prel;

^^P<0.005, ^p<0.05 dopa+Elt+Prel vs dopa+Elt.

Table 2. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on the double immunoreactivity for the ionized calcium binding adaptor molecule-1 (IBA-1) and interleukin (IL)-1β in the caudate–putamen (CPu) and substantia nigra pars compacta (SNc).

Experimental Group	Rr CPu	Rr SNc
Sham	0.694±0.007	0.627±0.009*
Vehicle	0.839±0.06$^{\$}$	0.818±0.081
L-dopa	0.807±0.03$^{\$}$	0.703±0.032
L-dopa+Elt	0.673±0.019$^{*\wedge}$	0.567±0.029***
L-dopa+Prel	0.650±0.02$^{**\wedge}$	0.616±0.039*
L-dopa+Elt+Prel	0.651±0.02$^{**\wedge}$	0.575±0.031***

The correlation of signal intensity was calculated as a Pearson's correlation coefficient (Rr). $ p < 0.05 vs Sham; *** p < 0.001, ** p < 0.005, * p < 0.05 vs Vehicle; ^ p < 0.05 vs L-dopa. N=7-10 for each experimental group.

Table 3. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on the double immunoreactivity for the ionized calcium binding adaptor molecule-1 (IBA-1) and interleukin (IL)-10 in the caudate–putamen (CPu) and substantia nigra pars compacta (SNc).

Experimental Group	Rr CPu	Rr SNc
Sham	0.501±0.016	0.369±0.036
Vehicle	0.567±0.044	0.456±0.033
L-dopa	0.547±0.031	0.469±0.066
L-dopa+Elt	0.555±0.021	0.486±0.079
L-dopa+Prel	0.504±0.038	0.550±0.069$^{\$*}$
L-dopa+Elt+Prel	0.539±0.038	0.581±0.098$^{\$*}$

The correlation of signal intensity was calculated as a Pearson's correlation coefficient (Rr). $ p < 0.05 vs Sham; * p < 0.05 vs Vehicle. N=7-10 for each experimental group.

Table 4. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on the double immunoreactivity for the ionized calcium binding adaptor molecule-1 (IBA-1) and tumor necrosis factor (TNF)-α in the substantia nigra pars compacta (SNc).

Experimental Group	Rr SNc
Sham	0.291±0.010
Vehicle	0.326±0.010
L-dopa	0.353±0.009
L-dopa+Elt	0.319±0.014$^{\wedge}$
L-dopa+Prel	0.315±0.028$^{*\wedge\wedge}$
L-dopa+Elt+Prel	0.318±0.014$^{*\wedge}$

The correlation of signal intensity was calculated as a Pearson's correlation coefficient (Rr). $ p < 0.05 vs Sham; * p < 0.05 vs Vehicle; ^^ p < 0.005, ^ p < 0.05 vs L-dopa. N=7-10 for each experimental group.

Table 5. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine plus preladenant on the immunoreactivity for tyrosine hydroxilase (TH) in the caudate–putamen (CPu) and substantia nigra pars compacta (SNc).

Experimental Group	Mean density of TH gray values in CPu	Number of TH-positive neurons in SNc
Sham	196.79±2.71	161.08±4.89
Vehicle	19.17±6.29$$$	7.80±1.29$$$
L-dopa	20.20±7.21$$$	12.33±2.58$$$
L-dopa+Elt	12.33±2.20$$$	14.32±2.98$$$
L-dopa+Prel	14.4±2.05$$$	10.72±3.08$$$
L-dopa+Elt+Prel	26.04±8.28$$$	19.38±4.34$$$

Densities of TH-positive fibers and number of neurons are expressed as mean ± SEM of the real quantified values. $$$p < 0.001 vs. Sham. n=7-10 for each experimental group.

Table 6. Effect of subchronic pretreatment with L-dopa, L-dopa plus eltoprazine, L-dopa plus preladenant or L-dopa plus eltoprazine and preladenant on the immunoreactivity for the dopamine transporter (DAT) in the caudate–putamen (CPu).

Experimental Group	Mean density of DAT gray values in CPu
Sham	183.02±2.16
Vehicle	142.27±7.35$
L-dopa	130.72±10.35$$
L-dopa+Elt	116.20±9.92$$$
L-dopa+Prel	143.44±13.11$
L-dopa+Elt+Prel	145.25±10.42$

Densities of DAT-positive fibers are expressed as mean ± SEM of the real quantified values. $$$ p < 0.001, $$ p < 0.005, $ p < 0.05 vs Sham. N=7-10 for each experimental group.

Table 7. Pearson's correlation coefficient (r) between Total AIMs and neuroinflammatory Markes in L-dopa- and L-dopa plus Eltoprazine and preladenant pretreated unilateral 6-OHDA-lesioned rats

Markers	Day 14 of Treatment	L-dopa Challenge
AIMs vs GFAP in CPu	r=0.66, p<0.005**	r=0.54, p<0.05*
AIMs vs GFAP in SNc	r=0.63, p<0.005**	r=0.48, p=0.05*
AIMs vs IBA-1 in CPu	r=0.83, p<0.0001***	r=0.57, p=0.05*
AIMs vs IBA-1 in SNc	r=0.62, p<0.005**	r=0.48, p<0.05*

Correlations between total AIMs and neuroinflammatory markers (GFAP and IBA-1 positive cells), in the CPu and SNc, expressed by unilaterally 6-OHDA-lesioned rats pretreated with L-dopa and the combined administration of L-dopa plus Eltoprazine plus preladenant. The correlation between total AIMs and neuroinflammatory markers was calculated as a Pearson's correlation coefficient (r). *** p < 0.001, ** p < 0.005, * p < 0.05.