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Research Paper

Parkinson's disease and pain: Modulation of nociceptive circuitry in a rat model of nigrostriatal lesion

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ABSTRACT

Parkinson's disease (PD) is a neurodegenerative disorder that causes progressive dysfunction of dopaminergic and non-dopaminergic neurons, generating motor and nonmotor signs and symptoms. Pain is reported as the most bothersome nonmotor symptom in PD; however, pain remains overlooked and poorly understood. In this study, we evaluated the nociceptive behavior and the descending analgesia circuitry in a rat model of PD. Three independent experiments were performed to investigate: i) thermal nociceptive behavior; ii) mechanical nociceptive behavior and dopaminergic repositioning; and iii) modulation of the pain control circuitry. The rat model of PD, induced by unilateral striatal 6-hydroxydopamine (6-OHDA), did not interfere with thermal nociceptive responses; however, the mechanical nociceptive threshold was decreased bilaterally compared to that of naive or striatal saline-injected rats. This response was reversed by apomorphine or levodopa treatment. Striatal 6-OHDA induced motor impairments and reduced dopaminergic neuron immunolabeling as well as the pattern of neuronal activation (c-Fos) in the substantia nigra ipsilateral (IPL) to the lesion. In the midbrain periaqueductal gray (PAG), 6-OHDA-induced lesion increased IPL and decreased contralateral PAG GABAergic labeling compared to control. In the dorsal horn of the spinal cord, lesioned rats showed bilateral inhibition of enkephalin and μ-opioid receptor labeling. Taken together, we demonstrated that the unilateral 6-OHDA-induced PD model induces bilateral mechanical hypernociception, which is reversed by dopamine restoration, changes in the PAG circuitry, and inhibition of spinal opioidergic regulation, probably due to impaired descending analgesic control. A better understanding of pain mechanisms in PD patients is critical for developing better therapeutic strategies to improve their quality of life.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder classically recognized by extensive dopaminergic loss in the nigrostriatal pathway (Hirsch and Hunot, 2009; Hornykiewicz and Kish, 1987) (Hirsch and Hunot, 2009), resulting in motor signs such as bradykinesia, resting tremor, rigidity and postural instability (Obeso et al., 2004) and non-motor signs such as autonomic disorders, and symptoms as sleep disturbances, depression and pain (Chaudhuri and Schapira, 2009). In most PD cases, pain appears before motor and cognitive symptoms and is reported by at least one-third of patients (Beiske et al., 2009; Goetz et al., 1986). Since the mechanisms of persistent pain in PD patients are poorly understood, painful symptoms are often neglected (Nandhagopal et al., 2010; Rana et al., 2013), contributing to deterioration of the quality of life of patients (Quittenbaum and Grahn, 2004; Visser et al., 2009). Despite the high incidence of pain in PD patients, few studies have explored the relationship between nigrostriatal injury and pain circuitry.

Modulation of the dopaminergic system in different nervous system structures, such as the basal ganglia, limbic areas, thalamus, midbrain periaquedutal gray (PAG) and spinal cord, plays a critical role in endogenous analgesia (Chudler and Dong, 1995; Hagelberg et al., 2002;

Abbreviations: APO, apomorphine; DHSC, dorsal horn of the spinal cord; ENK, enkephalin; GABA, gamma-aminobutyric acid; GAD, glutamic acid decarboxylase; MOR, μ-opioid receptor; PAG, periaqueductal gray; vlPAG, ventrolateral PAG; PD, Parkinson's disease; SN, substantia nigra; SNpc, SN *pars* compacta; SNpr, *SN pars* reticulata.

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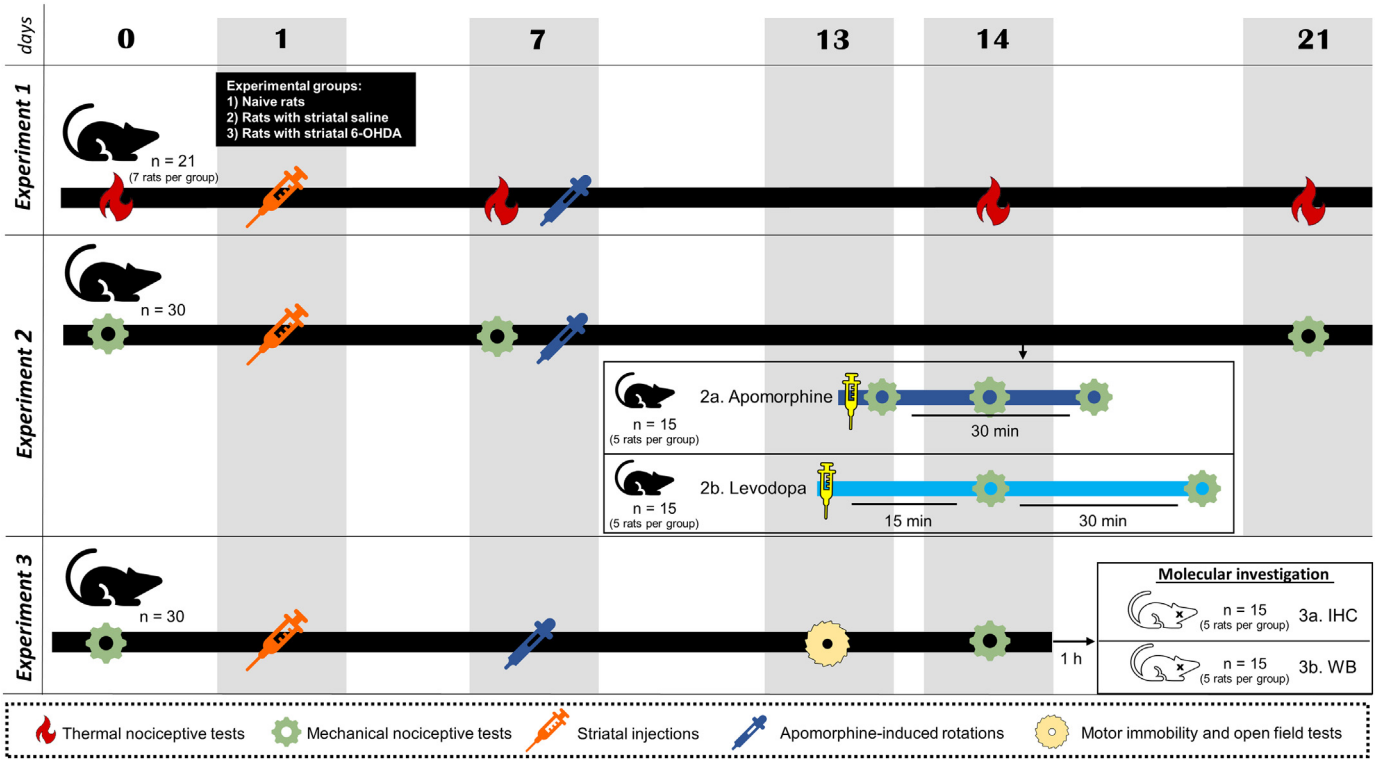

Fig. 1. Experimental design of the study. The rats were anesthetized, and 6-OHDA (to induce the PD model) or saline (control group) was injected into the left striatum. Naive animals were also investigated. Animals were evaluated by the apomorphine-induced rotation test 7 days after striatal injections to characterize the PD induction model in all 3 experiments. In the first experiment, the animals were evaluated to determine the thermal nociceptive threshold (tail-flick and plantar tests) before and 7, 14 and 21 days after the striatal injections and in naive rats. In the second experiment, the nociceptive threshold to nonpainful mechanical stimuli (von Frey filament test) was evaluated before, 7 and 14 days after the striatal injections and the mechanical nociceptive threshold (paw pressure test) was evaluated before and 7, 14 and 21 days after the striatal injections. Naive rats were also evaluated in these same times. On the 14th day, the animals were treated with the dopaminergic agonist, apomorphine (Experiment 2a) or levodopa (Experiment 2b), and immediately after or after 15 min, respectively, they were re-evaluated using the paw pressure test. The mechanical nociceptive test was repeated 30 min later to evaluate the time-response of dopaminergic repositioning. In the third experiment, motor immobility and open field tests were performed 13 days after striatal injections, and both tests were performed without any treatment or after haloperidol treatment to intensify motor deficits. The mechanical nociceptive threshold was evaluated before and 14 days after PD model induction. One hour after the last nociceptive evaluation, the brains and spinal cords were collected for immunohistochemistry (IHC, Experiment 3a) or western blotting (WB, Experiment 3b) assays. The immunoreactivity for tyrosine hydroxylase (a marker of dopaminergic neurons), c-Fos (a marker of activated neuronal nuclei) and GABA in the SN, for c-Fos and GABA in the PAG, and for enkephalin and MOR in the DHSC were evaluated by IHC. GAD 65/67 (enzymes that catalyze the conversion of glutamate to GABA) expression in the midbrain and spinal cord were evaluated by WB. DHSC: dorsal horn of the spinal cord; GABA: gamma-aminobutyric acid; GAD: glutamic acid decarboxylase; MOR: μ-opioid receptor. PAG: periaqueductal gray matter; PD: Parkinson's disease; SN: substantia nigra.

Melzack and Wall, 1965; Wood, 2008). In the ventrolateral PAG (vlPAG), dopaminergic neurons are interconnected with GABAergic neurons, which are crucial to pain control (Flores et al., 2004). Unilateral 6-hydroxydopamine (6-OHDA)-induced lesion of the nigrostriatal pathway in rats is widely used as a PD model (Blandini et al., 2008; Jackson-Lewis et al., 2012), which exhibit hypersensitivity to thermal and mechanical stimuli (Saadé et al., 1997; Takeda et al., 2014; Tassorelli et al., 2007) that is reversible via acute dopaminergic treatment (Nascimento et al., 2018) as observed in PD patients (Gerdelat-Mas et al., 2007). Even though dopamine has a critical role in pain sensitivity, other monoamines, such as noradrenaline and serotonin, are also very important for pain control (Bannister and Dickenson, 2016; Fields et al., 1991), which are depleted in PD patients (Delaville et al., 2011; Scatton et al., 1983). Descending analgesia mediated by noradrenergic and serotoninergic systems arises from the PAG, rostral ventromedial medulla and locus ceruleus, and disinhibition of GABAergic neurons in the vlPAG by the opioid system is required for its activation, resulting in inhibition of nociceptive transmission in the dorsal horn of the spinal cord (DHSC) (Lau and Vaughan, 2014; Millan, 2002; Ossipov et al., 2010). In addition to that, other direct or indirectly monoaminergic projections to the DHSC can also contribute to the inhibitory effect in the spinal cord (Millan, 2002). In this sense, dopaminergic circuitry could also influence nociceptive behavior direct in the DHSC by hypothalamic A11 projections acting in spinal D2 receptors (Kim et al., 2015; Taniguchi et al., 2011).

In the DHSC, the monoamines released by descending fibers directly or indirectly inhibit the nociceptive primary afferents, the spinothalamic projection neurons and the excitatory interneurons, leading to decreased output of nociceptive information (Millan, 2002; Ossipov et al., 2010). Concomitantly, inhibitory interneurons present in the DHSC, such as enkephalinergic, glycinergic and GABAergic interneurons, are depolarized, directly inhibiting spinal nociceptive neurons (Budai and Fields, 1998; Fields et al., 1991; Pertovaara and Almeida, 2006). GABAergic interneurons represent approximately 30% of spinal neurons in laminae I and II and 45% of neurons in lamina III (Todd, 2010), and their loss contributes to persistent pain (Guo and Hu, 2014). Enkephalinergic interneurons, which are found in the superficial laminae I-II (substantia gelatinosa), acting on the μ-opioid (MOR) and δ-opioid (DOR) receptor, are crucial to inhibition of nociceptive transmission (Budai and Fields, 1998; Millan, 2002; Raynor et al., 1994). Despite the importance of dopamine in the modulation and perception of pain, the relationship among PD, persistent pain and the circuitry involved in pain control remains unclear. The purpose of this study was to evaluate the nociceptive behavior of rats in a 6-OHDA-induced PD model and critical areas for nociceptive modulation to understand the mechanisms underlying pain hypersensitivity in PD. We hypothesized

that dopaminergic therapy may reduce the pain in these animals, and that the decrease in nociceptive threshold induced by PD model is accompany by inhibition of the descending analgesic pathway and spinal modulation.

2. Methods

2.1. Animals

A total of 81 adult male Wistar rats (200–250 g) were used in this work. The rats were housed in acrylic boxes (3 rats/box) for five days before the experimental procedures were initiated. The animals were maintained in a controlled environment under a 12/12-h light-dark cycle at room temperature (22 ± 2 °C) with wood shavings and free access to water and rat chow pellets. The experimental procedures were carried out considering the guidelines for the ethical use of animals in research involving pain and nociception (Zimmermann, 1983) and were reported in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). The study was approved by the Ethics Committee on the Use of Animals at Hospital Sírio-Libanês (São Paulo, Brazil) under protocol number CEUA 2009/06. The investigators were blind to group allocation in all behavioral analyses. The experimental outline is detailed in Fig. 1.

2.2. PD model induction

Rats were anesthetized with 2.5% 2,2,2-tribromoethanol (250 mg/kg, i.p., Sigma-Aldrich, MO, USA). Under stereotaxic conditions, 12 μg of 6-OHDA (Sigma-Aldrich) diluted in 2 μL of 0.9% saline with 0.2% ascorbic acid was injected in two different points into the left striatum (6 μg/μL of 6-OHDA in each point) (Chudler and Lu, 2008). The injection was performed at the following coordinates: 2.7 mm mediolateral (ML) and 4.5 mm dorsoventral (DV) (first point); and 3.2 mm ML, 0.5 mm anteroposterior (AP) and 4.5 mm DV (second point) (Paxinos and Watson, 2005). The control group was injected with 1 μL of saline in two different points into the left striatum. At the end of injection, the needle was held in place for an additional 5 min to avoid backflow of the solution. Then, the wound was closed and the animals were observed until fully recovered from anesthesia. A food supplement (Ensure, Abbott, SP, BRA) was added to the diet of the animals once a day for 2 days after the procedure, facilitating the recovery of the animals with nigrostriatal lesions.

2.3. Measurement of nociceptive responses

2.3.1. Thermal nociceptive threshold

The thermal nociceptive threshold was assessed in the tails of restrained rats using the tail-flick test (EFF 300-Light, Insight, SP, Brazil) as previously described (D'amour and Smith, 1941) and in the hindpaws of freely moving rats with the plantar test (plantar analgesia meter, IITC, CA, USA) as described by Hargreaves et al. (Hargreaves et al., 1988). In the tail-flick test, the nociceptive threshold was evaluated 3 times, and the mean of the 3 measurements was considered the baseline threshold. In the plantar test, the nociceptive threshold was measured 3 times bilaterally. A cutoff of 20 s was applied to avoid tissue damage. The results were analyzed by comparing the initial and final measurements. To reduce stress, the rats were habituated to the testing procedures one day before the experiment. The thermal nociceptive threshold for both tests was assessed before, 7, 14 and 21 days after the striatal injection. Naive rats that were not submitted to any surgical procedure were also evaluated in these same times (Experiment 1).

2.3.2. Mechanical nociceptive threshold

The mechanical nociceptive threshold was measured in response to tactile stimuli using von Frey filaments (Stoelting, IL, USA) with increasing force (from 407 mg to 15.136 g) (Chaplan et al., 1994). The threshold response was evaluated as previously described (Milligan et al., 2003). Briefly, the filaments were applied randomly to the planter surface of the hindpaw to determine the stimulus-intensity threshold required to elicit a paw-withdrawal response. The withdrawal response regarding the nociceptive threshold to nonpainful mechanical stimuli (to analyze the allodynia phenomenon) was evaluated before, 7 and 14 days after the striatal injection. Naive rats were also evaluated in these same times (Experiment 2).

The mechanical nociceptive threshold was also assessed using a pressure apparatus on the hind paws (EEF-440, Insight) as previously described (Randall and Selitto, 1957). Briefly, a mass (g) was applied continuously with increasing intensity over the hind paw of each animal. The mass required to induce a paw-withdrawal response represented the mechanical nociceptive threshold (to analyze the hyperalgesia phenomenon). 14 days after surgery, the animals were treated with the dopaminergic agonists, levodopa/benserazide (Prolopa, 50/12.5 mg/Kg, respectively, s.c., Hoffmann-La Roche, BSL, SUI) (Experiment 2a) or apomorphine (APO, 0.5 mg/Kg, s.c., Tocris Bioscience, BZ, UK) (Experiment 2b). The paw pressure test was performed 15 min after Prolopa treatment or immediately after APO treatment based on the time of these agonists' onset of action. All animals were re-evaluated in the nociceptive test after 30 min considering the clearance of the agonists. Naive rats and striatal saline-injected rats were also injected with the dopaminergic agonists. Additionally, other groups of animals were evaluated in the paw pressure test, before, 7 and 14 days after the striatal injection, followed by the molecular tests. Naive rats were also evaluated in these same times (Experiment 3).

The results of the thermal and mechanical nociceptive tests were analyzed by comparing the initial and final measurements. To reduce stress, the rats were habituated to the testing procedure 1 day before the experiment.

2.4. Measuring motor behaviors

2.4.1. Apomorphine-induced rotational behavior

Rotational asymmetric behavior was assessed using an automated rotometer system (Rota-Count 8, Columbus Instruments, OH, USA). The animals were injected with apomorphine (1 mg/Kg, s.c., Tocris Bioscience) and evaluated over 30 min as previously described (Zhang et al., 2008). The criterion for rotation was a 180° turn to the side contralateral to the injured hemisphere. To reduce stress, the rats were habituated for 30 min one day before the rotational test. Animals injected with 6-OHDA that did not present asymmetric rotational behavior were excluded. Apomorphine-induced rotational behavior was assessed seven days after the striatal injection in all 3 experiments. Naive rats were also evaluated in this same time.

2.4.2. Behavioral immobility and the open-field test

Since the nociceptive tests used here depend on the motor responses of animals to determine the nociceptive threshold, we selected an experimental PD model that does not induce exacerbated motor deficits. To induce motor impairment in the behavioral immobility and open-field tests in our PD model, the animals were injected with haloperidol (Haldol, 1 mg/Kg, s.c., Jansen-Cilag, SP, BRA), a dopaminergic antagonist, and then immediately evaluated in the bar test, followed by the open-field test. Control animals also received the dopaminergic antagonist. The behavioral immobility and open field tests were performed 13 days after the striatal injections. Naive rats were also evaluated in this same time (Experiment 3).

The immobility test, or the typical catalepsy or bar test, consists of placing an animal into an unusual posture and recording the time for the animal to correct this posture (Sanberg, 1980). Behavioral immobility was characterized by muscle rigidity and failure to correct an imposed posture for a prolonged period. In this test, the animals were positioned with both forepaws on a 9-cm-high horizontal bar (0.9-cm diameter). The time course during which the animal remained

motionless in this imposed posture was considered the bar test elapsed time (with a cutoff time of 120 s). The behavioral immobility endpoint was considered when both forepaws were removed from the bar or when the animal moved its head in an exploratory manner.

Locomotor activity and exploratory behavior were measured in an open-field arena (43.2 cm × 43.2 cm). Each animal was individually placed in the center of the arena and assessed for 20 min using an automatic infrared beam system (MED-OFA-RS, Med Associates, VT, USA). The following measures were taken: the distance traveled (locomotion) and vertical counts (the number of times the animal stood on its hind legs) (Broadhurst, 1960). The arena was cleaned with alcohol (5%) after each session to avoid possible biasing effects from olfactory cues. Animals without the haloperidol treatment were also assessed in the open field test to confirm the need to use dopaminergic antagonist to induce motor deficit in our PD model.

2.5. Immunohistochemistry

Immunohistochemical assays were performed as previously described (Pagano et al., 2012). Briefly, the rats from Experiment 3a were anesthetized with ketamine/xylazine (0.5/2.3 mg/Kg, respectively, intramuscularly) and then underwent transcardial perfusion with saline solution, followed by 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffer (PB). The animals were perfused 1 h after the paw pressure test because the peak expression of c-Fos protein occurs approximately 1 h after the stimulus and fade by 3 to 4 h post-stimulation (Herdegen and Leah, 1998).The brains and spinal cords (L2-L5 segments) were collected and postfixed in PFA for 4 h, followed by incubation with 30% sucrose solution in PB for 48 h at 4 °C. Coronal sections (30 µm) were obtained using a freezing microtome. Tissue sections were incubated with the following agents: 1) specific primary antibodies: mouse anti-tyrosine hydroxylase (TH, 1:1000, MAB5280, Millipore, MA, USA), rabbit anti-c-Fos (1:1000, PC38, Calbiochem), guinea-pig anti-GABA (1:500, NT 108, Protos Biotech, NY, USA), rabbit anti-ENK (1:500, MAB350, Millipore), or rabbit anti-MOR (1:500, sc-7488,Cell Signaling); 2) biotinylated secondary antibodies (1:200, Jackson ImmunoResearch, ME, USA); and 3) avidin-biotin-peroxidase complex (1100, ABC Elite kit, Vector Labs, CA, USA). Then, sections were visualized with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) and hydrogen peroxide. All antibodies were previously tested and compared to reactions that concealed the primary antibody to verify any cross-reactivity. The sections were mounted on glass slides, air-dried, dehydrated and coverslipped. Finally, images were captured utilizing a light microscope (Eclipse E1000, Nikon, NY, USA). Using ImageJ software (National Institutes of Health, MD, USA; http://rsbweb.nih.gov/ij/), the immunoreactivity (IR) intensity was evaluated to determine the relative densities of positively-labeled pixels for TH, GABA, ENK and MOR and the number of c-Fos-positive nuclei. Then, the IR results of the controls (the naive group) were normalized to 100 for comparison with the other groups. For each antibody, measurements were taken from 5 sections per animal and 5 animals per group. The regions of interest, including the substantia nigra (SN), vlPAG and DHSC, were identified based on specific atlases (Paxinos and Watson, 2005; Molander et al., 1984).

2.6. Western blotting

Rats from Experiment 3b were decapitated and their midbrains and spinal cords were immediately collected, separated into left and right hemispheres, frozen in liquid nitrogen, and stored at −70 °C. Tissues were then homogenized at 4 °C with extraction buffer (Tris 100 mM, pH 7.4; ethylenediaminetetraacetic acid 10 mM; phenylmethyl sulfonyl fluoride 2 mM; and aprotinin 0.01 mg/mL). The homogenates were centrifuged at 12,000 rpm at 4 °C for 20 min, the supernatant protein concentration was determined by the Bradford method (Bio-Rad, CA, USA), and the materials were stored at −70 °C. During electrophoretic separation, the samples were diluted in Laemmli sample buffer (Bio-Rad) with 2.5% 2-mercaptoethanol and boiled for 5 min. The samples were transferred to an 8% polyacrylamide SDS gel and subjected to electrophoresis. Then, the proteins were transferred to nitrocellulose membranes (0.2-µm diameters, Millipore), blocked for 2 h at room temperature with phosphate-buffered saline containing 0.05% Tween-20 (TTBS) and 5% nonfat milk, and incubated overnight at 4 °C with rabbit anti-GAD 65/67 (1:2000, AB1511, Millipore) diluted in 5% albumin in TTBS. The membranes were washed (3 × 10 min) with TTBS and then incubated with horseradish peroxidase IgG (1:10,000, Amersham Biosciences, NJ, USA) diluted in TTBS with 1% nonfat milk for 2 h at room temperature. After an additional wash cycle, the antigens were developed using a chemiluminescence kit (ECL, Amersham Biosciences) and analyzed for the density of the labeled bands using ImageJ software. The membranes were then incubated for 30 min at room temperature in stripping buffer (Abcam, UK, USA) and then with rabbit anti-β-actin (1:10,000, AC-74, Sigma-Aldrich) as a loading control. The data of the naive group were normalized to 100 for comparison with the other groups.

2.7. Data analysis

The results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed with GraphPad Prism. Statistical analysis of the nociceptive tests was performed using two-way analysis of variance (ANOVA) comparing group versus time, followed by Bonferroni's post hoc test. For the rotational behavior test, immobility test, open-field test, immunohistochemistry and western blotting assays, one-way ANOVA followed by Tukey's post hoc test was used. In all analyses, $p \leq .05$ was considered statistically significant.

3. Results

3.1. The 6-OHDA-induced PD model and the thermal nociceptive threshold

Rats were evaluated using the tail-flick and plantar tests before and 7, 14 and 21 days after 6-OHDA or saline injection. Striatal 6-OHDA injection did not induce changes in the thermal nociceptive threshold during the tail-flick test ($F_{(2,24)} = 2.021$, $p = .0817$, Fig. 2A) or the plantar test or in the left ($F_{(2,18)} = 0.61$, $p = .7203$, Fig. 2B) or right ($F_{(2,18)} = 0.1786$, $p = .9792$, Fig. 2C) paws compared with that of the control animals, including the naive and saline-injected rats.

3.2. The 6-OHDA-induced PD model, mechanical nociceptive threshold and dopaminergic repositioning

Rats injected with 6-OHDA showed a decreased (61%) withdrawal threshold to nonpainful mechanical stimuli (measured by von Frey filaments) in the ipsilateral paws 7 and 14 days after PD induction compared to the baseline measurement ($F_{(5,23)} = 7179$, $p < .001$, Fig. 3A). In the paws contralateral to the lesions, a decreased (87%) nociceptive threshold was observed only on day 7, which returned to the initial threshold 14 days after PD induction, compared to the baseline measurement ($F_{(5,22)} = 6.847$, $p < .01$, Fig. 3B).

Mechanical hyperalgesia (measured by paw pressure test) was observed bilaterally 7, 14 and 21 days after 6-OHDA injection in the PD rats, with a reduced pressure-elicited paw withdrawal threshold (56%) in both the left ($F_{(2,96)} = 18.50$, $p < .001$, Fig. 3C and E) and right ($F_{(2,96)} = 18.50$, $p < .001$, Fig. 3D and F) paws compared to that of the control groups. Apomorphine treatment transiently reversed the mechanical hyperalgesia in the left (Fig. 3C) and right (Fig. 3D) paws 14 days after PD induction. Thirty min after apomorphine injection, the nociceptive threshold for pressure decreased to 39% in the left paw (Fig. 3C) and 35% in the right paw (Fig. 3D) in relation to baseline and control group values. Similarly, levodopa transiently reversed the mechanical hyperalgesia in the left ($F_{(2,48)} = 25.16$, $p < .0001$; Fig. 3E)

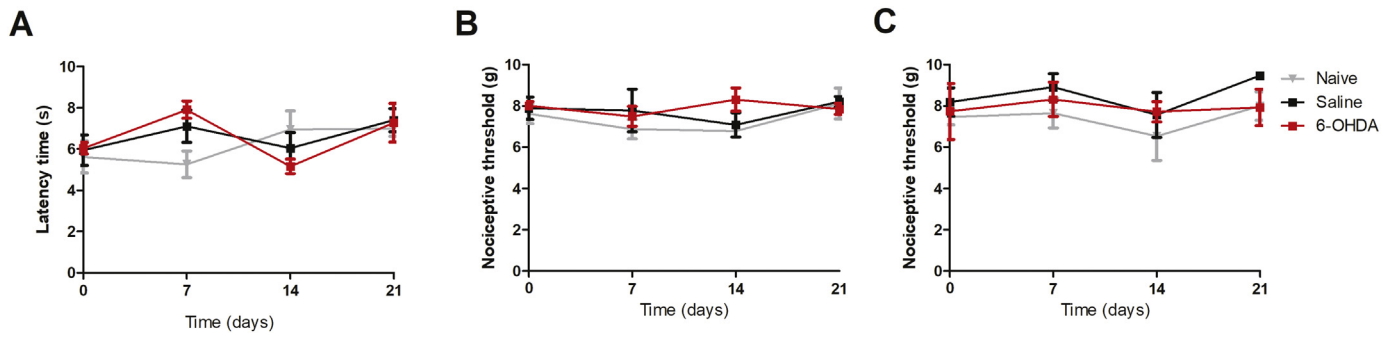

Fig. 2. Thermal nociceptive threshold and 6-OHDA-induced PD model. The tail-flick test (A) and plantar test in the left (ipsilateral to the lesion, B) and right (contralateral to the lesion, **C**) hind paws were performed before (day 0) and 7, 14 and 21 days after injection of saline or 6-OHDA into the left striatum. Naive rats without any surgical intervention were also evaluated. The values represent the mean ± SEM ($n = 7$ per group). 6-OHDA: 6-hydroxidopamine.

and right ($F_{(2,96)} = 18.50$, $p < .001$, Fig. 3F) paws 15 min after injection in the 6-OHDA-treated rats, and 45 min after levodopa treatment, the mechanical nociceptive threshold decreased again to 38% in the left paw (Fig. 3E) and 40% in the right paw (Fig. 3F) compared to those in the control groups.

3.3. Characterization of the PD model and the activation pattern in the SN

Behavioral immobility and open field tests were performed 13 days after the striatal injections and in naive rats treated or not with haloperidol. Increased latency on the bar test was observed in the 6-OHDA-treated animals compared to that of the naive and saline groups

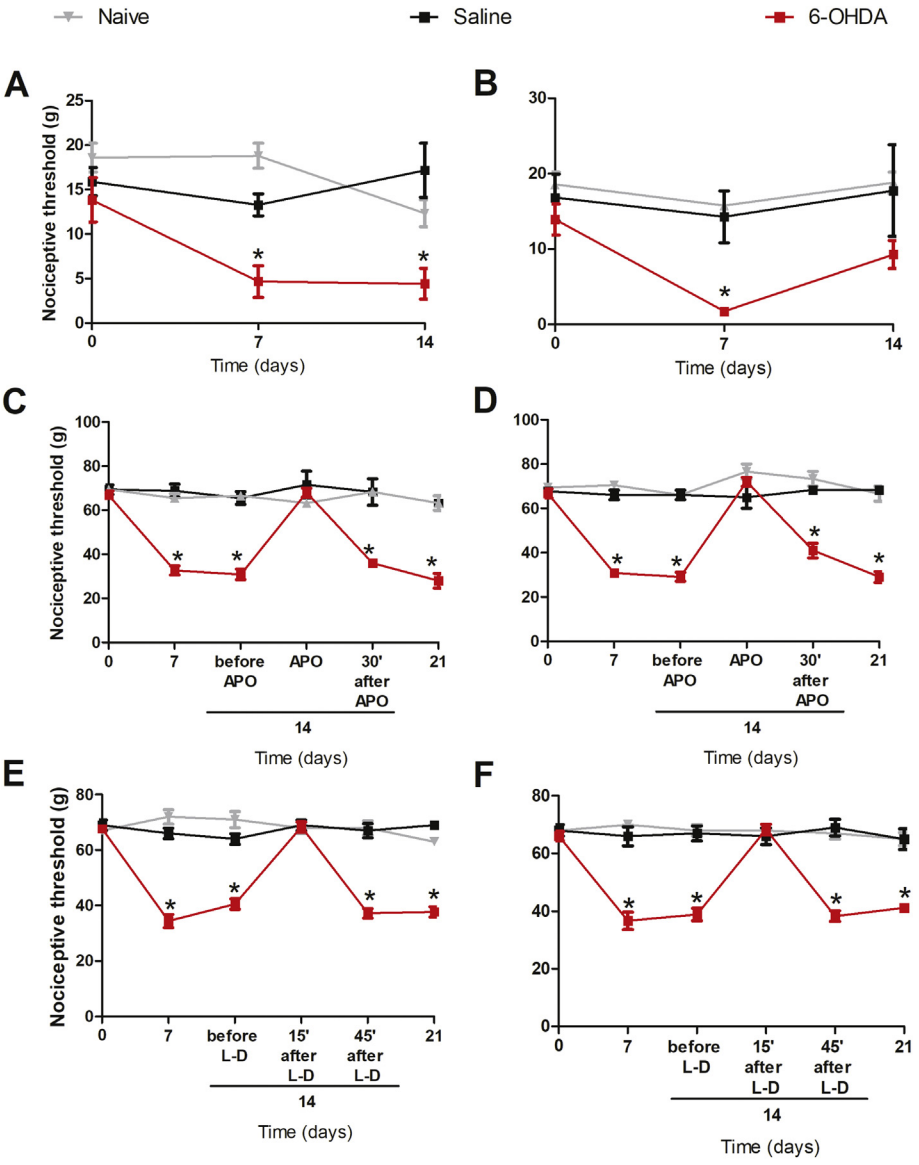

Fig. 3. Mechanical nociceptive threshold, 6-OHDA-induced PD model and dopamine involvement. Allodynia (A and B) and hyperalgesia (C–F) behaviors were evaluated in the hind paws of naive, saline-treated and 6-OHDA-treated animals. The test was performed in both paws, ipsilateral (IPL) to the lesion (A, C and E) and contralateral (CL) to the lesion (B, D and F), before (day 0) and 7 and 14 days after injection of the neurotoxin. 14 days after the procedure, the animals were injected with apomorphine (C and D) or levodopa (E and F), and immediately after or 15 min after, respectively, they were re-evaluated using the paw pressure test in the left (C and E) and right (D and F) paws. After 30 min, the animals were again evaluated. The values represent the mean ± SEM ($n = 5$ per group). *$p < .05$ compared to the control groups (naive or saline). 6-OHDA: 6-hydroxidopamine. APO: apomorphine. L-D: levodopa.

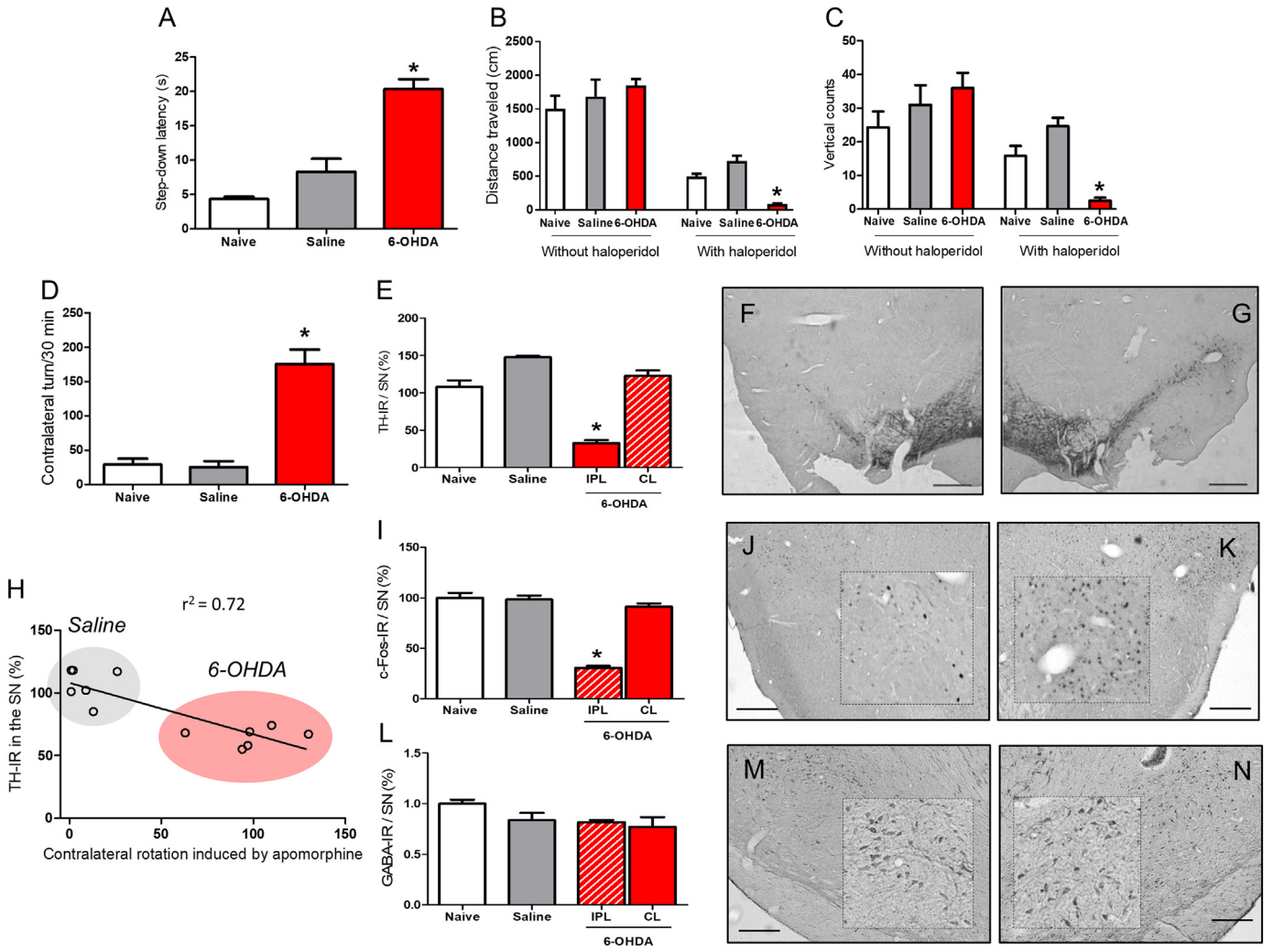

Fig. 4. Characterization of the 6-OHDA-induced PD model and the activation pattern of the substantia nigra. 13 days after the striatal injections, the animals were injected with haloperidol (Haldol, 1 mg/Kg, s.c.), and after 30 min, they were evaluated for behavioral immobility (catalepsy/bar test) (A). Animals injected or not with haloperidol were assessed for motor impairment in the open-field test by evaluating the distance traveled (cm) (B) and vertical counts (C). 7 days after the striatal injections, the rotational asymmetric behavior induced by apomorphine was evaluated (D). Quantification of immunoreactivity (IR) for tyrosine hydroxylase (TH, E), c-Fos (I) and GABA (L) in the substantia nigra (SN) of the animals injected with saline or 6-OHDA in the left striatum. Naive animals were used as controls. The values represent the mean ± SEM (n = 5 per group). The IR results of the controls (the naive group) were normalized to 100 for comparison with the other groups. Pearson correlation coefficient was determined comparing TH-IR in the SN (%) and contralateral rotation induced by apomorphine (H). *p < .05 compared to the naive group. Representative photomicrographs of TH-IR (F and G), c-Fos-IR (J and K) and GABA-IR (M and N) in the ipsilateral (F, J and M) and contralateral (G, K and N) SN of rats injected with 6-OHDA. Scale bars: 100 μm. 6-OHDA: 6-hydroxidopamine.

($F_{(2,8)}$ = 36.71, p = .0004, Fig. 4A). Our 6-OHDA-induced PD model per se did not induce changes in the parameters of distance traveled (Fig. 4B) and vertical counts (Fig. 4C) in the open-field test, when compared with naive and saline groups. However, the treatment with haloperidol was able to induce motor deficits in our PD model, decreasing the distance traveled ($F_{(2,13)}$ = 32.18, p < .0001, Fig. 4B) and vertical counts ($F_{(2,15)}$ = 32.01, p < .0001, Fig. 4C), compared with control groups also treated with the dopaminergic antagonist. In relation to apomorphine-induced rotational behavior, the rats subjected to unilateral striatal 6-OHDA injections exhibited pronounced contralateral rotation to the lesioned side in response to apomorphine ($F_{(2,29)}$ = 37.33, p = .0066, Fig. 4D), compared with the control groups. Fourteen days after striatal 6-OHDA injection, a significantly reduced number of dopaminergic neurons was observed in the ipsilateral SN *pars* compacta (SNpc) (62%) compared with that in the control groups ($F_{(3,41)}$ = 40.11, p < .0001, Fig. 4E–G). There was a strong negative correlation between the percentage of neurons in the SNpc and the contralateral rotations induced by apomorphine (r^2 = 0.72, p < .001, Fig. 4H). In addition, in the SNpc, the remaining neurons on the ipsilateral side of the lesion were less activated (less c-Fos-IR) than the neurons on the contralateral side of the lesion and the neurons in the SNpc of control rats ($F_{(3,11)}$ = 80.04, p < .0001, Fig. 4I–K), but no changes in GABA labeling were observed ($F_{(3,11)}$ = 2.415, p = .1418, Fig. 4L–N).

3.4. The 6-OHDA-induced PD model, descending pain control and spinal nociceptive circuitry

In the PAG, a critical nucleus of the descending analgesic pathway, 6-OHDA did not induce neuronal activation pattern changes compared with the control groups ($F_{(3,10)}$ = 8.488, p = .099, Fig. 5A, D, E). To better understand the activation pattern of the PAG, we evaluated the GABAergic system by analyzing GAD 65/67, enzymes that catalyze the conversion of glutamate to GABA, in the midbrain. We did not find any difference between the groups for GAD expression in the midbrain ($F_{(3,11)}$ = 80.04, p < .0001, Fig. 5B). Since both the SN and PAG are important GABAergic nuclei located in the midbrain, we immunohistochemically analyzed GABA labeling specifically in the vlPAG.

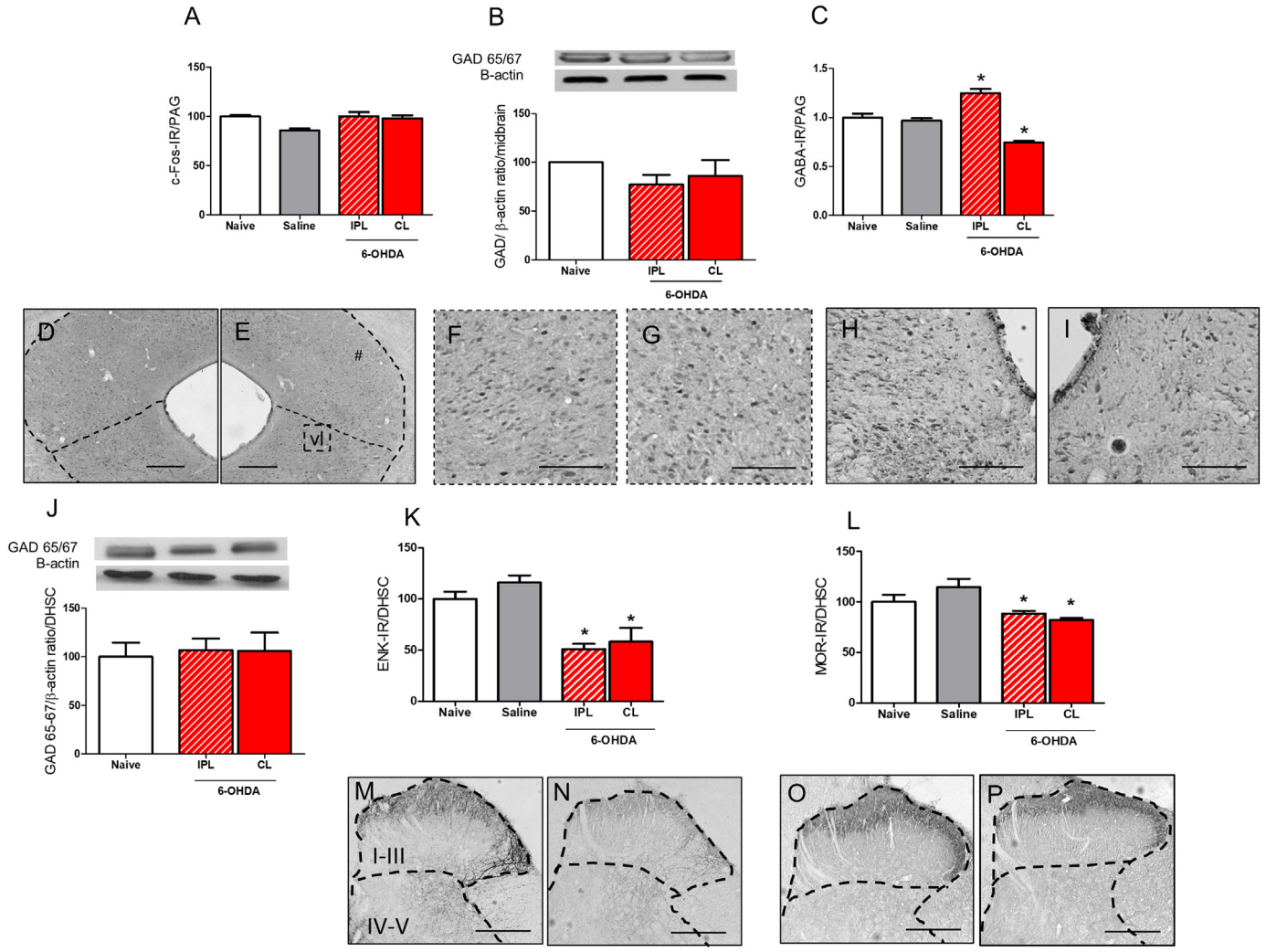

Fig. 5. Modulation of the circuitry of pain control and the 6-OHDA-induced PD model. Quantification of immunoreactivity (IR) for c-Fos (A) and GABA (C) in the ventrolateral periaqueductal gray (vlPAG) by immunohistochemistry, and for glutamic acid decarboxylase (GAD) 65/67 in the midbrain by western blotting (B) of the animals injected with saline or 6-OHDA in the left striatum. The IR results of the controls (the naive group) were normalized to 100 for comparison with the other groups. *p < .05 when compared to the naive group. Representative photomicrographs of c-Fos-IR ipsilateral (IPL, D) and contralateral (CL, E) PAG of 6-OHDA-injected rat. Figs. F and G represent higher magnification insets of the vlPAG of naive and 6-OHDA-injected rats, respectively. Representative photomicrographs of the GABA-IR in the lPL (H) and CL (I) vlPAG of 6-OHDA-injected rats. Quantification of IR for GAD 65/67 in the spinal cord (J), by western blotting, and for enkephalin (ENK, K) and for μ opioid receptor (MOR, L) in the dorsal horn of the spinal cord (DHSC), by immunohistochemistry, of naive, saline or 6-OHDA rats. Representative photomicrographs of the ENK-IR (M and N) and MOR-IR (O and P) in the CL DHSC of rats injected with striatal saline (M and O) or 6-OHDA (N and P). Laminae I–V of the DHSC are represented in figs. M-P. 6-OHDA: 6-hydroxidopamine. Scale bars: 50 μm (D and E) and 100 μm (F–I and M-P).

The PD model induced an increase in GABA-IR on the ipsilateral side of the lesion and a decrease on the contralateral side compared with that in the naive group ($F_{(3,11)} = 38.99$, $p < .0001$, Figs. 4C, F, G).

In the DHSC, our PD model did not affect GAD 65/67 expression compared with that in the naive rats ($F_{(2,8)} = 0.06059$, $p = .9418$, Fig. 5H). Both ENK-IR ($F_{(3,11)} = 13.00$, $p = .0019$, Figs. 4I, K, L) and MOR-IR ($F_{(3,11)} = 6.205$, $p = .0175$, Fig. 4J, M, N) were decreased after PD model induction in both sides of the DHSC compared with those in the control groups.

4. Discussion

Pain is among the most frequent nonmotor symptoms in PD and has been reported in all PD stages (Blanchet and Brefel-Courbon, 2018). Since the PD diagnosis mainly relies on motor signs, which first appear only after 40–60% SN dopaminergic neuron loss (Obeso et al., 2000), correlating pain and disease progression is difficult. Consistent with clinical findings, rat PD models exhibited reduced thermal and mechanical nociceptive thresholds (Cao et al., 2016; Charles et al., 2018; Chudler and Dong, 1995; Dieb et al., 2014; Gee et al., 2016; Kaszuba et al., 2017; Saadé et al., 1997; Takeda et al., 2014; Wang et al., 2017, 2018; Zengin-Toktas et al., 2013). The intensity of 6-OHDA-induced nigrostriatal lesions depends on both the site and dose of the neurotoxin, with injection in the medial prosencephalic bundle or SN causing more severe damage to the nigrostriatal pathway compared to striatal injection (Chen et al., 2013; Deumens et al., 2002; Hökfelt and Ungerstedt, 1973; Przedborski et al., 1995). Decreased thermal nociceptive thresholds have been demonstrated in rats 21 days after striatal 6-OHDA injection versus 7 days after injection (Chudler and Dong, 1995), justifying our findings and suggesting that more significant loss of dopaminergic neurons is crucial for inducing changes in thermal pain sensitivity. In our study, the 6-OHDA-induced PD model resulted in bilateral hyperalgesia, which is consistent with other studies (Chudler and Lu, 2008; Saadé et al., 1997; Takeda et al., 2014). The nociceptive threshold reduction was reversed by systemic administration of dopaminergic agonists (both apomorphine and levodopa), which

is consistent with previous data showing that dopamine replacement improves pain behavior in rat PD models and PD patients (Brefel-Courbon et al., 2005; Carta et al., 2007; Dellapina et al., 2011; Dolphin et al., 1976; Factor et al., 2000; Gerdelat-Mas et al., 2007; Nascimento et al., 2018). Considering that levodopa can be converted to noradrenaline (Dolphin et al., 1976) and influence serotonergic terminals (Carta et al., 2007), as well as the importance of these systems in pain modulation (Millan, 2002; Ossipov et al., 2010; Pertovaara, 2006), we suggest that levodopa may act as an analgesic agent not only in the dopaminergic system but also in the noradrenergic and serotonergic systems.

By exploring central sensitization in our model, we found that the 6-OHDA-induced PD model also resulted in mechanical allodynia, a phenomenon evidenced in persistent pain condition (Coderre et al., 1993; Takeda et al., 2014). Consistent with our results, mesostriatal 6-OHDA administration induced mechanical allodynia in the paw ipsilateral to the nigrostriatal lesion 35 days after PD model induction (Takeda et al., 2014). Taken together, our results demonstrate that the PD model studied interferes with mechanical nociceptive behavior; however, our model failed to mimic thermal nociceptive alterations.

For characterization of our PD model, we showed that striatal 6-OHDA injection causes apomorphine-induced asymmetric rotation, behavioral immobility and motor deficits in the open-field test, which were assessed after acute haloperidol treatment, and inhibition of TH staining in the SNpc as expected (Barnéoud et al., 2000; Blandini et al., 2008), suggesting that nigrostriatal lesions induced akinesia, bradykinesia, muscular rigidity, and gait and postural instability, similar to the signs described in PD patients (Agid, 1991). Importantly, we selected a PD model with minimal effects on motor deficits (Kirik et al., 1998), considering that the nociceptive tests used in this study depend on motor behavioral responses. Therefore, the dopaminergic system required manipulation to elicit motor impairment in our PD model, observed by comparing open field test with and without haloperidol, corroborating prior findings (Barnéoud et al., 2000; Jaskiw and Popli, 2004; Johnson and McFarland, 1993; Kirik et al., 1998). Disturbance of the GABAergic system has been suggested in the SN of hemiparkinsonian rats (di Michele et al., 2013; Galeffi et al., 2003; Rangel-Barajas et al., 2008) and PD patients (Anglade et al., 1995; Błaszczyk, 2016), resulting in overactivity of the remaining dopaminergic neurons in the SN (Błaszczyk, 2016; Zigmond et al., 1984). We did not observe these phenomena since GABA labeling did not change and decreased neuronal activation was observed in the SN in our PD model. Most dopaminergic neurons are localized in the SNpc, while GABAergic neurons are essentially restricted to the SN *pars* reticulata (SNpr) (González-Hernández and Rodríguez, 2000); therefore, hyperactivity of nigral GABAergic cells is largely localized in the SNpr after nigrostriatal degeneration (Floran et al., 1988; González-Hernández and Rodríguez, 2000; Wang et al., 2010). We investigated the SNpc, which may explain the discrepancy between our findings. Since dopaminergic neurons are involved in nociceptive response modulation (Chudler and Dong, 1995; Magnusson and Fisher, 2000; Taylor et al., 2016; Wood, 2008), inhibition of SN activation after striatal 6-OHDA injection can directly contribute to the decreased nociceptive threshold observed here.

The influence of dopaminergic circuitry damage on the PAG, the critical nucleus involved in the descending analgesic pathway (Basbaum and Fields, 1984; Tracey and Mantyh, 2007), is not clear. Striatal 6-OHDA did not change vlPAG activation, observed by c-Fos labeling, corroborating the findings of Tassorelli et al. (Tassorelli et al., 2007) who also did not identify c-Fos expression changes in the PAG of hemiparkinsonian rats. However, we observed increased GABA-positive neurons in the PAG ipsilateral to the 6-OHDA injection site and decreased GABA-positive neurons on the contralateral side. Although the number of c-Fos-positive neurons remained the same, suggesting no difference in the pattern of activation, those that were activated produced more GABA in the ipsilateral PAG. To activation of the descending analgesic system is required the inhibition of GABAergic interneurons within the vlPAG, which occurs via opioid action (Basbaum and Fields, 1984; Chiou and Huang, 1999; Kalyuzhny and Wessendorf, 1998; Lau and Vaughan, 2014). Hence, we may suggest that the increased labeling of GABAergic interneurons, in the PAG ipsilateral to the lesion, contribute to the inhibition of analgesic pathway in our PD model. On the other hand, increased pre-synaptic GABA release is observed in persistent pain models (Basbaum and Fields, 1984; Chiou and Huang, 1999; Hahm et al., 2011; Kalyuzhny and Wessendorf, 1998). Consistently, we suggest that increased GABA in the ipsilateral vlPAG is correlated with the nociceptive behavior induced by our PD model, and probably as a compensatory mechanism, the contralateral vlPAG showed decreased GABA-positive neurons. Dysregulation of the equilibrium between the descending inhibitory and facilitatory pain systems may explain the chronification of pain induced by our PD model (Vanegas and Schaible, 2004).

Descending pain modulation regulates pain-related signals in the DHSC, producing both hyperpolarization (inhibitory system) and depolarization (facilitatory system) of spinal relay nociceptive neurons (Bannister and Dickenson, 2016; Millan, 2002; Ossipov et al., 2010). The importance of serotonin and noradrenaline input in this regulation is well-characterized in the literature; however, the dopaminergic circuitry in the DHSC may also play an important role in controlling the nociceptive transmission (Millan, 2002; Vallone et al., 2000). Activation of GABAergic and enkephalinergic interneurons is fundamental for decreasing the output of nociceptive information (Budai and Fields, 1998; Ossipov et al., 2010; Ruda et al., 1986). In our 6-OHDA-induced PD model, no changes were observed in the spinal GABAergic system; however, both ENK and MOR immunostaining were decreased in the superficial laminae of the DHSC, reflecting downregulation of the spinal opioidergic system. Consistent with our findings, loss of spinal GABAergic neurons was not observed in a rat model of persistent pain (Polgár et al., 2003; Somers and Clemente, 2002). Regarding the opioidergic system, enkephalin acting on the MOR and DOR inhibits the release of excitatory pronociceptive neurotransmitters, such as substance P and calcitonin gene-related peptide, from nociceptive neurons in the DHSC, consequently reducing nociceptive transmission (Budai and Fields, 1998; Millan, 2002; Ossipov et al., 2010; Raynor et al., 1994). The MOR is present in lamina I projection neurons and in lamina II excitatory interneurons in the DHSC (Kemp et al., 1996; Marker et al., 2006). In the absence or with downregulation of spinal MOR and ENK expression, regulation of neurotransmission in pain circuits is compromised (Basbaum and Fields, 1984; Budai and Fields, 1998; Todd, 2010), which may be positively correlation with the hypernociceptive behavior observed. Supporting our finding, Charles et al. (2018) recently showed increased central sensitization by altering nociceptive integration in the DHSC in a rat model of PD. Furthermore, consistent with our data, disorder of the opioidergic system is suggested in PD patients (Sgroi and Tonini, 2018; Stefano et al., 2012). Therefore, we hypothesized that dysregulation of the vlPAG as well as the opioidergic deficit in the DHSC that may be responsible for the decreased nociceptive threshold observed in the 6-OHDA-induced PD model. Our findings in relation to the spinal opioidergic deficit in PD condition are promising to a further understanding of different therapeutic targets.

5. Conclusion

In summary, our data showed that a unilateral 6-OHDA-induced PD model induces bilateral mechanical hypernociception, which was reversed by dopamine restoration. Additionally, nigrostriatal lesions induced dysregulation of GABAergic levels in the vlPAG, suggesting impairment of the descending analgesic system and activation of the descending pain facilitatory system, causing downregulation of the spinal opioidergic system and therefore leading to nociceptive sensitization. Since pain is an important nonmotor symptom in PD, a better understanding of its pathophysiological mechanisms can lead to treatment and quality of life optimization in these patients.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RAD, ACPC, MBB, STM and KGR performed the nigrostriatal lesion induction procedure and the nociceptive and motor behavioral tests. RAD, ACPC and MBB performed the immunohistochemical assays. ACPC and KGR drafted the manuscript. ETF and MSH critically reviewed the work. RLP conceived the study, participated in its design and coordination, and drafted the manuscript. All authors read and approved the final manuscript.

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