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Research paper

Monoaminergic regulation of nociceptive circuitry in a Parkinson's disease rat model

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ABSTRACT

Pain is a common nonmotor symptom of Parkinson's disease (PD) that remains neglected and misunderstood. Elucidating the nondopaminergic circuitry may be key to better understanding PD and improving current treatments. We investigated the role of monoamines in nociceptive behavior and descending analgesic circuitry in a rat 6-hydroxydopamine (6-OHDA)-induced PD model and explored the resulting motor dysfunctions and inflammatory responses. Rats pretreated with noradrenaline and serotonin reuptake inhibitors were given unilateral striatal 6-OHDA injections and evaluated for mechanical hyperalgesia and motor impairments. Through immunohistochemistry, the number and activation of neurons, and the staining for astrocytes, microglia and enkephalin were evaluated in specific brain structures and the dorsal horn of the spinal cord. The PD model induced bilateral mechanical hyperalgesia that was prevented by reuptake inhibitors in the paw contralateral to the lesion. Reuptake inhibitors also prevented postural immobility and asymmetric rotational behavior in PD rats without interfering with dopaminergic neuron loss or glial activation in the substantia nigra. However, the inhibitors changed the periaqueductal gray circuitry, protected against neuronal impairment in the locus coeruleus and nucleus raphe magnus, and normalized spinal enkephalin and glial staining in lesioned rats. These data indicate that the preservation of noradrenergic and serotonergic systems regulates motor responses and nociceptive circuitry during PD not by interfering directly with nigral lesions but by modulating the opioid system and glial response in the spinal cord. Taken together, these results suggest that nondopaminergic circuitry is essential to the motor and nonmotor symptoms of PD and must be further investigated.

1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized by dopaminergic (DAergic) neuron loss in the nigrostriatal system, leading to motor and nonmotor features (Agid, 1991; Chaudhuri and Schapira, 2009; Obeso et al., 2004). Pain is a nonmotor symptom often described by PD patients, and its presence contributes to the deterioration of quality of life (Chaudhuri et al., 2006; Chaudhuri and Schapira, 2009). The prevalence of pain among PD patients is approximately 67% (Broen et al., 2012; Broetz et al., 2007; Defazio et al., 2008; Quittenbaum and Grahn, 2004) and is frequently reported prior to the onset of debilitating motor symptoms (Blandini et al., 2008; Cummings, 1992); however, the precise mechanisms involved in the pathogenesis of PD-induced pain are not fully understood.

Dopamine (DA) in the basal ganglia has been shown to modulate the pain response (Chaudhuri and Schapira, 2009; Chudler and Dong, 1995; Magnusson and Fisher, 2000; Mylius et al., 2009). However, despite the focus on the DAergic system and basal ganglia, PD also induces neuronal loss in other systems, including the noradrenergic (NAergic) locus coeruleus (LC) (Bertrand et al., 1997; Fornai et al., 2007) and the serotonergic (5-HTergic) raphe nuclei (Braak et al., 2003; Huot et al., 2011; Kish, 2003), which are critical for pain modulation through the descending analgesic pathways (Basbaum and Fields, 1984). In general, after the activation of the limbic system in response

Abbreviations: 5-HT, serotonin; 6-OHDA, 6-hydroxydopamine; C/D, citalopram + desipramine; DHSC, dorsal horn of the spinal cord; DA, dopamine; ENK, enkephalin; GABA, gamma-aminobutyric acid; IR, immunoreactivity; LC, locus coeruleus; NA, noradrenaline; NRM, nucleus raphe magnus; vlPAG, ventrolateral periaqueductal gray matter; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase.

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to painful stimuli, opioids disinhibit neurons in the ventrolateral periaqueductal gray matter (vlPAG), activating the LC and nucleus raphe magnus (NRM) (Fields et al., 1991; Sandkühler, 1996). Once these nuclei are activated, they release monoamines into the dorsal horn of the spinal cord (DHSC), directly inhibiting nociceptive neurons or activating spinal inhibitory interneurons such as GABAergic, glycinergic and enkephalinergic (ENKergic) interneurons, with consequent inhibition of the transmission of painful stimuli (Almeida et al., 2004; Budai and Fields, 1998; Jessel and Kelly, 1991; Malcangio and Bowery, 1996).

While the involvement of noradrenaline (NA) and serotonin (5-HT) is widely suggested in the pain process in PD (Skogar and Lokk, 2016; Tong et al., 2006), further studies are still necessary. In the present study, 6-hydroxydopamine (6-OHDA)-lesioned rats were used to further understand the role of these monoamines in PD-induced pain, motor impairment and nigrostriatal lesions. The animals were pretreated with a combination of a NA and a 5-HT reuptake inhibitor, desipramine and citalopram, respectively, before they received striatal injections of 6-OHDA in an attempt to protect the NAergic and 5-HTergic terminals against the neurotoxic action of 6-OHDA. Then, rats were subjected to pain and motor-related behavioral tests. The effect of the reuptake inhibitors was also investigated in the specific nuclei of the descending analgesic circuitry, including the LC, NRM and DHSC.

Given that neuroinflammation is also an important factor involved in PD-induced neurodegeneration (McGeer and McGeer, 2008; Rodrigues et al., 2001) and that the activation of spinal microglia and astrocytes is crucial for the maintenance of persistent pain (Cao and Zhang, 2008; Inoue et al., 2004), we also evaluated the effect of NA and 5-HT reuptake inhibitors on glial cells in the substantia nigra (SN) and DHSC. This study provides new insights into the roles of NA and 5-HT in pain modulation and their correlation with the nociceptive circuitry and inflammatory response in PD.

2. Methods

2.1. Experimental design

Under stereotaxic conditions, rats were injected with striatal 6-OHDA (to induce the PD model) or saline (as a control). A group of 6-OHDA animals received a combination of a 5-HT and a NA reuptake inhibitor intraperitoneally (i.p.; citalopram + desipramine, C/D) 30 min prior to the neurotoxin injection. Animals pretreated with saline i.p. were also evaluated. Hence, four experimental groups were evaluated, with 5 animals per group: 1) a saline i.p. + naive (S + N) group, 2) a saline i.p. + striatal saline (S + S) group, 3) a saline i.p. + striatal 6-OHDA (S + 6OH) group and 4) a C/D i.p. + striatal 6-OHDA (C/D + 6OH) group. Nociceptive behavior was evaluated by the paw pressure test before surgery (basal measurement, BM) and 7 and 14 days after surgery. As our mechanical nociceptive test depends on motor activity, we chose a dose and location for the neurotoxin injection that does not induce motor deficits in the absence of DAergic modulators. In this context, on the 12th day after striatal injection, the animals were treated with haloperidol (a DAergic antagonist) to intensify the DAergic deficit and after 1 h, were evaluated in postural immobility and open-field tests. The next day (the 13th day), rotational behavior was induced with apomorphine to validate the PD model. One hour after the last nociceptive test (on the 14th day), the animals were euthanized, and their brains and spinal cords were collected for evaluation of immunoreactivity (IR) for tyrosine hydroxylase (TH), Iba-1 (ionized calcium-binding adapter molecule 1, a microglial marker) and GFAP (glial fibrillary acidic protein, an astrocytic marker) in the SN; TH and NeuN (neuronal marker) in the LC; 5-HT and NeuN in the NRM; c-Fos (a marker of neuronal activation) and enkephalin (ENK) in the PAG; and GFAP, Iba-1, c-Fos and ENK in the DHSC (Fig. 2A).

2.2. Animals

A total of 28 male Wistar rats (200–250 g) were housed in acrylic boxes (3 rats per box) for at least one week before initiating the experimental procedures. The animals were maintained in appropriate rooms with a controlled light/dark cycle (12 h/12 h) and temperature (22 ± 2 °C) with wood shavings and free access to water and rat chow pellets. The animals were handled according to the guidelines for the ethical use of animals in research involving pain and nociception (Zimmermann, 1983), and this study is reported in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/arrive-guidelines). The protocols used during the execution of this project were approved by the Ethics Committee on the Use of Animals at the Hospital Sírio-Libanês (SP, BRA) under protocol number CEUA 2009/06.

2.3. NA and 5-HT reuptake inhibitors

Animals were injected intraperitoneally with the NA reuptake inhibitor desipramine hydrochloride (25 mg/Kg, Sigma-Aldrich, MO, USA) (Nishijima et al., 2016) concomitantly with the 5-HT reuptake inhibitor citalopram (2 mg/Kg, Sigma-Aldrich) (Flores et al., 2004) diluted in 0.9% saline. The treatment was performed 30 min prior to striatal injection of 6-OHDA or saline. Control animals were injected intraperitoneally with 0.9% saline.

2.4. Induction of the rat PD model

Animals were anesthetized with isoflurane (4% induction, 2.5% maintenance in 100% oxygen) combined with local anesthesia (2% lidocaine, 100 μL/animal). Under stereotaxic conditions, 12 μg of the neurotoxin 6-OHDA (Sigma-Aldrich) diluted in 2 μL of 0.9% saline with 0.2% ascorbic acid was injected at two different points (6 μg/μL of 6-OHDA at each point) into the left striatum (caudate/putamen) (Chudler and Lu, 2008). The injection was performed using a Hamilton syringe at two different coordinates: 2.7 mm mediolateral (ML), 0.0 mm anteroposterior (AP) (in the coronal suture) and 4.5 mm dorsoventral (DV) (first point); and 3.2 mm ML, 0.5 mm AP and 4.5 mm DV (second point) (Paxinos and Watson, 2005). The control animals were injected with 1 μL of saline at the same two points in the left striatum. At the end of the injection, the needle was left in place for an additional 5 min to prevent backflow of the solution. Then, the wound was closed, and the rats were returned to their home cages and monitored until they completely recovered from anesthesia. Their regular diet was supplemented with a dietary supplement (Ensure, Abbott, SP, BRA) once a day for 3 consecutive days to ensure full recovery of the animals after the nigrostriatal injury.

2.5. Evaluation of mechanical hyperalgesia: paw pressure test

The mechanical nociceptive threshold was evaluated using a pressure apparatus on the hindpaws (EEF 440, Analgesimeter, Insight, SP, BRA), as previously described (Randall and Sellito, 1957). Briefly, a force (in grams) of increasing intensity is continuously applied over the back of the hindpaws. The nociceptive response of the animal is represented by the withdrawal reaction of the pressed paw. The pain threshold is expressed as the grams of force necessary to induce the withdrawal reaction. The nociceptive test was performed before the striatal injection of saline or 6-OHDA (basal measurement) and 7 and 14 days after the striatal injections. The results were analyzed by comparing the basal and final measurements. To reduce stress, the rats were habituated to the testing procedure one day before the experiment.

2.6. Evaluation of apomorphine-induced rotational behavior

Rotational asymmetric behavior was evaluated using an automatic

rotameter system (Rota-Count 8, Columbus Instruments, OH, USA). Animals were injected subcutaneously (s.c.) with the DA agonist apomorphine (1 mg/Kg, Tocris Bioscience, Bristol, UK) (Zhang et al., 2008) dissolved in 0.9% saline and evaluated over a period of 30 min. The criterion for rotation was a 180° turn toward the side contralateral to the lesion. Eight animals were excluded from the study because they did not present rotational asymmetric behavior.

2.7. Evaluation of postural immobility: bar test

The postural immobility (akinesia) or typical catalepsy test is characterized by muscle rigidity and failure to correct an imposed posture for a prolonged period. Behavioral immobility was determined by evaluating the length of time for which the animals remained immobile in an upright position, supporting their front legs on the support bar without moving their hindpaws. To intensify the motor deficits in our PD model, the animals were injected with haloperidol, a D1 and D2 DAergic receptor antagonist (1 mg/Kg, s.c., Janssen-Cilag, SP, BRA), 1 h before being evaluated in the immobility test (Sanberg, 1980). During the test, the animals were positioned with both forepaws on a 9-cm-high horizontal bar (0.9-cm diameter). The duration for which the animal remained motionless in this imposed posture was considered the bar test elapsed time (with a cutoff time of 120 s). The endpoint of the immobility test was defined as the point at which both forepaws were removed from the bar or when the animal moved its head in an exploratory manner.

2.8. Evaluation of locomotor exploratory activity: open-field test

Immediately after the immobility test, while the rats were still under the effect of the DAergic antagonist, the locomotor exploratory activity of the animals was evaluated using an automated infrared beam system (MED-OFA-RS, Med Associates, VT, USA). The animals were individually placed in the center of the open-field chamber (43.2 cm × 43.2 cm), and their activity was recorded for 5 min. The behavioral parameters evaluated were distance traveled (cm) and number of rearings (number of times the animal stood on its hind legs) (Broadhurst, 1960). The open field was cleaned with a 5% alcohol/water solution before placing the animals to avoid possible bias due to odor clues left by previous rats.

2.9. Immunohistochemistry

At 1 h after the last nociceptive test, the animals were deeply anesthetized with ketamine/xylazine (0.5/2.3 mg/Kg, intramuscular) and then subjected to transcardial perfusion; tissue collection (brains and lumbar spinal cords) and immunohistochemical assay were subsequently performed as previously described (Dimov et al., 2016). Perfusion was performed 1 h after the last nociceptive stimulus because the peak expression of inducible transcription factor proteins (including c-Fos) occurs approximately 1 h after the stimulus and fades by 3 to 4 h post stimulation (Herdegen and Leah, 1998). Briefly, coronal sections (30 μm), obtained using a freezing microtome, were incubated with the following primary antibodies: mouse anti-NeuN (neuronal nucleus, 1:1000, MAB377B, Millipore, MA, USA), mouse anti-TH (1:1000, MAB5280, Millipore), rabbit anti-5-HT (1:1000, NT-102, Protos Biotech), mouse anti-GFAP (1:1000, G3893, Sigma-Aldrich), rabbit anti-Iba-1 (1:1000, 019–19,741, Wako Chemicals, VA, USA), mouse anti-ENK (1:500, MAB350, Millipore) or rabbit anti-c-Fos (1:1000, ABE457, Millipore). Then, the sections were incubated with biotinylated secondary antibodies (1:200, Jackson ImmunoResearch, ME, USA) and an avidin-biotin complex (1:100, ABC Elite kit, Vector Laboratories, CA, USA) and visualized with the chromogen diaminobenzidine (DAB) (Sigma-Aldrich) and hydrogen peroxide. The sections were mounted on glass slides, air-dried, dehydrated and coverslipped. Finally, images were captured using a light microscope, and the IR was analyzed using ImageJ software. Except for that of c-Fos (which was quantified as the number of positive neurons), IR was evaluated by optical density. The control (naive) group was normalized to 100% for comparison with the other groups. For each antibody, measurements were taken from 5 different sections per animal (for each one of the structures of interest) and from 5 animals per group. The regions of interest, the LC, NRM, SN, vlPAG and DHSC, were identified based on specific atlases (Molander et al., 1984; Paxinos and Watson, 2005).

2.10. Statistical analysis

The data are expressed as the mean ± standard error of the mean. Statistical analysis was performed with Prism software (GraphPad Software Inc., CA, USA) using two-way (2-w) analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test for the paw pressure test and apomorphine-induced rotational behavior and one-way (1-w) ANOVA followed by Tukey's *post hoc* test for the postural immobility, open-field test and immunohistochemistry results. Significance was defined as $p \leq .05$.

3. Results

3.1. NA and 5-HT reuptake inhibitors protect against neuronal impairment in the LC and NRM in a PD rat model

To verify the extent of the unilateral lesion induced by striatal 6-OHDA in the LC and NRM, NeuN, 5-HT and TH-positive staining in neurons was analyzed 14 days following the induction of the PD model. The S + 6OH group (6-OHDA-induced PD model) showed a significant reduction in NeuN-IR (1-w - $F_{(5,17)} = 74.62$, $p < .0001$ followed by Tukey's *post hoc* test; Fig. 1A-D) and TH-IR (1-w - $F_{(5,17)} = 40.37$, $p < .0001$, followed by Tukey's *post hoc* test; Fig. 1E-H) in the LC compared to the saline + naive (S + N) group. The citalopram- and desipramine-pretreated (C/D + 6OH) group exhibited decreased NeuN-IR in comparison to the S + N group and increased NeuN-IR in comparison to the S + 6OH group in the LC (Fig. 1A-D). Furthermore, the C/D + 6OH group displayed increased TH-IR in the LC compared to the S + N and S + 6OH groups (Fig. 1E-H). Additionally, the S + 6OH group (PD model) induced a decrease in NeuN-IR (1-w - $F_{(3,11)} = 13.37$, $p = .0018$, followed by Tukey's *post hoc* test; Fig. 1I-L) and 5-HT-IR (1-w - $F_{(3,11)} = 16.63$, $p = .0008$, followed by Tukey's *post hoc* test; Fig. 1M-P) in the NRM compared to the S + N group. The C/D + 6OH group showed increased NeuN-IR (Fig. I-L) as well as 5-HT-IR (Fig. M-P) in the NRM compared to the S + N and S + 6OH groups.

3.2. NA and 5-HT reuptake inhibitors protect against behavioral immobility and mechanical hyperalgesia in a rat model of PD

Compared with the control rats (S + N group), the 6-OHDA-lesioned rats presented an increase in haloperidol-induced postural immobility in the bar test (1-w - $F_{(3,11)} = 4.91$, $p = .007$, followed by Tukey's *post hoc* test; Fig. 2B) and a decrease in distance traveled (1-w - $F_{(3,11)} = 6.717$, $p = .0018$, followed by Tukey's *post hoc* test; Fig. 2C) and rearing behavior (1-w - $F_{(3,11)} = 5.126$, $p = .0057$, followed by Tukey's *post hoc* test; Fig. 2D) in the open-field test. Pharmacological pretreatment with the reuptake inhibitors decreased the behavioral immobility induced by striatal 6-OHDA without altering the distance traveled or the rearing behavior compared to treatment with 6-OHDA alone (Fig. 2B-D). The 6-OHDA-induced PD model induced a decrease in the nociceptive threshold (74%) in both hindpaws of the rats at 7 and 14 days after striatal injection of the neurotoxin (2-w - interaction factors $F_{(3,6)} = 229.0$, $p < .0001$, followed by Bonferroni's *post hoc* test; Fig. 2E and F) in comparison to the control condition (S + N group). Animals pretreated with C/D before 6-OHDA showed a decrease in the nociceptive threshold in the paw contralateral to the lesion (Fig. 2E), and they did not show any change in the nociceptive

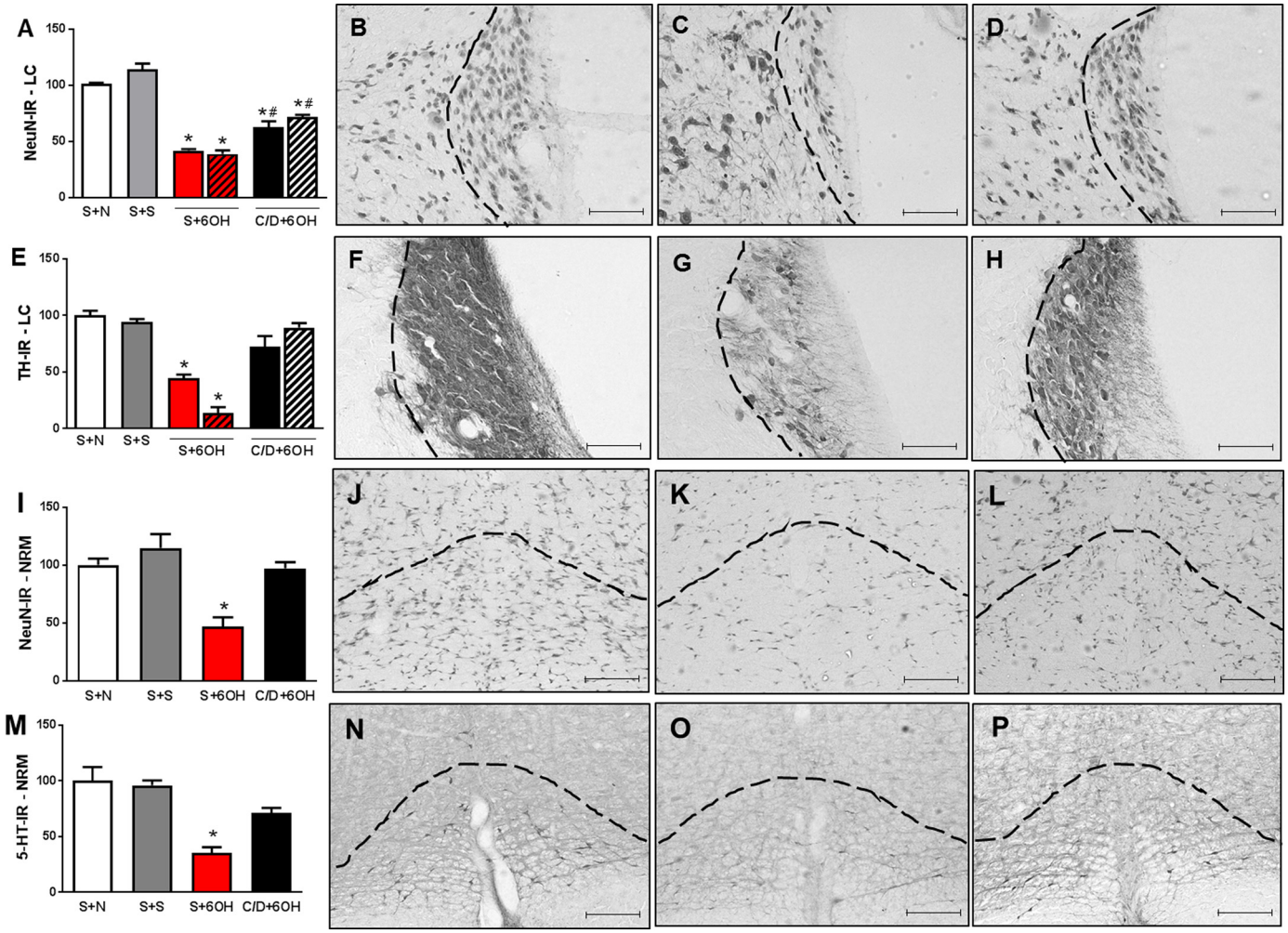

Fig. 1. Noradrenaline and serotonin reuptake inhibitors, Parkinson's disease (PD) model induction and the neuronal pattern in the locus coeruleus (LC) and nucleus raphe magnus (NRM). Quantification of the immunoreactivity (IR) of NeuN in the LC (A) and NRM (I), tyrosine hydroxylase (TH) in the LC (E) and serotonin (5-HT) in the NRM (M) in animals pretreated with citalopram/desipramine (C/D; 25 mg/Kg and 2 mg/Kg; intraperitoneally, i.p.) or saline (i.p.) 30 min prior to striatal saline or 6-hydroxydopamine (6-OHDA) injection. Control animals: naive rats and striatal saline rats, both pretreated with saline i.p. The values represent the mean ± standard error of the mean (SEM) (n = 5 per group). *p < .05 compared to the control animals (S + N group). $^{\#}$p < .05 compared to the saline + 6-OHDA animals (S + 6OH group). Photomicrographs illustrating NeuN-IR (B-D) and TH-IR (F–H) in the LC and NeuN-IR (J-L) and 5-TH-IR (N–P) in the NRM of saline + saline rats (S + S group; B, F, J and N), saline + 6-OHDA rats (S + 6OH group; C, G, K and O), and C/D + 6-OHDA rats (C/D + 6OH group; D, H, L and P). Scale bars: 100 μm.

threshold in the ipsilateral paw to the lesion (Fig. 2F) compared with control (S + N) animals and their own basal measurements.

3.3. NA and 5-HT reuptake inhibitors do not interfere with rotational behavior, DAergic neuron loss or glial activation in the SN in a PD rat model

The 6-OHDA-lesioned rats exhibited increased apomorphine-induced contralateral rotation to the lesioned side (2-w - interaction factors $F_{(3,6)} = 12.04$, $p < 0.0001$, followed by Bonferroni's *post hoc* test; Fig. 3A) compared with the control rats (S + N group). Additionally, lesioned rats (S + 6OH group) showed decreased TH-IR (1-w - $F_{(5,17)} = 89.62$, $p < 0.0001$, followed by Tukey's *post hoc* test; Fig. 3B-E), increased GFAP-IR (1-w - $F_{(5,17)} = 80.86$, $p < 0.0001$, followed by Tukey's *post hoc* test; Fig. 3F-I) and increased Iba-1-IR (1-w - $F_{(5,17)} = 88.30$, $p < 0.0001$, followed by Tukey's *post hoc* test; Fig. 3J-M) in the SN on the side ipsilateral to the lesion compared with control rats. Rats pretreated with 5-HT and NA reuptake inhibitors before striatal 6-OHDA injection showed asymmetric rotational behavior (Fig. 3A), decreased TH-IR (Fig. 3B), increased GFAP-IR (Fig. 3F) and increased Iba-1-IR (Fig. 3J) in the SN on the side ipsilateral to the lesion compared with control rats; however, compared with S + 6OH rats, no difference in these parameters was observed.

3.4. NA and 5-HT reuptake inhibitors were able to disinhibit the descending analgesic pathway and prevent changes in the spinal circuitry

No change in ENK-IR (Fig. 4A-C) or c-Fos-IR (Fig. 4E-G) was observed in the vlPAG in saline + 6-OHDA animals compared to control animals (saline + naive group). However, a significant increase in ENK-IR (1-w - $F_{(5,17)} = 6.57$, $p < .001$, followed by Tukey's *post hoc* test; Fig. 4A and D) and a decrease in c-Fos-IR (1-w - $F_{(5,17)} = 28.02$, $p < .0001$, followed by Tukey's *post hoc* test; Fig. 4E and H) were noted in the side of the vlPAG ipsilateral to the lesion in animals with striatal 6-OHDA that had been pretreated with C/D (C/D + 6-OHDA group) compared with control animals. The 6-OHDA-induced PD model bilaterally induced an increase in GFAP-IR (1-w - $F_{(5,17)} = 19.34$, $p < .0001$, followed by Tukey's *post hoc* test; Fig. 4I and Additional file 1: Fig. S1A and B), Iba-1-IR (1-w - $F_{(5,17)} = 22.87$, $p < .0001$, followed by Tukey's *post hoc* test; Fig. 4J and Additional file 1: Fig. S1D and E) and c-Fos-IR (1-w - $F_{(5,17)} = 12.33$, $p < .0003$, followed by Tukey's *post hoc* test; Fig. 4L and Additional file 1: Fig. S1J and K) in the DHSC compared with the control condition. However, the 6-OHDA-induced

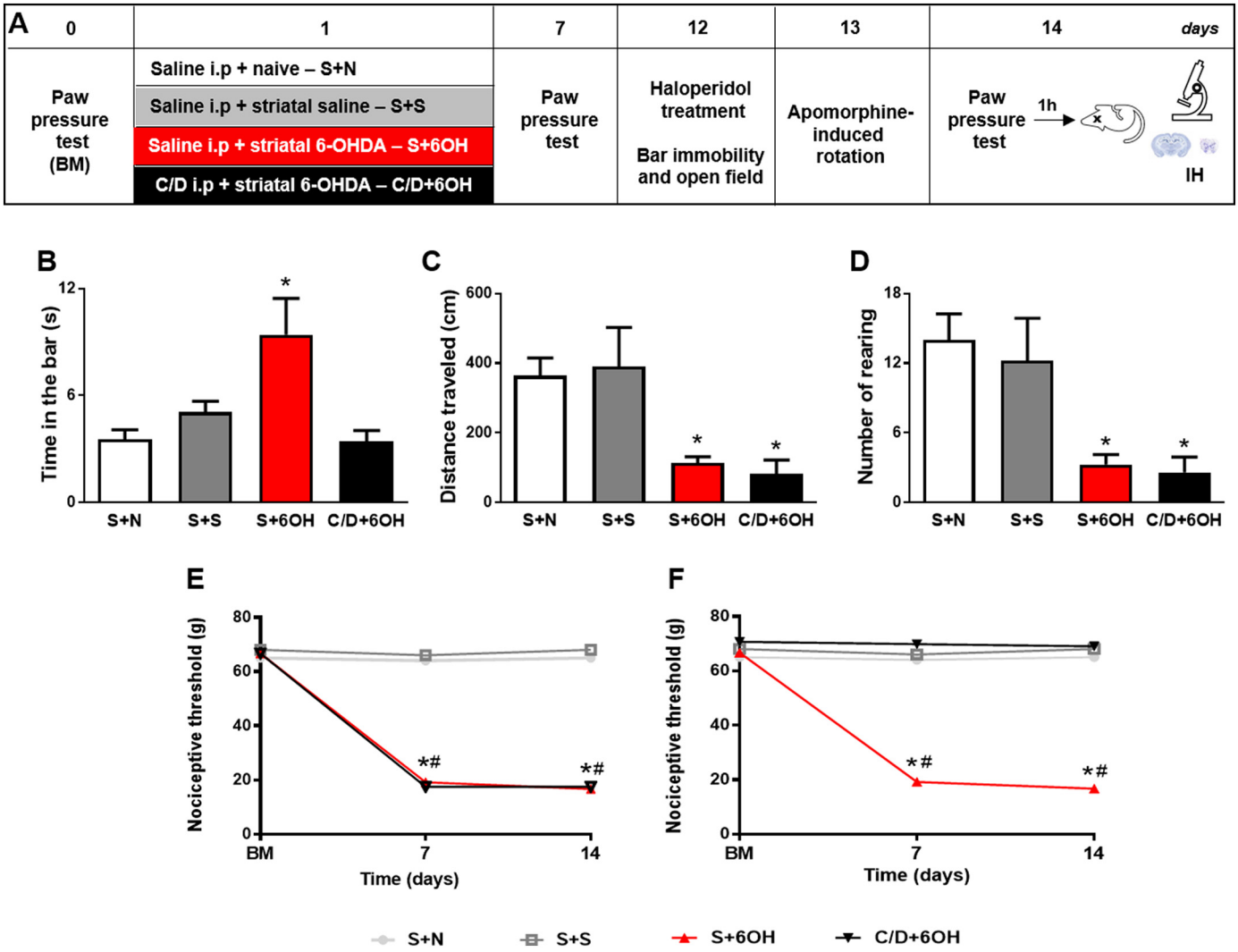

Fig. 2. Noradrenaline and serotonin reuptake inhibitors, the 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease (PD) model and motor and nociceptive symptoms. Experimental design (A). Motor impairments were induced with haloperidol (1 mg/Kg; subcutaneously, s.c.), and the rats were evaluated with a postural immobility test based on the time spent supported by a bar (B) and with an open-field test based on the distance traveled (C) and the number of rearings (D). Nociceptive behavior was evaluated before (basal measurement, BM) and 7 and 14 days after the striatal injections with a paw pressure test on the left (E) and right (F) hindpaws. The values represent the mean ± SEM (n = 5 per group). *p < .05 compared to the saline + naive group. #p < .05 compared to the basal measurements.

PD model significantly reduced ENK-IR (1-w - $F_{(5,17)} = 6.869$, $p = .0066$, followed by Tukey's *post hoc* test; Fig. 4K and Additional file 1: Fig. S1G and H) in the DHSC compared to the control condition. Pretreatment with reuptake inhibitors prior to the striatal administration of neurotoxin (C/D + 6-OHDA group) bilaterally inhibited the increases in GFAP-IR (Fig. I and Additional file 1: Fig. S1C) and Iba-1-IR (Fig. J and Additional file 1: Fig. S1F) and bilaterally increased the decrease in ENK-IR (Fig. K and Additional file 1: Fig. S1I) in the DHSC compared to 6-OHDA treatment alone. Additionally, rats pretreated with 5-HT and NA reuptake inhibitors before striatal 6-OHDA showed a decrease in increased c-Fos-IR in the DHSC on the side contralateral to the lesion compared to saline +6-OHDA rats (Fig. 4L and Additional file 1: Fig. S1L).

4. Discussion

Induction of PD models using neurotoxins has emerged as an invaluable tool for investigating the molecular basis of the disease and testing new therapeutic strategies. 6-OHDA is one of the most widely used neurotoxins for inducing PD models; this compound has high affinity for DA transporters and is subsequently transported into DAergic neurons, leading to nigrostriatal lesions (Blandini et al., 2008; Lane and Dunnett, 2008; Ungerstedt, 1968). Since this neurotoxin also has high affinity for the NA transporter (Luthman et al., 1989), desipramine, a tricyclic antidepressant that is a potent inhibitor of neuronal NA reuptake (Gillman, 2007), is usually administered to rodents 30 min prior to 6-OHDA injection to protect NAergic neurons (de la Fuente-Fernández et al., 2004; Mahmoudi et al., 2011; Schallert et al., 2000). Although 5-HT reuptake inhibitors are not as commonly used before 6-OHDA injection, the 5-HTergic system is also largely affected by this neurotoxin (Prinz et al., 2013; Reader and Gauthier, 1984; Santiago et al., 2014). Considering that desipramine has an indirect effect on 5-HT reuptake (Gillman, 2007; Hopwood and Stamford, 2001; Mantovani et al., 2009), the selective 5-HT reuptake inhibitor citalopram was used in our study to ensure the protection of 5-HTergic neurons following 6-OHDA injection. Thus, in an attempt to protect both 5-HTergic and NAergic systems, we combined the use of citalopram and desipramine prior to PD model induction. However, given that PD patients have shown NAergic and 5-HTergic degeneration in several different brain areas and in the lumbar spinal cord (Scatton et al., 1983, 1986),

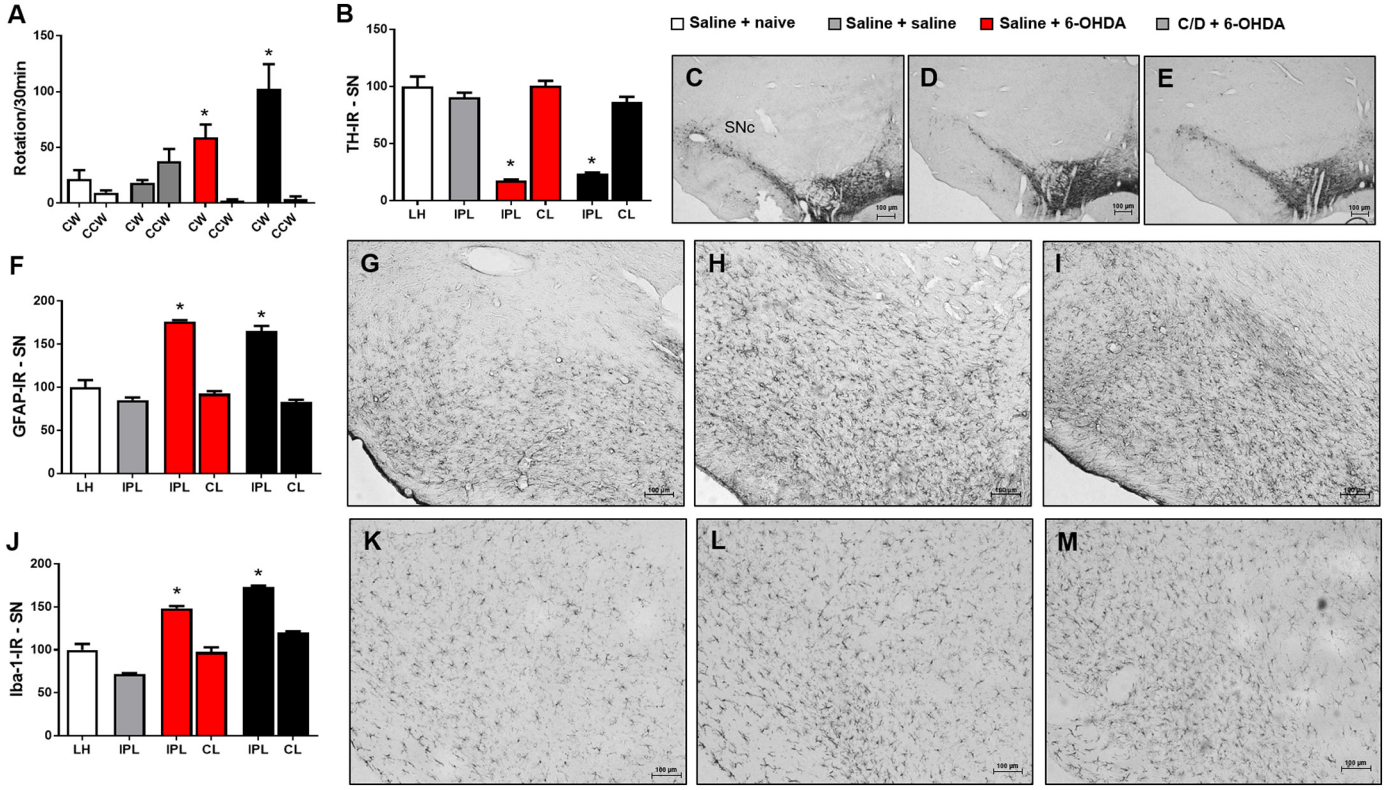

Fig. 3. Validation of the nigral lesions. Apomorphine-induced rotational behavior (A) and quantification of immunoreactivity (IR) for tyrosine hydroxylase (TH) (B), glial fibrillary acidic protein (GFAP) (F) and ionized calcium-binding adapter molecule 1 (Iba-1) (J) in the substantia nigra (SN) ipsilateral (IPL) and contralateral (CL) to the lesions in rats pretreated with citalopram/desipramine (C/D) or saline followed by striatal saline or 6-hydroxydopamine (6-OHDA). The values represent the mean ± SEM (n = 5 per group). *p < .05 compared to the control animals (saline + naive and saline + saline rats). Photomicrographs illustrating TH-IR (C-E), GFAP-IR (G-I) and Iba-1-IR (K-M) in the IPL SN in saline + saline (C, G and K), saline + 6-OHDA (D, H and L), and C/D + 6-OHDA (E, I and M) animals. Scale bars: 100 μm. CW: clockwise; CCW: counterclockwise; LH: left hemisphere; SNc: substantia nigra pars compacta.

protection of non-DAergic systems may not be optimal for understanding or treating PD with regard to both motor and nonmotor symptoms, as previously suggested (Tieu, 2011). For this reason, we analyzed the motor and nociceptive behavioral responses and neural circuitries in rats pretreated with NA and 5-HT reuptake inhibitors or saline in a PD rat model.

In our study, citalopram- and desipramine-pretreatment partially prevented 6-OHDA-induced neuronal loss and strongly prevented the neurotoxin-induced damage in NAergic TH-positive neurons in the LC. Additionally, this combined pretreatment totally prevented neuronal loss and 5-HTergic staining in the NRM. Taken together, these results indicated that citalopram- and desipramine-pretreated animals were protected against the loss of NA in the LC and the loss of 5-HT in the NRM induced by the striatal neurotoxin. The 6-OHDA-induced PD model caused DAergic loss and glial activation in the SN, asymmetric rotational behavior, postural immobility (akinesia) and decreases in locomotor exploratory activity, and motor impairments were observed only after DAergic pharmacological modulation. Pretreatment with NA and 5-HT reuptake inhibitors did not prevent the asymmetric rotational behavior, DAergic deficit or glial activation induced by the striatal 6-OHDA in the SN. The NA and 5-HT reuptake inhibitors prevented only the akinesia induced by striatal 6-OHDA, indicating that protecting 5-HT and NA signaling does not prevent nigrostriatal degeneration. Previous studies have suggested that modulation of NAergic and/or 5-HTergic transmission counters akinesia caused by DA deprivation after degeneration is already established (Loane and Politis, 2012; Matsubara et al., 2006; Nayebi et al., 2010; Prinssen et al., 1999); however, in our study, we have shown for the first time that the NA and 5-HT reuptake inhibitors prevented the induction of the akinesia phenomenon.

Our 6-OHDA-induced PD model decreased the mechanical nociceptive threshold, as previously shown in both human patients and rat models (Carvalho et al., 2013; Defazio et al., 2008; Gee et al., 2016; Goetz et al., 1986; Park et al., 2015; Domenici et al., 2019), and the NA and 5-HT reuptake inhibitors prevented hypernociception in the hindpaw ipsilateral to the lesion. The inhibition of pain hypersensitivity in 6-OHDA-lesioned rats was observed with the use of the NE and 5-HT reuptake inhibitor duloxetine five weeks after injection of neurotoxin (Cao et al., 2016); however, the novelty of our work is that NA and 5-HT reuptake inhibitors were able to prevent the development of 6-OHDA-induced mechanical pain hypersensitivity. In this sense, Chudler and Lu (2008) showed that striatal 6-OHDA did not induce mechanical hypernociception in hemiparkinsonian rats; however, in that study, desipramine was used before the striatal lesion was created, supporting our hypothesis that preserving NA or 5-HT neurons is not optimal for mimicking all features of PD pain. These results suggest that both the motor behavioral deficit (akinesia) and mechanical hyperalgesia in PD conditions are not mediated by nigral DA circuitry alone, emphasizing the importance of controlling other monoaminergic systems in parallel to provide more therapeutic benefits for patients.

Nonmotor symptoms are often correlated with mesolimbic dysfunction in PD (Frosini et al., 2015; Xu et al., 2012; Zhang et al., 2015). These symptoms not only start prior to motor dysfunction but also increase with disease progression (Barone et al., 2009; Shulman et al., 2001). The mesolimbic system is critical for the descending analgesic pathway, activating ENKergic interneurons that downregulate the GABAergic interneurons within the vlPAG, leading to NRM and LC activation (Fields et al., 1991; Sandkühler, 1996; Tobaldini et al., 2019). In our study, the 6-OHDA-induced PD model decreased 5-HT in the NRM and NA in the LC, and these alterations were prevented by pretreatment with citalopram and desipramine. Our findings suggest that the

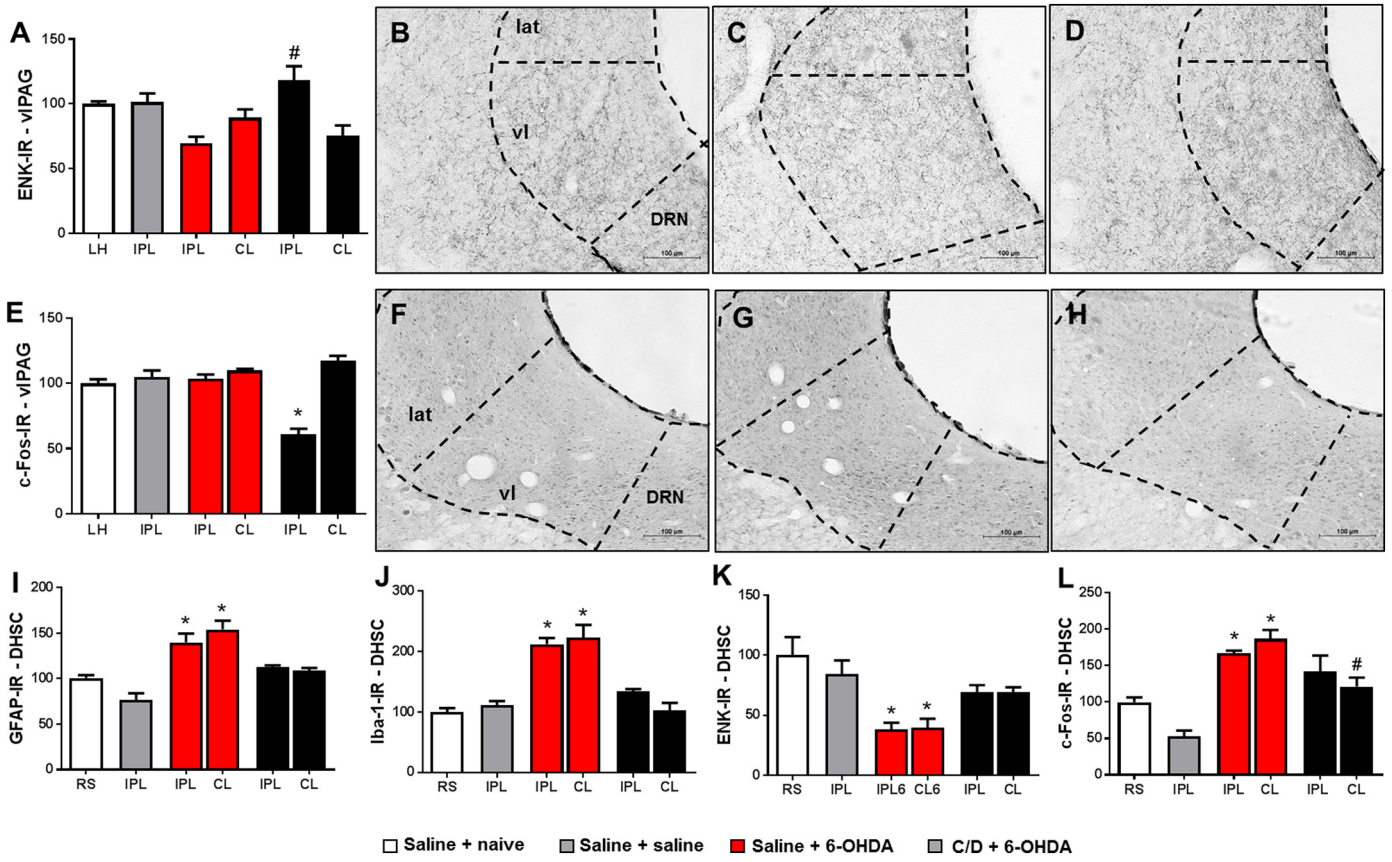

Fig. 4. Noradrenergic and serotonergic reuptake inhibitors, the Parkinson's disease (PD) model and the descending analgesic circuitry. Quantification of enkephalin (ENK) (A) and c-Fos-IR (E) immunoreactivity (IR) in the ventrolateral periaqueductal gray (vlPAG) and for glial fibrillary acidic protein (GFAP) (I), ionized calcium-binding adaptor molecule 1 (Iba-1) (J), ENK (K) and c-Fos (L) in the dorsal horn of the spinal cord (DHSC) of saline + naive rats, saline + saline rats, saline +6-hydroxydopamine (6-OHDA) rats and C/D + 6-OHDA rats. The values represent the mean ± SEM (n = 5 per group). *p < .05 compared to the control animals (saline + naive and saline + saline rats). #p < .05 compared to the saline +6-OHDA rats. Photomicrographs of ENK-IR (B-D) and c-Fos-IR (F–H) in the PAG IPL to the lesion in saline + saline rats (B and F), saline +6-OHDA rats (C and G), and C/D + 6-OHDA rats (D and H). C/D: citalopram and desipramine; CL: contralateral to the lesion; DRN: dorsal raphe nucleus; IPL: ipsilateral to the lesion; lat: lateral PAG; LH: left hemisphere; RS: right side; vl: ventrolateral PAG.

administration of reuptake inhibitors in the 6-OHDA-induced PD model is not optimal considering that preclinical and clinical studies have shown a decrease in NA and 5-HT in these areas involved with descending analgesic circuitry in PD conditions (Huot et al., 2011; Wang et al., 2009). Therefore, the importance of not protecting these areas when studying nonmotor symptoms in preclinical PD should be taken into consideration. The systemic use of reuptake inhibitors increased ENK and decreased c-Fos in the ipsilateral vlPAG, indicating disinhibition of this area and allowing activation of the descending analgesic system with consequent inhibition of the spinal nociceptive neurons. Persistent nociceptive input in chronic pain conditions leads to central sensitization, resulting in intrinsic changes in the DHSC, such as 1) increased excitability and synaptic efficacy of nociceptive neurons and interneurons; 2) loss of function of inhibitory interneurons and 3) glial activation (Latremoliere and Woolf, 2009; Woolf, 2011). Activation of astrocytes and microglia with subsequent release of cytokines is critical to the maintenance of the spinal activation of nociceptive neurons (Milligan et al., 2003; Watkins et al., 2003). In agreement with this concept, we recently demonstrated that the 6-OHDA-induced PD model increased staining for astrocytes and microglia in the DHSC (Domenici et al., 2019). In the present work, the data reinforce the idea of increased central sensitization by activation of spinal glial cells in PD conditions. Pretreatment with NA and 5-HT reuptake inhibitors, prior to the striatal neurotoxin, totally prevented the activation of spinal glial cells, presumably by activating the descending analgesic system, which was protected by the reuptake inhibitors. These findings suggest the importance of descending NAergic and 5-HTergic signaling systems in controlling the spinal inflammatory response, which is in line with previous results showing that NA and 5-HT release extrasynaptically possesses anti-inflammatory actions by directly interacting with receptors on microglia and astrocytes (Feinstein et al., 2002; Pocock and Kettenmann, 2007; Krabbe et al., 2012; Gyoneva and Traynelis, 2013; Miyazaki et al., 2013; Miyazaki and Asanuma, 2016).

Regarding spinal neuronal activation, Charles et al. (2018) showed that a status of chronic pain was associated with hyperexcitability of nociceptive neurons in the DHSC in a 6-OHDA rat model of PD, also suggesting an increase in central sensitization in PD. Our rat PD model equally induced neuronal hyperactivation in the DHSC, as observed by increased c-Fos staining accompanied by decreased ENK staining, which was consistent with the observed bilateral mechanical hyperalgesia. A correlation between c-Fos expression and the activation of nociceptive neurons in the DHSC has been suggested (Herdegen et al., 1991), and ENK inhibits these spinal nociresponsive neurons (Gogas et al., 1991). Most ENK present in the DHSC originates from local interneurons, is highly concentrated in the superficial laminae I and II (Bennett et al., 1982; Ruda et al., 1986), and acting on the mμ opioid receptor and delta opioid receptor, inhibits the release of pronociceptive neurotransmitters by the nociceptive primary afferents and the spinothalamic projection neurons (Ruda, 1982; Basbaum and Levine, 1991; Beaudry et al., 2011). The NA and 5-HT reuptake inhibitors partially prevented spinal c-Fos staining on the side contralateral to the lesion and preserved the ENKergic system in the DHSC, and this change was correlated with the prevention of hyperalgesia in the right hindpaw of rats injected with striatal 6-OHDA. Our findings offer stronger

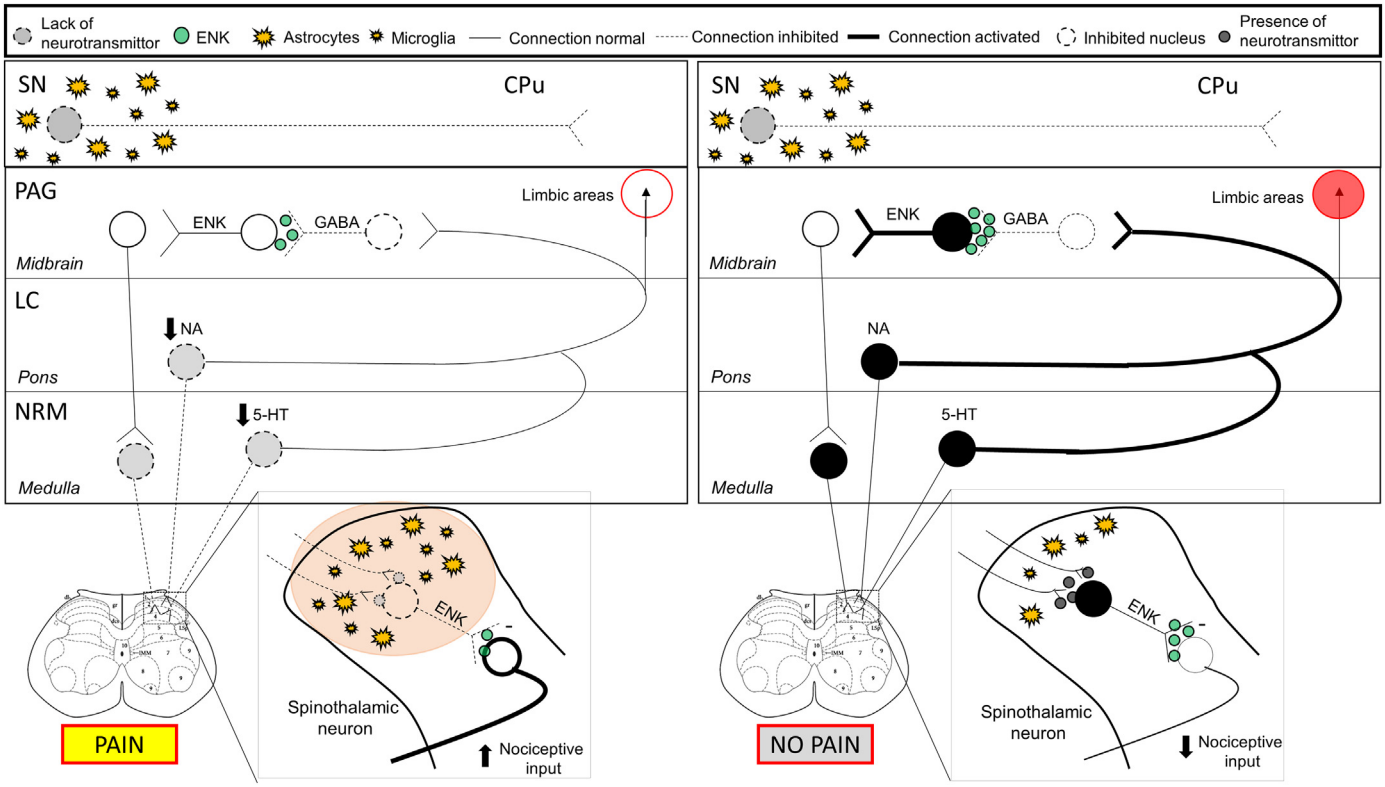

Fig. 5. Representative scheme of the control of the nociceptive circuitry in a 6-hydroxydopamine (6-OHDA)-induced Parkinson's disease (PD) model comparing animals pretreated with NA and 5-HT reuptake inhibitors or saline. The striatal 6-OHDA injection caused a dopaminergic deficit and glial activation in the substantia nigra (SN), and the reuptake inhibitors did not interfere with the nigral lesion. The 6-OHDA-induced PD model did not alter the PAG circuitry *per se* based on the evaluation of ENK and GABA; however, the model caused noradrenergic (NAergic) and serotonergic (5-HTergic) deficits in the midbrain nuclei, suggesting as a consequence a decrease in the release of monoamines in the DHSC. In parallel with or as an outcome of these events, the PD model induced spinal activation of astrocytes, microglia and nociceptive neurons and inhibition of enkephalinergic (ENKergic) interneurons that, combined with the lack of spinal monoaminergic release, resulted in increased transmission of the nociceptive stimulus. In contrast, protection of the NAergic and 5-HTergic systems activated the descending analgesic circuitry. Systemic pretreatment with NA and 5-HT reuptake inhibitors activated the ENKergic interneurons in the vlPAG, suggesting an inhibition of the GABAergic interneurons and thus disinhibiting the PAG. Once the reuptake inhibitors protected the NAergic and 5-HTergic systems, the ENKergic interneurons were activated, and subsequently, spinal glia and nociceptive neurons were inhibited after activation of the descending pathways. Taken together, the results suggest that despite the lack of nigrostriatal DA, prevention of NA and 5-HT deficits can protect against central sensitization in the DHSC, which is consistent with the prevention of hyperalgesia observed in the animals pretreated with the reuptake inhibitors. The discontinued lines represent a lack of transmission, and the thicker lines represent accentuated transmission. 5-HT: serotonin; DA: dopamine; DHSC: dorsal horn of the spinal cord; ENK: enkephalin; LC: locus coeruleus; NA: noradrenaline; NRM: nucleus raphe magnus; PAG: periaqueductal gray.

evidence that spinal nociceptive circuitry, *via* the endogenous opioid system, is crucial for controlling the pain response in PD conditions. Descending NAergic and 5-HTergic axons promote their antinociceptive effects, at least in part, *via* synapses with ENK-containing neurons of the DHSC (Basbaum et al., 1983; Basbaum and Fields, 1984). Given these results, we hypothesize that the prevention of hyperalgesia in the hindpaw contralateral to the lesion, observed with pretreatment with the NA and 5-HT reuptake inhibitors, may occur due to a specific disinhibition of the vlPAG ipsilateral to the 6-OHDA injection site with consequent activation of the descending analgesic system in the spinal cord contralateral to the lesion. In addition, even though the majority of monoaminergic fibers cross the midline of the DHSC in pyramid decussation (Basbaum and Fields, 1984), evidence has shown that after unilateral 6-OHDA lesions, dopamine output in the ipsilateral spinal cord decreases, suggesting that an uncrossed nigrospinal pathway may also be present (Commissiong et al., 1979; Molochnikov and Cohen, 2014). Considering that protection of the NAergic and 5-HTergic system did not alter the degeneration in the SN, the uncrossed nigrospinal pathway may also explain the hyperalgesia in the hind paw ipsilateral to the lesion in animals pretreated with reuptake inhibitors.

Rat PD model-induced hyperalgesia has been partially attributed to decreases in 5-HT in the NRM and DHSC (Wang et al., 2017) and to LC impairment (Wang et al., 2009). In support of this suggestion, our findings showed that preservation of the NAergic and 5-HTergic systems in the rat PD model of nigrostriatal lesions results in the inhibition of spinal nociceptive transmission. However, to the best of our knowledge, this is the first time that the descending NAergic and 5-HTergic system has been associated with the pain threshold and circuitry regarding the opioidergic system and neuronal/glial activation within the DHSC in PD conditions. We suggest a positive correlation between PD-induced pain and changes in the vlPAG circuitry together with glial activation, dysregulation of the ENKergic system and hyperexcitability of nociceptive neurons in the DHSC. Additionally, NA and 5-HT seem to be involved in controlling the nociceptive response in PD without involvement in nigral degeneration. A schematic representation of this hypothesis is shown in Fig. 5.

5. Conclusion

PD model-induced pain was correlated with inhibition of the descending analgesic system along with concomitant increases in glial and neuronal activation and inhibition of opioidergic interneurons in the spinal cord. NA and 5-HT reuptake inhibitors protected against pain-related behavior in the side ipsilateral to the lesions without changing motor behavior. We hypothesize that the protection of these monoamines disinhibited PAG, decreasing the central sensitization in the

DHSC. Thus, even though desipramine is widely administered before 6-OHDA lesions are induced, the use of reuptake inhibitors should be carefully considered and addressed when studying the nonmotor symptoms of PD, especially pain, so that we can better understand the different roles of DA, NA and 5-HT in persistent pain-related behavior in PD.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2019.04.015.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ACPC and MBB performed the nigrostriatal injections, nociceptive and motor behavioral tests and immunohistochemistry assays. ACPC drafted the manuscript. MSH and ETF 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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