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**Behavioral, cellular and molecular responses to cold and mechanical stimuli in rats with bilateral dopamine depletion in the mesencephalic dopaminergic neurons.**

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**Author contributions:**

All authors contributed substantially to this study: conception and design of the study (M.E, O.O., A.H); acquisition and interpretation of data (M.E., O.O, A.H., Y.A); drafting of the article (F.D., S.Y., S.S.Z), intellectual revision of the article (M.E., O.O., A.H); and approval of the last version of the manuscript (M.E., O.O., A.H., F.D., S.S.Z., S.Y., Y.A).

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## **Abstract**

Pain is the major non-motor symptom in Parkinson's disease (PD). Preclinical studies have mostly investigated mechanical pain by considering the decrease in a nociceptive threshold. Only a few studies have focused on thermal pain in animal models of PD. Therefore, the goal of this study was to assess the thermal nociceptive behavior of rats subjected to 6-hydroxydopamine (6-OHDA) administration, which constitutes an animal model of PD. Thermal plate investigation demonstrated significant thermal sensitivity to cold temperatures of 10 °C and 15 °C, and not to higher temperatures, in 6-OHDA-lesioned rats when compared with sham. 6-OHDA-lesioned rats also showed cold allodynia as demonstrated by a significant difference in the number of flinches, latency and reaction time to acetone stimulus. Ropinirole administration, a dopamine receptor 2 (D2R) agonist, blocked the acetone-induced cold allodynia in 6-OHDA-lesioned rats. In addition, mechanical hypersensitivity and static allodynia, as demonstrated by a significant difference in the vocalization threshold and pain score respectively, were noticed in 6-OHDA-lesioned rats. Acetone stimulus induced a significant increase in extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) phosphorylation, a pain process molecular marker, in the spinal dorsal horn (SDH), the insular and cingulate cortices in 6-OHDA-lesioned rats when compared to sham. In 6-OHDA-lesioned rats, there was a significant augmentation in the expression of both protein kinase C gamma (PKC $\gamma$ ) and glutamate decarboxylase 67 (GAD67) in the SDH. This highlighted an increase in excitation and a decrease in inhibition in the SDH. Overall, the present study demonstrated a clear cold thermal hypersensitivity, in addition to a mechanical one, in 6-OHDA-lesioned rats.

## Introduction

Parkinson's disease (PD), which is the second most prevalent neurodegenerative disorder, results from the death of dopamine-containing cells in the substantia nigra pars compacta (SNc). The disease is characterized by motor symptoms, such as resting tremor, rigidity, akinesia and postural instability, and non-motor symptoms.

The latter are often reported by patients before the diagnosis of PD (Skogar and Lokk, 2016; Tseng and Lin, 2017; Jankovic and Tan, 2020). Pain represents the main non-motor symptom of PD and epidemiological studies have estimated its prevalence between 68 % and 95 % (Buhmann et al., 2020). All of the symptoms generally occurred in the off-period of the treatment (Barcelo et al., 2010; Fil et al., 2013; Blanchet and Brefel-Courbon, 2018) and disappeared after levodopa treatment (Young Blood et al., 2016). A decrease in chemical, mechanical and thermal thresholds have been stated in both PD patients and animal models (Takeda et al., 2005; Chudler and Lu, 2008; Marques et al., 2013; Zenkin-Toktas et al., 2013; Dieb et al., 2015; 2016 a-b; Buhidma et al., 2020). After electrical stimulation, electromyogram recording showed that the heat pain threshold had decreased and the spinal nociceptive threshold had changed in PD patients (Mylius et al., 2015). The mechanism of pain is not yet well understood in PD patients, although, in different studies, (Wood, 2006; Chudler and Lu 2008; Borsook, 2012; Young Blood et al., 2016), it was noted that the basal ganglia were involved in pain pathogenesis. For example, neuropathic pain (NP) was exaggerated due to the loss of dopamine (DA) in the striatum (Takeda et al., 2005; Taylor et al., 2014). On the other hand, the pain was diminished when amphetamine was infused into the nucleus accumbens (Altier and Stewart, 1999; Harris and Peng, 2020). The administration of the dopamine receptor 2 (D2R) agonist to the striatum reduced the sensation of pain (Magnusson and Fisher, 2000; Wawrzczak-Bargieła A, 2020).

Spontaneous and evoked pains clinically characterize NP. It can occur as a dysfunction in the peripheral or the central nervous systems (Viana, 2018). NP can be characterized by allodynia or hyperalgesia (Sandkühler, 2009; Jensen and Finnerup, 2014). Only a few studies, in which the 6-hydroxydopamine (6-OHDA) rat constituted the PD model, have explored pain in bilateral denervation (Paillé et al., 2007) of the nigrostriatal pathway (Zenkin-Toktas et al., 2013; Dieb et al., 2014; 2016 a-b). Therefore, the present study explored mechanical and thermal hyperalgesia and cold allodynia (reduced pain threshold) in the hind paw of a rat model of PD with a bilateral 6-OHDA lesion of the medial forebrain bundle (MFB). In

addition, the effect of ropinirole, a D2R agonist, was investigated pharmacologically in mechanical hyperalgesia and thermal cold allodynia. Many of the NP symptoms, such as allodynia, are a result of changes within the spinal dorsal horn (SDH) (Sandkühler, 2009). Therefore, two markers were used to investigate segmental sensitization at both the cellular and molecular levels. Protein Kinase C © (PKC©), which labels cells within the SDH lamina II and plays an important function in pain chronicity (Dieb et al., 2015; Todd, 2017); and the phosphorylation of extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), which is expressed under noxious stimuli in the SDH (Ji et al., 1999; Polgár, 2007). Active ERK1/2 (pERK1/2) represents a molecular marker for pain processes (Ji et al., 1999; Liu et al., 2012; Dieb and Hafidi, 2015). In addition, the insular and anterior cingulate cortices are involved in the neuropathic pain process (Ohara et al., 2005) and both structures receive dopaminergic projections from the SNc (Chudler and Dong, 1995; Zhang et al., 2017). Their involvement in the pain process, in the rat model of PD, has been explored by observing pERK1/2 expression.

## MATERIALS AND METHODS

### Animals

Experiments were performed using 65 adult male Sprague-Dawley rats (Charles River, France) initially weighing 280–350 g. The Rats were housed three per cage in a Specific Pathogen Free facility under standard laboratory conditions (12/12 light/dark cycle, light on at 8:00 am) and were allowed free access to food pellets and water. The Rats were acclimatized for one week after transport to the animal house. 6-OHDA-lesioned rats included in the experiments were the rats showing signs of motor impairment (hind limb rigidity), aphagia, and/ or adipsia. Rats with signs of deterioration, for example, having lost more than 20 % of their bodyweight over the 6 days after surgery, were euthanized and excluded from the experiments. The experiments complied with the ethical guidelines of the animal ethics committee of Clermont Auvergne University (APAFIS#19965-20190325052285).

### Surgery

6-OHDA lesion:

The Rats were anesthetized with Ketamine 60 mg/kg and xylazine 10 mg/kg, intraperitoneal (i.p) and were positioned on a stereotaxic frame (David Kopf Instrument, CA, USA) to undergo a bilateral injection of 6-OHDA into the MFB [concentration of 3  $\mu\text{g}/\mu\text{L}$ ] with the rate (0.25  $\mu\text{L}/\text{min}$ ) in two deposits (2.25 and 2.85  $\mu\text{L}$ ). The 6-OHDA was dissolved in a vehicle solution (0.02% ascorbate saline) (Sigma-Aldrich, France, # H4381). The Injections were done at the following coordinates (Table 1) under aseptic conditions (Dieb et al., 2016):

**Table 1.** Coordinates of the 6-OHDA lesion surgery:

Deposit	Anterior	Lateral	Ventral	Tooth bar
2.25 $\mu\text{L}$ ,	-4.0	$\pm 0.8$	-8.0	+3.4
2.85 $\mu\text{L}$ ,	-4.4	$\pm 1.2$	-7.8	-2.4

To protect the adrenergic neurons from 6-OHDA toxicity, the animals were injected with desipramine (25 mg/kg, i.p., Sigma-Aldrich, Saint-Quentin-Fallavier, France, # D3900) 30 minutes before the 6-OHDA injection. Desipramine was used in our animal model to focus on dopaminergic depletion in pain and to limit the noradrenergic effect. Sham-lesioned rats were only injected with the vehicle at the same coordinates. The injection syringe was retracted

slowly 1 mm/minute. The physical appearance of the rats and their body temperature were monitored regularly, as were the surgical sutures to ensure they remained in place and that the head incision was well coated. 6-OHDA-lesioned rats were fed with wet food pellets, milk, and water until they were able to feed themselves.

## **Study Design**

From our previous studies, we know that the 6-OHDA-lesioned rat had pain hypersensitivity for up to three months (Dieb et al., 2016 b). To decrease the number of rats used, experiments were organized to test the same animals within a certain timeframe and add some when it was needed. Also, experiments were done over time to make sure that the animals had no effects from previous experiments. The D2R agonist was investigated in all tests when it could be done easily without harming the rats (intrathecal administration and von Frey filament test, intraperitoneal injection, and acetone test). Due to the half-life of ropinirole [30 minutes in rats, (Ramji et al., 1999)], we were not able to do the paw pressure test. In addition, since the thermal place preference test showed hypersensitivity to cold temperatures, the D2R was tested in the acetone drop.

## **The thermal place preference test**

The thermal place preference (TPP) test was used (Balayssac et al., 2014) one week after surgery. The apparatus consisted of two hot/cold plates (165X165 mm; Bioseb, Chaville, France). Every plate was regulated at  $\pm 0.1$  °C. The reference plate was set at 25 °C, while the test plate at (10 °C to 50 °C) according to the required temperature. For each session (3 minutes), the rat was positioned in the middle of the apparatus and was permitted free movement between the two plates. The time the rat passed on each test plate was calculated as a percentage. To avoid any possibility of habituation, the reference and test plates were permuted between each session. The animals were acclimatized to the experimental conditions, using two plates set at 25 °C, before the test.

## **Mechanical hyperalgesia**

Hypersensitivity to mechanical stimuli was conducted by applying a gradually increasing pressure stimulus using the Ugo Basile analgesiometer (Randall-Selitto apparatus, Bioseb, France), (Randall and Selitto, 1957), at two and four weeks after surgery. The rats left and right hind paws were subjected to an incremental linear mechanical force (measured in grams)

until a squeak was observed marking a painful threshold. To habituate the rats, the experimenter handled them for 20 seconds two or three times beginning, three days before the experiment. This was also repeated on the day of the experiment before putting the rats on the Ugo Basile apparatus. An increasing pressure was then exerted by applying the tip of the apparatus on the paw until the rat's vocalization was obtained. It was easier and more reliable to monitor vocalization than motor response. The vocalization threshold was measured three times to obtain two consecutive values that differed < 10 %, with a minimum of 10 minutes between each measurement and the following one. The pressure threshold of vocalization was obtained at a cut-off value of 250 g.

### **Von Frey filament test**

The rats were subjected to mechanical stimulation with a von Frey filament to test allodynia in the hind paw (Chaplan et al., 1994) at two and four weeks after surgery. Each rat was acclimatized for 1 hour on a metal mesh floor, under a small plastic cage (35x20x15 cm). Tactile allodynia was set with the use of non-noxious 10 g von Frey filament (Bioseb, Chaville, France) (Chaplan et al., 1994). Painful behavior was noted when there was paw withdrawal and/or an escape trial. All experiments were done relatively at the same time of the day. Ropinirole was injected intrathecally two weeks after surgery into a 6-OHDA-lesioned group and the pain score was calculated relative to another 6-OHDA-lesioned group, which had the saline intrathecal injection.

### **Intrathecal injection**

Intrathecal injection of ropinirole was done according to the method described by Mestre et al., 1994. Briefly, the rats were tightly restrained. Ropinirole (20 nmol) (Tang et al., 2021) or 10 µL of saline solution using a 30-gauge needle was injected intrathecally (i.t.) in between L4 and L5 of the spinal cord. The success of the procedure was confirmed by tail-flick on injection of ropinirole as indicated by Mestre et al., 2009. Pain score (paw withdrawal) was evaluated pre-injection and every 6 minutes up to 36 minutes after ropinirole injection. Spinal cord L3-L4 constitute the segments regulating the sensory process at the level of posterior paws (Takahashi et al., 1994). In addition, acetone drop and von Frey filaments were tested in the posterior paw. For these reasons, ropinirole was administered at the level of L3-L4.



## **Acetone drop test**

The acetone test (Choi et al., 1994) was performed at ten days after surgery for four days successively to test cold hyperalgesia-like (cold allodynia) reactions in the hind paws of three animal groups: sham, 6-OHDA-lesioned and 6-OHDA-lesioned rats with acute intraperitoneal administration (for 4 days, starting at day 10 post-surgery) of ropinirole-hydrochloride (D2R agonist, 5 mg/Kg/ml, Sigma-Aldrich, Saint-Quentin-Fallavier, France, # R2530) (Mavrikaki et al., 2014, Tremblay et al., 2017). The ropinirole treated group is referred to as the 6-OHDA +D2R agonist group, a behavioral test was done after 15 minutes post-injection (Mavrikaki et al., 2014) for this group. The Rats were acclimatized for 15 minutes on a metal mesh floor, under small plastic cages (35x20x15 cm). An acetone drop from a 1 ml syringe was gently applied to the hind paw plantar surface (without touch). Three parameters were tested using the acetone stimulus. Each parameter was considered after the application of acetone 5 times (with a 5 minute interval between successive applications). The first one was the number of flinches, for which the number of hind paw withdrawals was counted for five consecutive applications. The second parameter was the latency, for which the duration between the application of acetone and the hind paw reaction was measured. While the third parameter was the reaction time which was assessed for the duration of two minutes. The test was repeated for four successive days in the second week after surgery and the median + interquartile range values were calculated.

The behavioral experiments were done with a blind design so that the experimenter could not recognize the different animal groups.

## **Immunohistochemistry**

At the end of the behavioral experiments, ketamine (60 mg/kg) and xylazine (10 mg/kg) i.p. were used to deeply anesthetise the rats which were perfused transcardially with a warm (37 °C) heparinized saline solution (25 IU heparin/ml) followed by a cold (10 °C) phosphate-buffered solution (0.1 M, pH 7.6) containing 4% paraformaldehyde and 0.03% picric acid for 15 minutes. Brains and spinal cords were then removed and placed in a solution of 30% sucrose and 0.05% sodium azide at 4 °C overnight. Coronal sections (40 µm) were collected with a using a freezing microtome and placed in a 0.05 M Tris-buffered saline (TBS) solution. Free-floating sections were placed in 1% normal goat serum at room temperature for 1 hour before overnight incubation in primary mouse monoclonal anti-TH antibody solution (1:5000, Sigma-Aldrich, Saint-Quentin-Fallavier, France, #T2928), rabbit monoclonal anti-

pERK1/2 (1:200, Cell Signaling Technology, Ozyme Saint-Cyr-l'École, France, #4370), rabbit monoclonal anti-GAD1 (1:1000, Cell Signaling Technology, #41318) and mouse monoclonal anti-PKC $\gamma$  (1:500, Santa-Cruz Biotechnology, France, #sc-166385). After several rinses, sections were incubated with a secondary antibody (1:500 for goat anti-mouse 488 #4408; goat anti-rabbit 555 #4413, goat anti-mouse 555 #4409, goat anti-rabbit 488 #4412, Cell Signaling Technology) for 2 hours at room temperature. All the antibodies were diluted in TBS containing 0.25 % bovine serum albumin and 0.3 % TritonX-100. The sections were finally rinsed in TBS, mounted onto Thermo Scientific Superfrost Plus slides (Fisher Scientific, UK), and coverslipped with Dako Fluorescence Mounting Medium (Agilent, United States). The specificity of immunostaining was assessed by omitting the primary antibody, which resulted in the absence of the signal. Photomicrographs of immuno-stained sections were captured using a motorized Zeiss Axioplan 2 microscope equipped with a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics France SARL, Massy, France) (switching between FITC and Texas Red filter sets) driven by MetaMorph 5.4 software. In each rat, image acquisition was done using multiple sections depending on the structure taken (Table 2). The Measurement of PKC $\gamma$  positive cell number was taken from the whole lamina Ili of SDH, as it contains the majority of the PKC $\gamma$  positive neurons (Dieb et al., 2016 a-b), using sections that were taken from the Lumbar (L3 - L6) spinal cord region. The final data were presented as a median + interquartile range value of the total sections of each group. The pERK1/2-positive cells were counted in all of the superficial laminae in the multiple sections at the level of the Lumbar (L3 – L6) spinal cord region. While for the insular and cingulate cortices, rostro-caudal slices from (2.04 mm to – 0.48mm relative to Bregma) were taken, a square area of equal dimensions was applied to all counted sections. Tyrosine hydroxylase (TH) immunolabeling was quantified in the whole sections, at different slices, at the level of SNc and ventral tegmental area (VTA). Rostro-caudal sections from (-5.16mm to -6.12mm) were analyzed.

The cells for all PKC $\gamma$ , pERK1/2, and TH were quantified using the multipoint cursor of ImageJ Software (ImageJ v1.6, National Institutes of Health, Bethesda, MD) where the numbers of cells were displayed in the results window. Cell count was done with a blind observer who did not know the different animal groups.

**Table 2.** The number of sections of each group used for each antibody:

Cell Count Measurement			
Antibody	Structure	Group	Number of sections
PKC $\gamma$	Spinal cord	Sham	18
		6-OHDA-lesioned	25
		6-OHDA +D2R agonist	33
pERK1/2	Spinal cord	Sham	28
		6-OHDA-lesioned	32
		6-OHDA +D2R agonist	38
	Insular cortex	Sham	9
		6-OHDA-lesioned	10
		6-OHDA +D2R agonist	18
	Cingulate cortex	Sham	10
		6-OHDA-lesioned	16
		6-OHDA +D2R agonist	24
TH	SNc and VTA	Sham	27
		6-OHDA-lesioned	13

GAD67 and PKC $\gamma$  fluorescence intensities were compared throughout different spinal cord sections at the level of the Lumbar (L3 – L6) region.

### Western Blot

At the end of behavioral experiments, sham, 6-OHDA-lesioned and 6-OHDA +D2R agonist groups were anesthetized with ketamine (60 mg/kg) and xylazine (10 mg/kg) and sacrificed by decapitation. Fresh spinal cord tissues (spinal cord from L3 to the end of the spinal cord) and fresh pieces from insular and cingulate cortices bilaterally (2mm) were punched, snap-frozen in liquid nitrogen, and stored at -80 °C until use. Each sample was homogenized with a glass homogenizer in 200  $\mu$ l of lysis buffer (50 mM HEPES, pH 7.5, 1 % TritonX-100, 10mM EDTA, 150mM NaCl, 10mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.1M NaF, 2mM vanadate) supplemented with PMSF 0.5 mM, complete™, Protease Inhibitor Cocktail (Sigma Aldrich, #5892970001) and Triton 1%, sonicated for 3 minutes and incubated on ice for 20 minutes. 100- $\mu$ l of extraction

buffer was added to each sample before centrifugation at 14,000 g for 15 minutes at 4 °C. The supernatants were collected in clean tubes and stored at -80 °C until use. The total amount of protein in each sample was measured using the BCA Protein Assay Kit (Pierce-Thermo Scientific, Illkirch-Graffenstaden, France) before loading on polyacrylamide gels. Samples (40 µg) were separated using 4-20 % Mini-PROTEAN TGX polyacrylamide gels (BioRad, Roanne, France) and transferred to nitrocellulose membranes using the Wet/Tank Blotting System (BioRad). After the blots had been washed with TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.01% Tween-20), they were blocked with 5% BSA in TBS-T for 1 hour at room temperature. Then the membranes were incubated at 4 °C overnight with primary antibodies diluted in 5% BSA in TBS-T. The Primary antibodies used were rabbit monoclonal anti-pERK1/2 (1:500, Cell Signaling Technology, #4370), rabbit monoclonal anti-GAD1 (1:1000, Cell Signaling Technology, #41318), mouse monoclonal anti-PKC $\gamma$  (1: 400, Santa-Cruz Biotechnology, France, #sc-166385) and rabbit polyclonal anti-GAPDH (0.2 µg/ml, Sigma-Aldrich, #G9545). After washing with TBS-T, membranes were incubated for 1 hour with HRP-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (1:5000, Invitrogen-Thermo Scientific #31460, #31430, respectively) diluted in 5% BSA in TBS-T. The bands were visualized with Clarity™ western ECL substrate solution (Bio-Rad, USA) and the positive pixel areas of specific bands were measured with a computer-assisted image analysis system (ChemiDoc XRS, Bio-Rad, USA) and normalized against the corresponding GAPDH loading control bands. The 6-OHDA-lesioned group and the 6-OHDA +D2R agonist one were normalized against the sham group of the corresponding gel to be able to compare the different gels. Results are expressed as the median  $\pm$  interquartile range of expression levels of the ratio between 6-OHDA-lesioned and 6-OHDA +D2R agonist to the sham group of each gel.

### **Body weight analysis**

Because bilateral lesions of the MFB may cause aphagia and/or adipsia, the animals' weight was checked daily after surgery. 6-OHDA-lesioned rats were not able to self-feed. They were fed (wet food pellets + milk + water) by syringe feeding twice a day, for 8 to 10 days.

## **Statistical analysis**

The results are expressed as median  $\pm$  interquartile range. Statistical analysis was performed using one-way ANOVA to compare the three groups, followed by Tukey's posthoc multiple comparisons test for parametric data and the Kruskal-Wallis test, followed by Dunn's posthoc multiple comparisons test for non-parametric data. Normality tests were done using the Shapiro-Wilk normality test, if parametric, Levene's test was done to check for equal variances (supplementary material). Two-way ANOVA, followed by Sidak's posthoc multiple comparisons test and Friedman's test were used when convenient. In addition, Student's T-test was used when the comparison between only two groups was required for parametric data and the Mann-Whitney test for non-parametric data. The level of significance was determined at a P-value  $\leq 0.05$ . Data were analyzed using GraphPad Prism Software (v.8.0.2) and SPSS (v. 1.0.0.1447). Fixed-effect model ANOVA was used as variables were fixed factors in the experiment. Also, in the two-way ANOVA analysis, variables were fixed factors in both rows and columns factors.

## **Results**

### **Thermal hyperalgesia (Figure 1)**

Thermal sensitivity was tested one week after surgery; the rats were tested for thermal place preference (Fig.1) at different temperature degrees (10 °C, 15 °C, 25 °C, 35 °C, and 50 °C). The 6-OHDA-lesioned rats showed a significantly higher thermal sensitivity when compared with the sham ones. This increase was observed with the temperatures 10 °C and 15 °C, as the 6-OHDA-lesioned rats spent less time on the test plate than sham ones. While at 50 °C, the sham rats spent less time than 6-OHDA-lesioned rats on the plate. For the other temperatures, 25 °C and 35 °C; there was no significant difference observed between the groups.

### **Cold allodynia (Acetone test, Figure 2)**

Cold allodynia was tested during the second week in the three groups; sham, 6-OHDA-lesioned, and 6-OHDA +D2R agonist. 6-OHDA-lesioned rats had a significant increase in cold allodynic behavior, which was shown by an increasing number of flinches, the reaction time and latency in comparison with the sham group. Ropinirole had a significant role in decreasing allodynic behavior, which was evident from the significant decreases of reaction time and latency between the 6-OHDA +D2R agonist group and the 6-OHDA lesioned one.

### **Mechanical hyperalgesia and static allodynia (Figure 3)**

Mechanical hyperalgesia and mechanical static allodynia were tested at two and four weeks after surgery using the Randall-test (Fig. 3A) and non-noxious 10 g von Frey filament (Fig. 3B), respectively. The 6-OHDA-lesioned rats had greater mechanical hyperalgesia and mechanical static allodynia than the sham group, as was substantiated by the increase in vocalization at 50 % of the baseline score and the increase in paw withdrawals at two and four weeks after surgery, respectively.

### **pERK1/2 expression in the spinal dorsal horn (Figure 4)**

pERK1/2 labeling expression upon paw acetone stimulus was observed in the superficial laminae of the SDH in the sham (Fig. 4A), 6-OHDA-lesioned (Fig. 4B), and 6-OHDA +D2R agonist rats (Fig. 4C). pERK1/2-positive cells were quantified in the SDH superficial laminae in the three animal groups. The 6-OHDA-lesioned rats had a significant increase in pERK1/2 in comparison with both the sham and 6-OHDA +D2R agonist groups. Ropinirole intraperitoneal administration led to a significant decrease in pERK1/2 in the 6-OHDA +D2R

agonist rats when compared with the 6-OHDA-lesioned ones, while the sham group showed a less significant decrease. Western blot (Fig. 4E) revealed a significant pERK1/2 protein difference between the 6-OHDA-lesioned rats and both the sham and 6-OHDA +D2R agonist rats (Fig. 4F).

### **PKC $\gamma$ expression in the spinal dorsal horn (Figure 5)**

PKC $\gamma$  labeling is localized in the SDH superficial laminae cells in the sham (Fig. 5A), 6-OHDA-lesioned (Fig. 5B), and 6-OHDA +D2R agonist rats (Fig. 5C). PKC $\gamma$  labeling was quantified using the number of positive cells (Fig. 5D). The 6-OHDA-lesioned rats had more PKC $\gamma$  expression than both the sham and 6-OHDA +D2R agonist groups regarding the number of cells. Also, the 6-OHDA-lesioned rats had more intense fluorescent labeling than both the sham and 6-OHDA +D2R agonist rats. Ropinirole intraperitoneal administration led to a significant decrease in PKC $\gamma$  expression in the 6-OHDA +D2R agonist rats when compared with the 6-OHDA-lesioned ones regarding the fluorescence intensity and the number of cells. While there was a non-significant increase in western blot band intensities between the 6-OHDA-lesioned and both the sham and 6-OHDA +D2R agonist groups.

### **GAD67 expression in the spinal dorsal horn (Figure 6)**

GAD67 labeling was expressed throughout the SDH laminae in the sham (Fig. 6A), 6-OHDA-lesioned (Fig. 6B), and 6-OHDA +D2R agonist animals (Fig. 6C). The 6-OHDA-lesioned groups demonstrated more of an augmentation in the GAD67 expression more than either of the sham or 6-OHDA +D2R agonist groups did, as shown by the increase in fluorescence intensity of the 6-OHDA-lesioned group over the other two groups. In addition, there was an increase in the western blot band intensity of the 6-OHDA lesioned group in comparison with the sham and 6-OHDA +D2R agonist animals. Ropinirole intraperitoneal administration led to a significant decrease in GAD67 expression in the 6-OHDA +D2R agonist rats in relation to the 6-OHDA-lesioned ones.

### **pERK1/2 expression in the Insular and the Cingulate cortices (Figures 7-8)**

In the insular cortex, pERK1/2 labeling was expressed in the cell bodies and dendrites of somata located across the different cortical layers in the sham (Fig. 7A), 6-OHDA-lesioned (Fig. 7B), and 6-OHDA+D2R agonist (Fig. 7C) groups. These cells had mostly pyramidal cell morphology. The 6-OHDA-lesioned group showed an increase in pERK1/2 as revealed by its quantification and the increase in western blot band intensity when compared with both the

sham and the 6-OHDA +D2R agonist rats. Ropinirole intraperitoneal administration led to a significant decrease in pERK1/2, as was substantiated by the diminution in pERK1/2 expression in the 6-OHDA +D2R agonist rats more than in the 6-OHDA lesioned ones, when taking into account the number of cells and band intensity.

In the cingulate cortex, pERK1/2 labeling was observed in the cell bodies and dendrites of the cells located mostly within the deep layers in the sham (Fig. 8A), 6-OHDA-lesioned (Fig. 8B), and 6-OHDA +D2R agonist groups. (Fig. 8C). The 6-OHDA-lesioned group showed a significant increase in pERK1/2, as was revealed by the increase in pERK1/2 quantification when compared with both the sham and 6-OHDA +D2R agonist rats, in addition to its increase in western blot band intensity in the 6-OHDA-lesioned group compared with the sham rats. Ropinirole intraperitoneal administration led to a significant decrease in pERK1/2 expression in the 6-OHDA +D2R agonist rats more than in the 6-OHDA-lesioned ones, when taking into account the number of cells and band intensity.

### **Intrathecal ropinirole administration (Figure 9)**

To test segmental involvement in the pain process, ropinirole was intrathecally administered and the rats were then subjected to a non-noxious 10 g von Frey filament test. Intrathecal administration of ropinirole led to a decreasing pain score (paw withdrawal) in the 6-OHDA-lesioned rats in comparison with saline-injected, 6-OHDA-lesioned rats. The pain score decrease remained evident for up to 25 minutes.

### **TH immunostaining in the 6-OHDA-lesioned rats (Figure 10)**

TH labeling was explored in the SNc and ventral tegmental area (VTA) in both the sham (Fig. 10A) and 6-OHDA-lesioned groups (Fig. 10B) in order to highlight the effects of 6-OHDA toxin in the MFB lesion. TH labeling was observed in the somata and processes in the SNc and VTA. A general extreme significant decrease in the TH staining was observed in the 6-OHDA-lesioned rats when compared to sham confirming the degeneration of dopaminergic cells. The Quantification of TH cells in SNc and VTA (Fig. 10C) demonstrated a 67.8 % decrease in cell number between the 6-OHDA-lesioned and sham groups. A significant 56.7 % decrease in TH positive cell number was also observed between the 6-OHDA-lesioned and sham rats in the VTA (Fig. 10D). As well, a significant 89.9 % decrease was obtained in TH positive cell number between the 6-OHDA-lesioned and sham groups in the SNc (Fig. 10E).



## **Body weight**

After surgery, the 6-OHDA-lesioned group had a significantly decreased body weight due to aphagia and adipsia. A considerable weight difference was observed in the 6-OHDA-lesioned rats in comparison with the sham ones (Fig. 10F).

## Discussion

The main results of this study showed that bilateral 6-OHDA-lesioned rats demonstrated an increase in thermal sensitivity to cold temperatures. This sensitivity was supported by the presence of cold allodynia, which was exhibited by the acetone test. In addition, lesioned rats demonstrated the presence of both mechanical hyperalgesia and mechanical static allodynia. Acetone stimulation promoted the expression of pERK1/2 in the SDH, the insular, and cingulate cortices, thus implicating these structures in the pain-process in both 6-OHDA-lesioned rats and sham ones, nevertheless, pERK1/2 expression in the 6-OHDA-lesioned rats was considerably elevated when compared with the sham ones. In addition, the 6-OHDA lesion induced an increase in the expression of PKC $\gamma$  and GAD67 (glutamate decarboxylase) in the SDH. These two proteins may highlight an increase in excitation (PKC $\gamma$ ) and a decrease in inhibition (GABA), respectively. Moreover, the use of ropinirole, a D2R agonist, reversed the expression of PKC $\gamma$  and GAD67 in the SDH and alleviated the pain sensitivity. These results demonstrated a clear implication of the SDH and dopamine in the pain process in 6-OHDA-lesioned rats.

Although a study has shown that ketamine had a neuroprotective role in the 6-OHDA PD model (Ferro et al., 2007), according to our previous work, (Ouachikh et al., 2013; Ouachikh et al., 2014; Dieb et al., 2014; Dieb et al., 2016a-b), there was always a neuronal cell death percentage over 60% while using ketamine. This allowed us to consider that we are in an animal model of PD. Therefore, the use of ketamine as an anesthetic did not constitute an obstacle to neuronal degeneration.

Pain is measured through the motor and non-motor reflexes. Different studies using 6-OHDA-lesioned animals showed a reduction in mechanical and thermal nociceptive thresholds or allodynia in both hind paws without the affection of their motor functions (Saadé et al., 1997; Takeda et al., 2005; Paillé et al., 2007; Dieb et al., 2015; 2016 a-b; Campos et al., 2019; Tang et al., 2021). In addition, 6-OHDA lesions generally showed a contralateral motor deficit along with bilateral pain (Chudler and Lu, 2008; Dieb et al., 2015; 2016 a-b). After the lesion, while there was motor improvement, pain scores remained similar over time. This showed the probable occurrence of compensatory modulation in motor circuits, which did not apply to the pain pathway. In PD patients, pain occurs before the onset of motor symptoms (O'Sullivan et al., 2008; Tseng and Lin, 2017), and increases with disease progression (Stamey et al., 2008; Barone et al., 2009). Together, these data infer that in the 6-

OHDA-lesioned animal, pain may be associated with dopamine depletion rather than motor deficit.

The 6-OHDA-lesioned rats developed sensitivity to cold temperature (TPP test) and cold allodynia (acetone test). In addition, these rats also developed mechanical hyperalgesia and allodynia. Noxious cold stimuli activate nociceptive C and A $\delta$  afferents, some of which are of a polymodal subtype and respond to a mechanical stimulus (Simone and Kajander, 1996; Dubin and Patapoutian 2010; Sneddon, 2018). These afferent subtypes are probably under central dopaminergic regulation and may be involved in both the mechanical and cold sensitizations observed in 6-OHDA-lesioned rats. SNc probably modulates pain through the inhibition of nociceptive inputs at the spinal cord neuron level (Pasquier et al., 1977; Barnes et al., 1979; Beckstead et al., 1979; Von Krosigk and Smith, 1991) or ascending projections to cortical areas such as insular and / or cingulate cortices (Beckstead et al., 1979; Burkey et al., 1999; Ohara et al., 2003). This is highlighted here by the expression of pERK1/2 at the level of the SDH and the insular and cingulate cortices. pERK1/2 was expressed under noxious and not non-noxious stimuli (Ji et al., 1999; Dieb et al., 2015; 2016 a-b). pERK1/2 was highly expressed in these structures in the 6-OHDA-lesioned rats and the administration of D2R agonist ropinirole decreased its expression. This result agrees with previous studies showing an elevation of pERK1/2 expression and its decrease using bromocriptine, another D2R agonist in 6-OHDA-lesioned rats (Dieb et al., 2016 a-b). Although the intraperitoneal administration of ropinirole may have affected a variety of brain structures, here, it is demonstrated to affect pain processing structures such as insular and cingulate cortices, in addition to SDH. The latter is clearly involved in the pain process as demonstrated by the changes in the different markers at this level. In addition, the effect of intrathecal ropinirole administration on pain (von Frey test) is in support of the segmental involvement of the pain process in 6-OHDA-lesioned rats.

Most of the data in animals (Domenici et al., 2019; Tang et al., 2021) and humans (Brefel-Courbon et al., 2005) showed that dopamine or dopamine agonists did not affect pain in the control or sham animals. For this reason, and to decrease the number of animals needing to be used, a dopamine agonist was not tested on the sham animals.

The use of specific inhibitors of ERK phosphorylation demonstrated that ERK played a role in allodynia and hyperalgesia caused by spinal cord injury or chronic inflammatory and neuropathic pain (Cruz and Cruz, 2007). Since the question here was the phosphorylation of

pre-existing ERK and not the expression of ERK, the total ERK (phosphorylated and non-phosphorylated) was not taken into consideration. In addition, if the total ERK/pERK had been taken into consideration a difference might not have been obtained between the groups. Also, the time between stimulus and sacrifice of the animal was too short for protein synthesis. ERK phosphorylation is induced because of glutamate release (English and Sweatt 1996; 1997), and its binding to both NMDA and metabotropic receptors. It is also induced by neurotrophins through their binding to their tyrosine kinase receptors and by neurotransmitters, such as NPY. Phosphorylation of ERK modulates glutamate receptor and potassium channel activities (Adams et al., 2000; Hu et al., 2006) leading to increased neuronal excitability (Hu and Gereau, 2003). ERK phosphorylation contributes to LTP (Jones et al., 1999; Martin et al., 2000; Schafe et al., 2000; Wei et al., 2006) probably through the expression of different NMDA subunits. Phosphorylation of ERK mediates the windup phenomenon, which is a kind of central sensitization evoked during repetitive C-fiber stimulation (Fukui et al., 2007). More recent studies have also demonstrated a decrease in the mechanical and thermal thresholds in 6-OHDA-lesioned rats, thus implying a segmental pain process (Dieb et al., 2016; Charles et al., 2018; Campos et al., 2019). Moreover, the windup was higher in the 6-OHDA-lesioned when compared to the sham rats (Charles et al., 2018). The windup is modified by an endogenous opioid mechanism at the level of the SDH (Guan et al., 2006). This opioidergic system has been involved in the mechanical allodynia observed in the 6-OHDA model of PD (Campos et al., 2019). Together, these data imply a segmental pain process in PD animal models.

The present study also demonstrated an increase in the expression of PKC $\gamma$ , a protein that is involved in pain chronicity (Malmberg et al., 1997; Dieb and Hafidi, 2015; Dieb et al., 2016 a-b). Inhibiting PKC $\gamma$  also depressed the expression of pERK1/2 in the superficial laminae of the medullary dorsal horn and alleviated the pain in the rats (Dieb and Hafidi, 2015). pERK1/2 positive cells receive PKC $\gamma$  ending innervations (Dieb and Hafidi, 2015). In the present study, ropinirole administration decreased both PKC $\gamma$  and pERK1/2 expressions. Therefore, dopamine depletion in 6-OHDA-lesioned rats produced sensitization (increase in excitation) at the spinal dorsal horn level (Charles et al., 2018) increasing PKC $\gamma$  cells excitation (increase in PKC $\gamma$  staining) leading to the activation of pERK1/2 cells. The latter transfer non-painful stimuli to the pain processing pathway (Dieb and Hafidi, 2015). It is to be noted that, in our case, we have never found pERK1/2 or PKC $\gamma$  in glial cells. A previous

report of double immunostaining using NeuN antibody and pERK1/2 showed that they both colocalize in the same neurons (Dieb et al., 2015).

The administration of the D2R agonist, bromocriptine, lowered the expression of PKC $\gamma$ , decreased the expression of pERK1/2 in the superficial laminae of the medullary dorsal horn, and alleviated the pain in 6-OHDA-lesioned rats (Dieb et al., 2016 a-b). Although the present study confirmed the important role of ropinirole (intraperitoneal and intrathecal administrations) in the pain process, other non-dopaminergic processes should not be excluded. Ropinirole is D3/D2 receptor agonist with very little affinity for D4 and no affinity for D1/D5, serotonin, histamine, acetylcholine, muscarinic, opioids, or adrenergic receptors (Wood, 2008). Ropinirole binds to D2 like receptors with avoiding ergot-like side effects. Ropinirole activates D2 receptors in the central and peripheral nervous systems. The efficiency of ropinirole was better than that of bromocriptine after 3 years of treatment. In different animal models, ropinirole had an antiparkinsonian effect (Fukuzaki et al., 2000). The ropinirole antiparkinsonian effect depends on both D2R and D3R (Matsukawa et al., 2007). To sum up, Ropinirole -as dopamine agonist- is effective as a therapy for early and progressing PD symptoms. This includes pain, as some studies have shown that, in PD models, unilateral injury led to bilateral dorsal horn spinal cord neuron hyperexcitability and augmented D2R function bilaterally (Shill & Stacy, 2009; Tang et al., 2021).

The present study confirms that of Dieb and Hafidi, 2015, except that, here, the stimulus is acetone. Although, to date, there is no proof in the literature of direct projections from SNc/VTA to the spinal cord. There is, however, some evidence that SNc/VTA may influence brainstem descending inhibitory pathways (Beckstead et al., 1979; Comissiong et al., 1979; Loughlin and Fallon, 1984; Ikemoto, 2007; Halliday et al., 2012). For example, the stimulation of SNc inhibits nociceptive input by activating spinal cord neurons through dopaminergic descending pathways (Barnes et al., 1979; Burkey et al., 1999).

Together, these data indicate that dopamine probably regulates the pain process by decreasing excitation in the dorsal horn PKC $\gamma$  cells. PKC $\gamma$  cells represent a key cell for switching non-noxious stimuli to the nociceptive pathway (Dieb and Hafidi, 2015; Dieb et al., 2015; Dieb et al., 2016 a-b).

PKC $\gamma$  is expressed only in the excitatory interneurons in the rat spinal cord (Todd, 2017) and these neurons contribute to the SDH sensitization. PKC $\gamma$  protein expression up-regulation was observed in different pain models (Zhou et al., 2012; Dieb et al., 2014; Dieb et

al., 2016). PKC $\gamma$  mutant mice displayed normal responses to acute painful stimuli but did not display the exaggerated response to noxious stimuli usually seen after partial sciatic nerve section (Malmberg et al., 1997). Glutamate release and the activation of NMDA receptor-induced both upregulation of PKC $\gamma$  protein and its translocation from the plasma membrane to the cytosol. PKC $\gamma$  positive cells project endings to pERK1/2 positive cells within the medullary dorsal horn (Dieb et al., 2015). The inhibition of PKC $\gamma$  decreased the expression of pERK1/2 and alleviated the allodynia (Dieb et al., 2015). Moreover, several studies have shown that repetitive morphine exposure increased the level of PKC $\gamma$  protein in the SDH (Mao et al., 1995). PKC $\gamma$  mutant mice failed to exhibit opioid tolerance (Zeititz et al., 2001).

Another way of increasing excitation at the level of SDH is by decreasing the inhibition or, by disinhibition. Such mechanisms have been described in medullary and spinal dorsal horns where a decrease in GABA transmission promoted pain (Castro-Lopes et al., 1993; Ibuki et al., 1997; Eaton et al., 1998; Dieb and Hafidi, 2015). In the present study, both immunohistochemistry and western blots demonstrated an increase in GAD67. There is a converse correlation between the synthesis of GABA and the expression of GAD67 (Rimval and Martin, 1992). GAD67 activity is regulated by GABA feedback, and this activity declined when GABA levels were elevated. Therefore, the high expression of GAD67 in the dorsal horn of 6-OHDA-lesioned rats may have reflected a decrease in GABA levels. This decrease, in turn, increased the excitation of PKC $\gamma$  cells as reported previously (Dieb and Hafidi, 2015). The 6-OHDA-lesioned rats presented both mechanical allodynia and hyperalgesia, in addition to thermal hyperalgesia.

GAD67 expression is controlled by dopamine. For example, dopamine depletion or D2R antagonists produced an increase in GAD67 expression (Soghomonian and Chesselet, 1992; Périer et al., 2003; Winkler et al., 2003; Billings and Marshall, 2004). Moreover, levodopa treatment reversed GAD67 mRNA overexpression in 6-OHDA-lesioned rats (Bacci et al., 2002; Périer et al., 2003). Although there is no previous report in the literature on this at the spinal cord level, to the best of our knowledge, the administration of ropinirole decreased GAD67 expression in the spinal dorsal horn, as demonstrated by immunohistochemistry and western blot. Dopamine depletion (SNc/VTA) could regulate GAD67 expression via brainstem descending inhibitory pain pathways or, directly, via dopaminergic hypothalamic nuclei (A11). SNc/VTA project to A11 nucleus. The latter is known to project directly to the spinal cord (Björklund and Skagerberg, 1979; Hokfelt et al., 1979; Skagerberg and Lindvall, 1985; Ozawa et al., 2017).

In the present study, 6-OHDA targeted both SNc and VTA, two nuclei that almost project to similar ascending (cortical and subcortical) or descending (brainstem) structures (Beckstead et al., 1979; Loughlin and Fallon 1984; Ikemoto, 2007; Halliday et al., 2012), which are part of the medial pain system. The neuronal activity in this system is regulated by nociceptive stimuli (Chudler and Dong, 1995; Cortelli et al., 2013) and by dopamine (Schultz and Romo, 1987; Mitsi and Zachariou, 2016). Therefore, the impact of the SNc/VTA lesion on pain is considered as a whole and not each nucleus separately. Both nuclei are affected by the 6-OHDA injection to the MFB. First, the primary target of these structures is the striatum (dorsal and ventral) which plays an important role in pain regulation. Striatal administration of D2R agonists had an anti-nociceptive effect (Lin et al., 1981; Cobacho et al., 2010) mediated by the rostral ventromedial medulla (RVM) (Ansah et al., 2007). Insular and cingulate cortices regulate pain (Chudler and Dong, 1995; Mitsi and Zachariou, 2016; Alles and Smith, 2018). However, a major difference between the SNc and the VTA is that the latter modulates the perception of the affective pain and the reward related pain (Chudler and Dong 1995). Acetone stimulation increased the expression of pERK1/2 in the insular and cingulate cortices, hence demonstrating their implication in the pain process in 6-OHDA-lesioned rats. The administration of ropinirole significantly decreased the number of pERK1/2 positive cells in both structures, demonstrating the role of dopamine regulation in this expression. A decreased cold pain threshold has been shown in PD patients, and Levodopa administration normalized it (Brefel-Courbon et al., 2005; 2013). Similarly, over-activation in the insular and cingulate cortices was observed in pain-free PD subjects who were off levodopa at the time in comparison to healthy subjects (Brefel-Courbon et al., 2005).

In conclusion, the present study demonstrated cold and mechanical allodynia and mechanical hyperalgesia in 6-OHDA-lesioned rats. Acetone stimulus activated neurons within superficial spinal dorsal laminae, cingulate and insular cortices.

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## Figure's legend

**Figure 1:** Thermal place preference behavioral test. The graph represents time spent on thermal test plates of the sham and 6-OHDA-lesioned groups. There were significant differences; Student's t-test, between the groups for temperatures 10 °C ( $t_{10} = 4.02$ ,  $p = 0.002$ ,  $**P \leq 0.01$ ), 15 °C ( $t_{10} = 8.31$ ,  $p = 0.00001$ ,  $***P \leq 0.001$ ) and a non-significant difference for 35 °C ( $t_{10} = 0.66$ ,  $p = 0.53$ ,  $p > 0.05$ ). While Mann-Whitney U test was used for 25 °C ( $U_{6,6} = 10$ ,  $p = 0.22$ ,  $p > 0.05$ ) and 50 °C ( $U_{6,6} = 4$ ,  $p = 0.03$ ,  $*P \leq 0.05$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 6$ ) and 6-OHDA-lesioned groups ( $n = 6$ ).

**Figure 2:** Acetone cold allodynia behavioral test (Intraperitoneal administration of Ropinirole for 6-OHDA +D2R agonist group). Graph (A) represents the number of flinches for the three groups; Kruskal-Wallis H test ( $H_2 = 17.23$ ,  $p = 0.0002$ ,  $***P \leq 0.001$ ): 6-OHDA-lesioned rats had more significant numbers than the sham ones (Dunn's multiple comparisons test,  $p = 0.001$ ,  $***P \leq 0.001$ ) also, the 6-OHDA +D2R agonist group had an increase in significance over the sham group (Dunn's multiple comparisons test,  $p = 0.01$ ,  $**P \leq 0.01$ ). Graph (B) represents the latency time of the three groups; Kruskal-Wallis H test ( $H_2 = 15.13$ ,  $p = 0.0004$ ,  $***P \leq 0.001$ ), the 6-OHDA-lesioned rats had a more significant increase in latency time than both the sham and 6-OHDA +D2R agonist groups (Dunn's multiple comparisons tests,  $p = 0.006$ ,  $p = 0.04$ , respectively,  $**P \leq 0.01$ ,  $*P \leq 0.05$  respectively). Graph (C) represents the reaction time of the three groups; Kruskal-Wallis H test ( $H_2 = 28.58$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): the 6-OHDA-lesioned group had a more significant increase in reaction time than both the sham and 6-OHDA +D2R agonist groups (Dunn's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as box-and-whiskers plots, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 11$ ), 6-OHDA-lesioned ( $n = 23$ ), and 6-OHDA+D2R agonist groups ( $n = 9$ ).

**Figure 3:** Graph (A) represents mechanical hyperalgesia (Randall-test) at two and four weeks after surgery; Two-way ANOVA test with significant interaction, row and column factors ( $F_{2, 30} = 479.6$ ,  $F_{2, 30} = 695.4$ ,  $F_{1, 30} = 197$  respectively,  $p < 0.0001$ ,  $***P \leq 0.001$ ): followed by (Sidak's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as a line plot of the median  $\pm$  interquartile range of sham ( $n = 6$ ) and 6-OHDA-lesioned rats ( $n = 6$ ). Graph (B) shows similar results using the non-noxious 10 g von Frey filament test at two and four weeks after surgery; Friedman's test ( $\chi^2_2 = 16.84$ ,  $p < 0.001$ ,  $***P \leq 0.001$ ) followed by (Wilcoxon Signed-Rank Test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 6$ ) and 6-OHDA-lesioned groups ( $n = 6$ ).

**Figure 4:** pERK1/2 protein expression. The Figure shows pERK expression in the SDH of the sham (A), 6-OHDA-lesioned (B), and 6-OHDA +D2R agonist groups (C). Graph (D) represents pERK1/2 positive cells within the SDH superficial laminae; Kruskal-Wallis H test ( $H_2 = 55.64$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): 6-OHDA-lesioned rats had a more significant number than both the sham and 6-OHDA +D2R agonist groups (Dunn's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ) also, the 6-OHDA +D2R agonist group had an increase in significance over the sham (Dunn's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 5$ ), 6-OHDA-lesioned ( $n = 6$ ), and D2R agonist groups ( $n = 6$ ). The Scale bar represents 50  $\mu\text{m}$ . Figure (E) shows western blot bands of pERK1/2 for the three groups. Graph (F) represents western blot band intensities for the three groups; one-way ANOVA ( $F_{2, 6} = 10.29$ ,  $p = 0.012$ ,  $*P \leq 0.05$ ): The 6-OHDA-lesioned rats had a more significant increase in expression than both the sham and 6-OHDA +D2R agonist groups (Tukey's multiple comparisons test,  $p = 0.02$ ,  $p = 0.02$  respectively,  $*P \leq 0.05$ ). Data are represented as a barplot of the median  $\pm$  interquartile range of the sham ( $n = 3$ ), 6-OHDA-lesioned ( $n = 3$ ) and 6-OHDA +D2R agonist groups ( $n = 3$ ), GAPDH was used as the loading control.

**Figure 5:** PKC $\gamma$  protein expression. The Figure shows PKC $\gamma$  expression in the SDH of the sham (A), 6-OHDA-lesioned (B) and 6-OHDA +D2R agonist groups (C). PKC $\gamma$  positive cells were observed within the SDH mostly in cells of lamina Iii and scattered cells within lamina III. Figure (B) has more intense fluorescent labeling than both Figures (A) and (C). The Scale bar represents 50  $\mu\text{m}$ . Graph (D) represents PKC $\gamma$  cell number count; Kruskal-Wallis H test

( $H_2 = 29.42$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): the 6-OHDA-lesioned rats had a higher significant number than both the sham and 6-OHDA +D2R agonist groups (Dunn's multiple comparisons test,  $p = 0.0001$ ,  $p < 0.0001$  respectively,  $***P \leq 0.001$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles, and the whiskers show the 5th and 95th percentile of the sham ( $n = 6$ ), 6-OHDA-lesioned ( $n = 7$ ), and D2R agonist group ( $n = 6$ ). Figure (E) shows the western blot bands of PKC $\gamma$  for the three groups. Graph (F) represents western blot band intensities for the three groups, one-way ANOVA ( $F_{2, 9} = 1.11$ ,  $p = 0.37$ ,  $p > 0.05$ ): the 6-OHDA-lesioned group had a less significant increase in expression than both the sham and 6-OHDA +D2R agonist groups (Tukey's multiple comparisons tests,  $p = 0.35$ ,  $p = 0.65$ , respectively,  $p > 0.05$ ). Data are represented as a barplot of the median  $\pm$  interquartile range of the sham ( $n = 4$ ), 6-OHDA-lesioned ( $n = 4$ ) and 6-OHDA +D2R agonist groups ( $n = 4$ ), GAPDH was used as the loading control.

**Figure 6:** GAD67 protein expression. The Figure shows GAD67 expression in the SDH of the sham (A), 6-OHDA-lesioned (B), and 6-OHDA +D2R agonist groups (C). Figure (B) has more intense fluorescent labeling than both figures (A) and (C). The Scale bar represents 50  $\mu\text{m}$ . Figure (D) shows the western blot bands of GAD67 for the three groups. Graph (E) represents western blot band intensities for the three groups; Kruskal-Wallis test ( $H_2 = 15.79$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): The 6-OHDA-lesioned rats had a more significant increase in expression than both the sham and 6-OHDA +D2R agonist groups (Dunn's multiple comparisons test,  $p = 0.03$ ,  $p = 0.0004$ , respectively,  $*P \leq 0.05$ ,  $***P \leq 0.001$ ). Data are represented as a barplot of the median  $\pm$  interquartile range of the sham ( $n = 4$ ), 6-OHDA-lesioned ( $n = 8$ ) and 6-OHDA +D2R agonist groups ( $n = 8$ ), GAPDH was used as the loading control.

**Figure 7:** pERK1/2 protein expression. The Figure shows pERK expression in the insular cortex of the sham (A), 6-OHDA-lesioned (B), and 6-OHDA +D2R agonist groups (C). Graph (D) represents pERK1/2 positive cells within the insular cortex; Kruskal-Wallis test ( $H_2 = 25.71$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): the 6-OHDA-lesioned animals had more significant numbers than both the sham and 6-OHDA +D2R agonist groups (Tukey's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 6$ ), 6-OHDA-lesioned ( $n = 6$ ), and 6-OHDA +D2R agonist groups ( $n = 7$ ). The Scale bar represents 50  $\mu\text{m}$ . Figure (E) shows western blot bands of pERK1/2 for the three groups. Graph (F) represents western blot band intensities for the

three groups; one-way ANOVA ( $F_{2, 6} = 6.60$ ,  $p = 0.031$ ,  $*P \leq 0.05$ ): The 6-OHDA-lesioned group had a more significant increase in expression than both the sham and 6-OHDA +D2R agonist groups (Tukey's multiple comparisons test,  $p = 0.044$ ,  $p = 0.046$  respectively,  $*P \leq 0.05$ ). Data are represented as a barplot of the median  $\pm$  interquartile range of the sham ( $n = 3$ ), 6-OHDA-lesioned ( $n = 3$ ) and 6-OHDA +D2R agonist groups ( $n = 3$ ), GAPDH was used as the loading control.

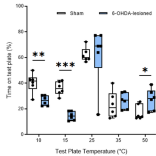
**Figure 8:** pERK1/2 protein expression. The Figure shows pERK expression in the cingulate cortex of the sham (A), 6-OHDA-lesioned (B), and 6-OHDA +D2R agonist groups (C). Graph (D) represents pERK1/2 positive cells within the cingulate cortex; one-way ANOVA ( $F_{2, 47} = 14.36$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ): the 6-OHDA-lesioned rats had more significant numbers than both the sham and 6-OHDA +D2R agonist groups (Tukey's multiple comparisons test,  $p < 0.0001$ ,  $***P \leq 0.001$ ). Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 6$ ), 6-OHDA lesioned ( $n = 6$ ), and 6-OHDA +D2R agonist groups ( $n = 7$ ). The Scale bar represents 100  $\mu\text{m}$ . Figure (E) shows western blot bands of pERK1/2 for the three groups. Graph (F) represents western blot band intensities for the three groups; one-way ANOVA ( $F_{2, 6} = 5.34$ ,  $p = 0.047$ ,  $*P \leq 0.05$ ): the 6-OHDA-lesioned group had a more significant increase in expression than the sham group (Tukey's multiple comparisons test,  $p = 0.048$ ,  $*P \leq 0.05$ ). Data are represented as a barplot of the median  $\pm$  interquartile range of the sham ( $n = 4$ ), 6-OHDA-lesioned ( $n=4$ ) and 6-OHDA + D2R agonist groups ( $n = 4$ ), GAPDH was used as the loading control.

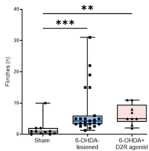
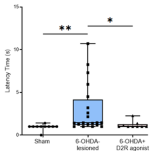
**Figure 9:** A significant allodynic behavior was observed in the hind paws in the 6-OHDA-lesioned rats. The allodynic behavior decreased almost immediately with the intrathecal administration of ropinirole in the 6-OHDA-lesioned rats; two-way ANOVA test with significant interaction, row and column factors ( $F_{6, 70} = 14.08$ ,  $F_{6, 70} = 17.80$ ,  $F_{1, 70} = 147.9$ , respectively,  $p < 0.0001$ ,  $***P \leq 0.001$ ): followed by (Sidak's multiple comparisons test:  $p < 0.0001$ ,  $***P \leq 0.001$ ). This effect lasted for almost 25 minutes. Data are represented as a line plot of the median  $\pm$  interquartile range of the sham ( $n = 6$ ) and 6-OHDA-lesioned rats ( $n = 6$ ).

**Figure 10:** TH immunostaining in the sham (A) and 6-OHDA-lesioned animals (B) shows a profound decrease in the intensity of staining, in both the SNc and the VTA in the 6-OHDA-lesioned rats when compared with the sham ones. The Cell count of the TH positive cells in

both the SNc and the VTA together; Mann-Whitney test ( $U_{27,15}=18$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ) (C) or in the VTA only; Mann-Whitney test ( $U_{28,13}=45$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ) (D) or in the SNc only; Mann-Whitney test ( $U_{28,13}=2$ ,  $p < 0.0001$ ,  $***P \leq 0.001$ ) (E) revealed a significant difference between the 6-OHDA-lesioned and the sham groups. Cell death percentage in both the SNc and the VTA was 67.8 %, in VTA only, it was 56.7 % and in the SNc, it only was 89.9 % after 6-OHDA lesion. Data are represented as a box-and-whiskers plot, where the box depicts the median and the 25th and 75th quartiles and the whiskers show the 5th and 95th percentile of the sham ( $n = 5$ ) and 6-OHDA-lesioned groups ( $n = 6$ ). The Scale bar represents 200  $\mu\text{m}$ . The graph (F) represents data from two animal groups: sham ( $n = 11$ ) and 6-OHDA-lesioned ( $n = 18$ ). A significant decrease in weight difference (Student's T-test) was observed in the 6-OHDA-lesioned group in comparison to the sham rats after surgery ( $p = 0.027$ ,  $* P \leq 0.05$ ).

**Figure 11:** Behavioral Experiments Time Course. 6-OHDA (6-hydroxydopamine) and VFT (von Frey test).



**A****B****C**