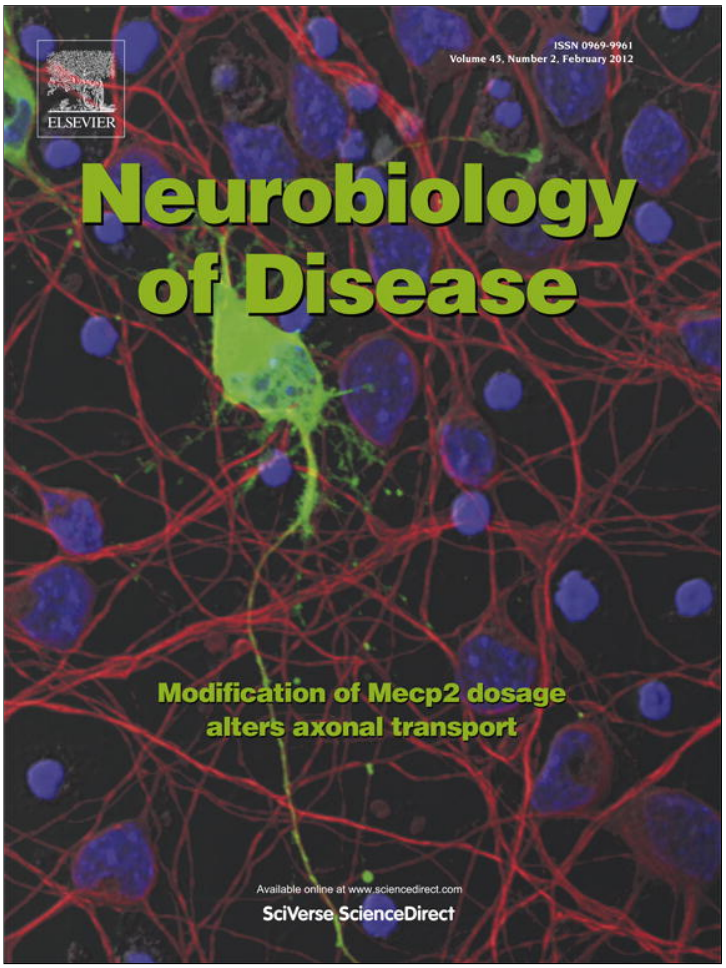

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Emerging dysfunctions consequent to combined monoaminergic depletions in parkinsonism

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ABSTRACT

The loss of dopamine (DA) neurons has been the pathophysiological focus of the devastating conditions of Parkinson's disease, but depletion of DA alone in animal models has failed to simultaneously elicit both the motor and non-motor deficits of PD. The present study aimed to investigate, in rats, the respective role of dopamine (DA), noradrenaline (NA) and serotonin (5-HT) depletions on motor and non-motor behaviors and on subthalamic (STN) neuronal activity. We show that NA or DA depletion significantly decreased locomotor activity and enhanced the proportion of bursty and irregular STN neurons. Anxiety-like states required DA depletion plus the depletion of 5-HT or NA. Anhedonia and "depressive-like" behavior emerged only from the combined depletion of all three monoamines, an effect paralleled by an increase in the firing rate and the proportion of bursty and irregular STN neurons. Here, we provide evidence for the exacerbation of behavioral deficits when NA and/or 5-HT depletions are combined with DA depletion, bringing new insight into the combined roles of the three monoamines in PD.

Introduction

Parkinson's disease is a neurological disorder characterized by the manifestation of motor symptoms, attributed to the degeneration of dopamine (DA) neurons of the substantia nigra pars compacta (SNc) (Ehringer and Hornykiewicz, 1960). Although the motor symptoms of PD are well defined, the non-motor features, such as depression, anhedonia and anxiety, are under-studied and, consequently, under-treated.

Despite the focus on DA, PD is a multi-system disorder characterized also by the loss of noradrenaline (NA) neurons of the locus coeruleus (Bertrand et al., 1997; Fornai et al., 2007) and serotonin (5-HT) cells of the dorsal raphe (Kish, 2003). Although NA and 5-HT depletions have been suggested as other landmarks of the disease, a specific role for each neurotransmitter in the pathophysiology of PD is still not clearly determined. NA and 5-HT are widely recognized in the development of depression and anxiety both of which have been reported in PD patients (Delaville et al., 2011; Halliday et al., 1990; Murai et al., 2001). On the other hand, total inhibition or destruction of the 5-HTergic system alone in the rat does not induce any "depressive-like" behavior or anxiety (Cervo et al., 1991; Wieland et al., 1990). Considering these findings together, we hypothesized that these symptoms could be a consequence of dysfunction of some combination of DAergic, NAergic and 5-HTergic pathways.

Many studies have identified the subthalamic nucleus (STN) as a basal ganglia nucleus playing a key role in the pathophysiology of PD. After DA depletion, STN neurons, which normally exhibit a tonic discharge pattern, become bursty in animal models of PD (Bergman et al., 1994; Ni et al., 2001). This pathological bursty pattern has also been reported in PD patients (Benazzouz et al., 2002; Hutchison et al., 1998). Moreover, the motor symptoms of PD are alleviated by either STN ablation (Bergman et al., 1990; Guridi and Obeso, 2001) or high frequency stimulation (Benabid et al., 2000; Benazzouz et al., 1993). In addition to its role in motor regulation, the STN plays a pivotal role in associative and limbic functions (Temel et al., 2005). Furthermore, within the basal ganglia, the STN is one of the structures most heavily innervated by NAergic (Boyajian et al., 1987; Canteras et al., 1990) and 5-HTergic (Steinbusch, 1981) terminals. Functionally, NAergic as well as 5-HTergic agents, can modulate STN neuronal activity with an impact on motor behavior in the rat (Belujon et al., 2007; De Deurwaerdere and Chesselet, 2000). From these studies, and in

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view of its important role in the basal ganglia and in PD, we hypothesized that defective NAergic and/or 5-HTergic transmission could influence the electrical activity of STN neurons in the context of PD.

Thus the present study aimed to investigate the effects of DA, NA and 5-HT depletions one by one or combined upon (i) motor and non-motor functions including locomotor activity, depressive-like behavior and anxiety, and (ii) on the electrical activity of STN neurons in the rat.

Material and methods

Animals

Adult male Wistar rats, weighing 280–380 g were used for behavioral and *in vivo* electrophysiological experiments. They were housed five per cage under artificial conditions of light (light/dark cycle, light on at 7:00 a.m.), temperature (24 °C), and humidity (45%) with food and water available *ad libitum*. All animal experiments were carried out in accordance with the European Communities Council Directive (*EU Directive 2010/63/EU*).

Monoamine depletion procedures

The present study was carried out on a total of 151 rats distributed in eight groups as summarized in Fig. 1. Each group was subdivided into subgroups to perform one or two behavioral tests. The number of animals per group appears in the figure legends.

Each rat received either 6-hydroxydopamine (6-OHDA-lesioned rats) or NaCl (0.9%; sham-lesioned rats) into the right medial forebrain bundle (MFB). Two weeks later, 6-OHDA-lesioned rats and sham-lesioned rats received intraperitoneal (i.p.) injection of N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP-4) or NaCl (0.9%) followed, one week later, by two i.p. injections of 4-Chloro-L-phenylalanine (pCPA) or NaCl (0.9%) on two consecutive days. The rationale for waiting 2 weeks after 6-OHDA injection to deliver the NA- and 3 weeks for 5-HT-depleting drugs was based on the fact that the stable stage of behavioral deficits, as well as the pathological activity in basal ganglia nuclei, appears at least 2 weeks after the injection of 6-OHDA (Neve et al., 1982; Ni et al., 2001; Orieux et al., 2000). Furthermore, behavioral or electrophysiological experiments were then performed 4 weeks after 6-OHDA, 1 week after DSP-4 and/or the day following the last pCPA injection. Animals exposed to 2 or 3 depletions did not develop any weight loss and had a good general welfare.

6-OHDA, DSP-4 and pCPA were purchased from Sigma (Saint-Quentin Fallavier, France). NA depletion has been performed using DSP-4, a neurotoxin highly selective for NAergic fibers arising from the LC that does not affect other NAergic systems, such as the sympathetic nervous system (Fornai et al., 2001; Fritschy and Grzanna, 1989, 1991). DSP-4 was used at a dose of 50 mg/kg according to the work of Grzanna et al. (1989). It was dissolved in NaCl 0.9% immediately before use. pCPA, a selective inhibitor of 5-HT synthesis, was also used at a dose of 50 mg/kg during two successive days as previously determined in the laboratory (data not shown). 6-OHDA was stereotaxically injected into the MFB as previously described (Belujon et al., 2007). Thirty minutes prior to surgery, animals were given an i.p. injection of desipramine (25 mg/kg, Sigma) dissolved in 0.9% NaCl and injected in a volume of 5 ml/kg body weight. Desipramine was used in order to protect the NAergic system. Rats were then placed in a stereotaxic frame (Kopf, Unimecanique, France) under chloral hydrate anesthesia (400 mg/kg, i.p., Sigma). Each animal received a unilateral injection of 2.5 µl 6-OHDA (Sigma, 5 mg/ml in sterile NaCl, 0.9%) with 0.1% ascorbic acid into the right MFB at coordinates 2.8 mm posterior to bregma, 2 mm lateral to the midline and 8.4 mm below the skull according to the brain atlas of Paxinos and Watson (1996). 6-OHDA injection was made over a 5 min period using a 10 µl Hamilton microsyringe. At the end of each injection, the syringe needle was left in place for an additional 5 min and then withdrawn slowly to prevent reflux of the solution.

Evaluation of motor activity

Spontaneous motor activity (open-field)

Spontaneous horizontal motor activity was measured in an isolated room between 8:00 a.m. and 1:00 p.m. using a photoelectric actimeter (Actitrack, Panlab, S.L., Barcelona, Spain), as previously described (Chetrit et al., 2009).

Evaluation of catalepsy scores (bar test)

The degree of catalepsy resulting from monoamine depletions was assessed using the bar tests as previously reported (Chetrit et al., 2009). It consisted of positioning the rat's forepaws on a horizontal bar (0.7 cm diameter) placed at 9 cm above the ground and measuring the latency for the forepaw contralateral to the 6-OHDA-lesioned

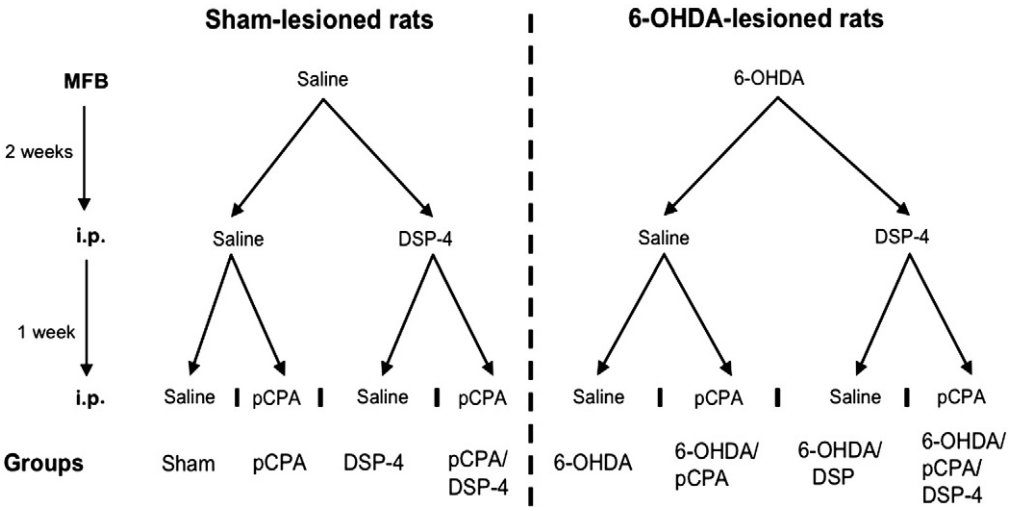

Fig. 1. Schematic presentation of different groups of drug-treated animals and their respective controls. MFB: medial forebrain bundle; i.p.: intra-peritoneal injection. Sham: rats treated with 0.9% NaCl (n = 17); pCPA: rats treated with 4-Chloro-L-phenylalanine (n = 19); DSP-4: rats treated with N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (n = 10), pCPA/DSP-4: n = 21; 6-OHDA: rats treated with stereotactic injection of 6-hydroxydopamine (6-OHDA) into the MFB (n = 8), 6-OHDA/pCPA: n = 23, 6-OHDA/DSP-4: n = 13, 6-OHDA/pCPA/DSP-4: n = 19.

hemisphere to be withdrawn from the bar and touch the floor (descent latency). The rats that did not maintain their position on the bar received a score of 0 s and were considered non-cataleptic. The latency cut-off time was set at 120 s.

Evaluation of 6-OHDA-induced motor asymmetry

The stepping test was used to assess the asymmetry in forelimb motor activity induced by the unilateral lesion of the nigro-striatal pathway after 6-OHDA injection into the right MFB. This test was done as previously reported (Olsson et al., 1995) in order to select only animals with an effective depletion of DA before their submission to another depleting drug (DSP-4 and/or pCPA) or NaCl. Unilateral dopamine depletion led to a deficit in contralateral paw use compared to ipsilateral paw use in all our DA depleted animals (4.2 ± 1.1 *versus* 12.4 ± 1.1 respectively, Wilcoxon test, $p<0.01$).

Evaluation of "depression-like behavior"

Sucrose preference

Published procedures were used (Sclafani and Ackroff, 2003; Tordoff and Bachmanov, 2003). Rats were placed in individual cages with food and water *ad libitum*. Over the course of three days, rats were housed in the presence of two bottles of water and the position of the water bottles was randomly changed. When the lights went out at 7:00 p.m., pre-weighed water and 1% sucrose bottles were placed on the home cage and rats were allowed to drink for 2 h. During these 2 h the position of the bottles was changed. The 2 h intake was measured by weighing the bottles before and after the test. Sucrose preference was calculated as follow: $100 \times$ [sucrose intake (g)/(sucrose intake (g) + water intake (g))].

Forced swim test (FST)

The FST was conducted as previously described (Porsolt et al., 1978). Off line analyses were performed as previously reported (Detke et al., 1995). A rat was judged to be immobile when it remained floating in the water without struggling and was making only those movements necessary to keep its head above water.

Evaluation of anxiety

Animals were tested in the elevated plus maze (EPM) to assess the anxiety-related behavior. The EPM consisted of two open arms (50 cm long × 10 cm wide) and two walled arms (50 cm long × 10 cm wide × 38.5 cm high) with an open roof, arranged around a central platform (10 cm × 10 cm), with the two arms of each type placed opposite to each other. A camera was mounted 1.5 m above the EPM. Animals were placed onto the central platform, facing one of the open arms. The animal was allowed to explore the maze for 5 min. The following variables were measured: time (s) spent in the open and closed arms and number of entries into the open and closed arms (an entry was counted when the animal entered an arm with all four paws).

Extracellular single unit recordings

Extracellular single-unit recordings in the right STN, ipsilateral to the 6-OHDA-lesioned MFB, were made in rats anesthetized with urethane (1.2 g/kg, i.p.) as previously reported (Belujon et al., 2007; Ni et al., 2001). A single glass micropipette electrode (impedance: 8–12 MΩ; aperture 0.5 μm) was filled with 4% Pontamine sky blue in 3 M NaCl. The electrode was lowered into the STN according to the coordinates given in the brain atlas (Paxinos and Watson, 1996) (AP: −3.8 mm, L: −2.5 mm, D: 6.8–8.2 mm). Extracellular neuronal activity was amplified, bandpass-filtered (300–3000 Hz) using a preamplifier (Neurolog, Digitimer, UK) and transferred *via* a Powerlab interface (AD Instruments, Charlotte, NC, USA) to a computer equipped with Chart 5 software (AD Instruments, Charlotte, NC, USA). Only neuronal activity with a signal-to-noise ratio >3:1 was recorded and used for further investigation. Basal firing of neurons was recorded for 20 min to ascertain the stability of the discharge activity. At the end of each session, the recording site was marked by electrophoretic injection (Iso DAM 80, WPI, Hertfordshire, UK) of Pontamine sky blue through the micropipette at a negative current of 20 μA for 7 min. The location of the Pontamine sky blue dots was histologically verified as previously reported (Belujon et al., 2007) and only brains in which the dot was clearly visible in the STN were used for data analysis.

Data analysis

The activity of each neuron was analyzed with a spike discriminator using a spike histogram program (AD Instruments, Charlotte, NC, USA) and firing parameters (interspike interval (5 ms bin)) were calculated using Neuroexplorer program (AlphaOmega, Nazareth, Israel). Firing patterns were analyzed using the method developed by Kaneoke and Vitek (1996) as previously described (Labarre et al., 2008).

Validation of the experimental model

Selected animals for final analysis went through a series of validation steps that were all mandatory for final inclusion. The displayed n refers to this final inclusion.

Validation of DA lesion

DA cell loss in the SNc was verified by immunohistochemistry of tyrosine hydroxylase (TH) as previously described (Bouali-Benazzouz et al., 2009; Ni et al., 2001). TH-immunohistochemistry was performed for all rats submitted to 6-OHDA-lesion with or without additional NA and/or 5-HT depletion. It was used as an inclusion criterion and only rats with almost total loss of TH-immunoreactivity were considered for the analysis of behavioral and electrophysiological data.

Biochemical assessment of monoamine depletion

In order to quantify the level of DA depletion, a part of 6-OHDA animals, validated by immunohistochemistry, were used for quantitative HPLC analysis. In addition, this technique was used to quantify also the extent of NA and 5-HT depletions in all the tested groups, as it was not possible to use immunohistochemistry for these two monoamines. There were no criteria for NA and 5-HT depletions, as the injections were done intraperitoneally with a relative homogeneity in the depletion of these two monoamines. In all our animals used in the present study NA depletion was ≥70% and 5-HT ≥60%. Only a few animals were excluded from the study because they did not show NA and/or 5-HT depletions.

Tissue content of DA was measured in the anterior striatum and that of NA and 5-HT in the rostral part of the frontal cortex as previously reported (Hoyer et al., 1994; Loughlin et al., 1982). The choice of these brain structures was based on their extensive monoaminergic innervations (De Deurwaerdere et al., 1995; Navailles et al., 2010). Tissue concentrations were measured by HPLC with electrochemical detection in these brain areas of control and treated rats as previously described (De Deurwaerdere et al., 1995). Rats were decapitated and their brains were removed rapidly and frozen in cold isopentane. Both right and left portions of the anterior striatum and the frontal cortex were dissected and stored at −80 °C until their use in biochemical assays. The tissues were homogenized in 200 μl of 0.1 N HClO4 and centrifuged at 13,000 rpm for 30 min at 4 °C. Aliquots of the supernatants were diluted in the mobile phase (1/2 for the cortex and 1/4 for the striatum) and injected into the HPLC column (Chromasyl C8, 150 × 4.6 mm, 5 μm) protected by a Brownlee-Newgard precolumn (RP-8, 15 × 3.2 mm, 7 μm). The mobile phase, delivered at 1.2 ml/min flow rate, was as follows (in mM): 60 NaH2PO4, 0.1 disodium EDTA, and 2 octane sulfonic acid plus 7% methanol, adjusted to pH 3.9 with

orthophosphoric acid and filtered through a 0.22 μm Millipore filter. Detection of compounds was performed with a coulometric detector (CoulochemI, ESA) coupled to a dual-electrode analytic cell (model 5011). The potential of the electrodes was set at +350 and −270 mV. Results are expressed as ng/g of tissue, and each value is the mean ± SEM. The percentage of monoamine depletions in drug-treated animals was calculated with regard to sham-lesioned animals.

Statistical analysis

Statistical analyses were done using Prism (GraphPad Software, San Diego, CA). Biochemical data were compared using a Student's *t*-test in rats receiving 6-OHDA *versus* NaCl to determine the impact of DA lesion alone compared to its control. Then, sham-lesioned and 6-OHDA-lesioned groups were separated and the effects of DSP-4 and pCPA on monoamine tissue contents were analyzed using a one-way ANOVA followed, when significant, by the Tukey's test for adequate multiple comparisons. Behavioral data in sham and 6-OHDA rats were first compared using the Mann–Whitney test. Then, the effect of DSP-4 and pCPA were compared separately in sham-lesioned or 6-OHDA-lesioned groups (see Fig. 1) using a Kruskal–Wallis test followed, when significant, by Dunn's multiple comparison test. For electrophysiological analysis, changes in the proportion of different firing patterns were analyzed using a Chi^2 test. Firing rates from sham and 6-OHDA rats were compared using a Student's *t*-test. The effects of DSP-4 and pCPA on firing rates were separately analyzed from the sham and 6-OHDA groups using a one way ANOVA followed, when significant, by the post-hoc Newmann–Keuls test. A p value <0.05 was considered significant.

Results

Monoamine tissue content is reduced by selective drugs in specific brain regions

Table 1 summarizes the tissue content of DA in the anterior striatum and NA as well as 5-HT in the frontal cortex. 6-OHDA, DSP-4 and pCPA selectively depleted their respective monoamine system. The n of animals in Table 1 corresponds to the number of rats per group used in the study and in which monoamine (DA, NA and 5-HT) tissue contents were quantified by post-mortem HPLC.

Dopamine level

As expected, 6-OHDA injection into the right MFB dramatically decreased by almost 95% tissue level of DA in the ipsilateral striatum compared to sham-lesioned animals ($p<0.001$, Student's *t*-test). DSP-4 and/or pCPA treatments did not modify by themselves striatal DA tissue content either in sham-lesioned rats (one-way ANOVA $F(3,55)=1.32$, ns) or in 6-OHDA-lesioned rats ($F(3,57)=0.44$, ns). Notably, the dramatic decrease in striatal DA content observed in 6-OHDA rats was similar in 6-OHDA/pCPA group (84% compared to their respective control), in 6-OHDA/DSP-4 group (93%) and in 6-OHDA/DSP-4/pCPA group (97%). It is noteworthy that DA levels in the unlesioned-side were not significantly modified in sham (one-way ANOVA $F(3,55)=1.98$, ns) and 6-OHDA groups (one-way ANOVA $F(3,56)=2.51$, ns) (Table 1).

Noradrenaline level

6-OHDA administration into the right MFB did not affect NA tissue content in the frontal cortex (Student's *t*-test, $p>0.05$). As expected, DSP-4 induced a dramatic decrease in NA tissue content in both sham-lesioned (one-way ANOVA; $F(3,67)=27.19$, $p<0.001$,) and 6-OHDA-lesioned ($F(3,58)=65.61$, $p<0.001$) rats. The magnitude of the depletion provoked by DSP-4 was similar in both sham (−81.6%) and 6-OHDA rats (−79.4%) compared to their respective controls. Similarly, pCPA did not modify the NA depleting action of DSP-4 in both sham-lesioned (−84.6%) and 6-OHDA-lesioned rats (−92.2%). DSP-4 had a similar efficacy in the left side (unlesioned for DA) in both sham-lesioned ($F(3,58)=41.11$, $p<0.001$,) and 6-OHDA-lesioned ($F(3,55)=2.51$, $p<0.001$) rats (Table 1).

Serotonin level

The administration of 6-OHDA into the right MFB did not modify 5-HT tissue content in the frontal cortex when compared to its respective sham group (Student's *t*-test, $p>0.05$). As expected, pCPA induced moderate to severe depletion (73–82%) of 5-HT tissue content in the frontal cortex that was comparable in both sham-lesioned (one-way ANOVA; $F(3,63)=39.94$, $p<0.001$,) and 6-OHDA-lesioned ($F(3,55)=40.68$, $p<0.001$) groups. The addition of DSP-4 to pCPA did not change the depleting action of pCPA on 5-HT tissue content in both sham-lesioned (−82.8%) and 6-OHDA-lesioned rats (−89.2%). The decrease in 5-HT tissue content was similar in the left side (unlesioned for DA) in both sham-lesioned ($F(3,60)=31.10$, $p<0.001$) and 6-OHDA-lesioned rats ($F(3,56)=32.31$, $p<0.001$) (Table 1).

NA depletion as well as DA depletion reduced locomotor activity

In agreement with previous data (Belujon et al., 2007) 6-OHDA-lesion significantly decreased the score of spontaneous movements by 56.4% compared to sham-lesioned animals (Mann–Whitney test, $p<0.001$, Fig. 2A).

Table 1
Neurochemical analysis of 6 O-HDA, DSP-4 and pCPA treatments induced selective depletion of DA, NA and 5-HT respectively. Tissue contents of striatal DA and frontal cortex NA and 5-HT measured by HPLC in different groups of animals. Values are concentrations in ng/g of wet tissue presented as the mean ± SEM. (n): number of rats; Statistical analysis using one way ANOVA followed by Tukey's multiple comparison test was performed.

	DA Anterior striatum		NA Frontal cortex		5-HT Frontal cortex	
	Left	Right	Left	Right	Left	Right
Sham-lesioned groups						
Sham (n = 17)	5738.2 ± 566.6	5995.4 ± 577.7	147.2 ± 17.3	151.7 ± 14.3	160.3 ± 18.8	166.3 ± 10.6
pCPA (n = 19)	5133.4 ± 317.0	6193.4 ± 561.6	150.1 ± 9.3	142.7 ± 8.8	24.2 ± 6.7***	33.6 ± 9.6***
DSP-4 (n = 10)	6674.0 ± 1043.2	6350.8 ± 1017.1	31.3 ± 10.5***	23.5 ± 5.0***	146.6 ± 31.0	163.6 ± 27.9
DSP-4/pCPA (n = 21)	4835.0 ± 369.3	5030.0 ± 464.3	20.0 ± 4.0***	25.0 ± 5.6***	31.0 ± 6.9***	25.0 ± 4.5***
6-OHDA-lesioned groups						
6-OHDA (n = 8)	7340.8 ± 1339.7	307.9 ± 132.2	146.9 ± 14.2	139.1 ± 15.4	155.1 ± 20.9	144.5 ± 14.2
6-OHDA/pCPA (n = 23)	7349.5 ± 656.7	976.0 ± 253.7	194.7 ± 14.0	161.4 ± 11.9	33.8 ± 8.3†††	53.3 ± 8.5†††
6-OHDA/DSP-4 (n = 13)	6518.8 ± 935.2	436.1 ± 75.3	40.4 ± 15.6†††	20.8 ± 6.0†††	144.0 ± 14.4	132.3 ± 16.8
6-OHDA/DSP-4/pCPA (n = 19)	4957.1 ± 558.9	185.1 ± 48.2	17.1 ± 5.0†††	6.1 ± 2.0†††	20.9 ± 7.3†††	14.0 ± 4.9†††

*** $p<0.001$ in comparison with sham group.
††† $p<0.001$ in comparison with 6-OHDA group.

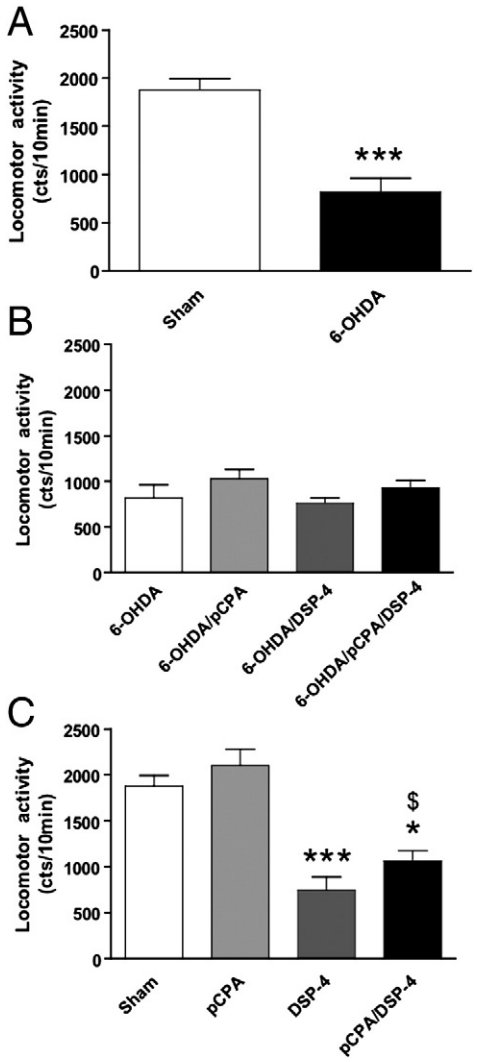

Fig. 2. DA depletion as well as NA depletion, but not that of 5-HT, reduced locomotor activity measured by the "open field" actimeter. Histograms represent the number of horizontal movements recorded during 10 min on day 4 after habituation. Values are the mean ± SEM in sham and 6-OHDA rats (A); in 6-OHDA-lesioned groups (B) and in sham-lesioned groups (C). *: $p<0.05$, ***: $p<0.001$ in comparison with sham. $: $p<0.05$ in comparison with pCPA group (Dunn's multiple comparison test). n = 11 rats in sham group, n = 9 rats in 6-OHDA group, n = 5 rats in pCPA group, n = 9 rats in DSP-4 group, n = 5 rats in pCPA/DSP-4 group, n = 6 rats in 6-OHDA/pCPA group, n = 8 rats in 6-OHDA/DSP-4 group and n = 6 rats in 6-OHDA/pCPA/DSP-4 group.

The impact of additional depletions of NA and 5-HT differed between 6-OHDA-lesioned and sham-lesioned rats. In 6-OHDA-lesioned rats, even if they were still able to move, additional 5-HT and/or NA depletions did not induce any potentiation of the motor impairment induced by DA depletion (Kruskal–Wallis test, $F=4.52$, $p=0.20$; Fig. 2B). Nevertheless, in sham-lesioned rats NA depletion by itself dramatically decreased locomotor activity (Kruskal–Wallis test, $F=20.87$, followed by Dunn's post-hoc test, $p<0.001$; Fig. 2C). Additional 5-HT depletion did not affect the decrease in locomotor activity induced by DSP-4 ($p>0.05$). 5-HT depletion alone had no locomotor effect ($p>0.05$).

In addition to the locomotor activity, we determined whether monoaminergic depletions could promote catalepsy using the bar test. 6-OHDA-lesion did not induce catalepsy compared to sham-lesioned animals (0.6 ± 0.3 s *vs* 1.6 ± 0.7 s descent latency; Mann–Whitney test, $p=0.44$). NAergic and/or 5-HTergic depletions did not trigger catalepsy in either 6-OHDA-lesioned rats (0.6 ± 0.2 s for 6-OHDA/DSP-4 group, 1.8 ± 0.6 s for 6-OHDA/pCPA group and 0.7 ± 0.3 s for 6-OHDA/pCPA/DSP-4 group, Kruskal–Wallis, $F=2.96$, $p=0.40$) or sham-lesioned rats (0.7 ± 0.3 s for DSP-4 group, 0.9 ± 0.5 s for pCPA group and 1.2 ± 0.5 s for pCPA/DSP-4 group *vs* 1.6 ± 0.7 s for sham group, Kruskal–Wallis, $F=0.72$, $p=0.87$).

DA depletion is necessary, but not sufficient alone, to reduce the number of entries and time spent in open arms

Anxiety behavior was assessed using the elevated plus maze. DA depletion did not induce any anxiety behavior as the number of entries, as well as the time spent in open arms, did not change compared to sham-lesioned animals (Mann–Whitney test, $p>0.05$ for the two parameters, Figs. 3A and D). In 6-OHDA-lesioned rats, additional depletion of 5-HT or NA alone or combined dramatically decreased the number of entries and time spent in open arms (Figs. 3B and E, Kruskal–Wallis test, $F=18.15$, $p=0.0004$ for entries in open arms and $F=20.69$, $p=p=0.0004$ for time spent in open arms, followed by Dunn's post-hoc test, $p<0.05$ for 5-HT depletion and $p<0.01$ for NA depletion). In contrast, anxiety behavior was not affected by 5-HT or NA depletion alone or combined in sham-lesioned rats (Figs. 3C and F, Kruskal–Wallis test, $F=0.42$, $p=0.94$ for entries in open arms and $F=3.28$, $p=0.35$ for time spent in open arms).

Only combined depletion of the three monoamines decreased sucrose consumption and increased immobility time in the forced swim test

To evaluate mood behavioral disabilities we used two validated tests. The sucrose consumption is a widely used test for anhedonia and the forced swim test for depressive-like behavior.

Sucrose consumption

6-OHDA-lesioned rats did not show any change in sucrose consumption compared to sham-lesioned animals (Mann–Whitney test, $p>0.05$, Fig. 4A). Interestingly, when NA and 5-HT depletions were combined with DA depletion, a significant decrease in sucrose consumption was observed (Kruskal–Wallis test, $F=13.48$, $p=0.004$ followed by Dunn's post-hoc test, $p<0.01$, Fig. 4B). NA and 5-HT depletion did not induce any anhedonia behavior by themselves in 6-OHDA-lesioned animals (Dunn's post-hoc test, $p>0.05$, Fig. 4B). However, 5-HT depletion and NA depletion, alone or combined together did not affect anhedonia behavior as the sucrose consumption was not significantly different from that of sham-lesioned animals (Kruskal–Wallis test, $F=2.41$, $p=0.49$, Fig. 4C).

Forced swim test

6-OHDA depletion alone did not produce any change in "depressive-like" behavior compared to sham-lesioned animals (Mann–Whitney test, $p>0.05$, Fig. 5A). However, as for sucrose consumption, combining 5-HT and NA depletions with DA depletion induced a significant increase in immobility time (Kruskal–Wallis test, $F=15.30$, followed by Dunn's post-hoc test, $p=0.016$, Fig. 5B), whereas 5-HT depletion or NA depletion in 6-OHDA-lesioned animals did not induce by themselves any "depressive-like" behavior (Dunn's post-hoc test, $p>0.05$, Fig. 5B). In the same manner, pCPA and DSP-4 depletions alone or combined together in sham-lesioned animals did not induce "depressive-like" behavior ($p>0.05$, Fig. 5C).

Effects of monoamine depletions on the firing activity of STN neurons

We investigated whether NA, DA and 5-HT depletion alone or combined induced changes in the firing rate and patterns of STN neurons.

As previously reported (Belujon et al., 2007; Ni et al., 2001), 6-OHDA-lesion did not change the spontaneous firing rate of STN neurons compared to the sham-lesioned animals (Student *t-test*, $p=0.12$,

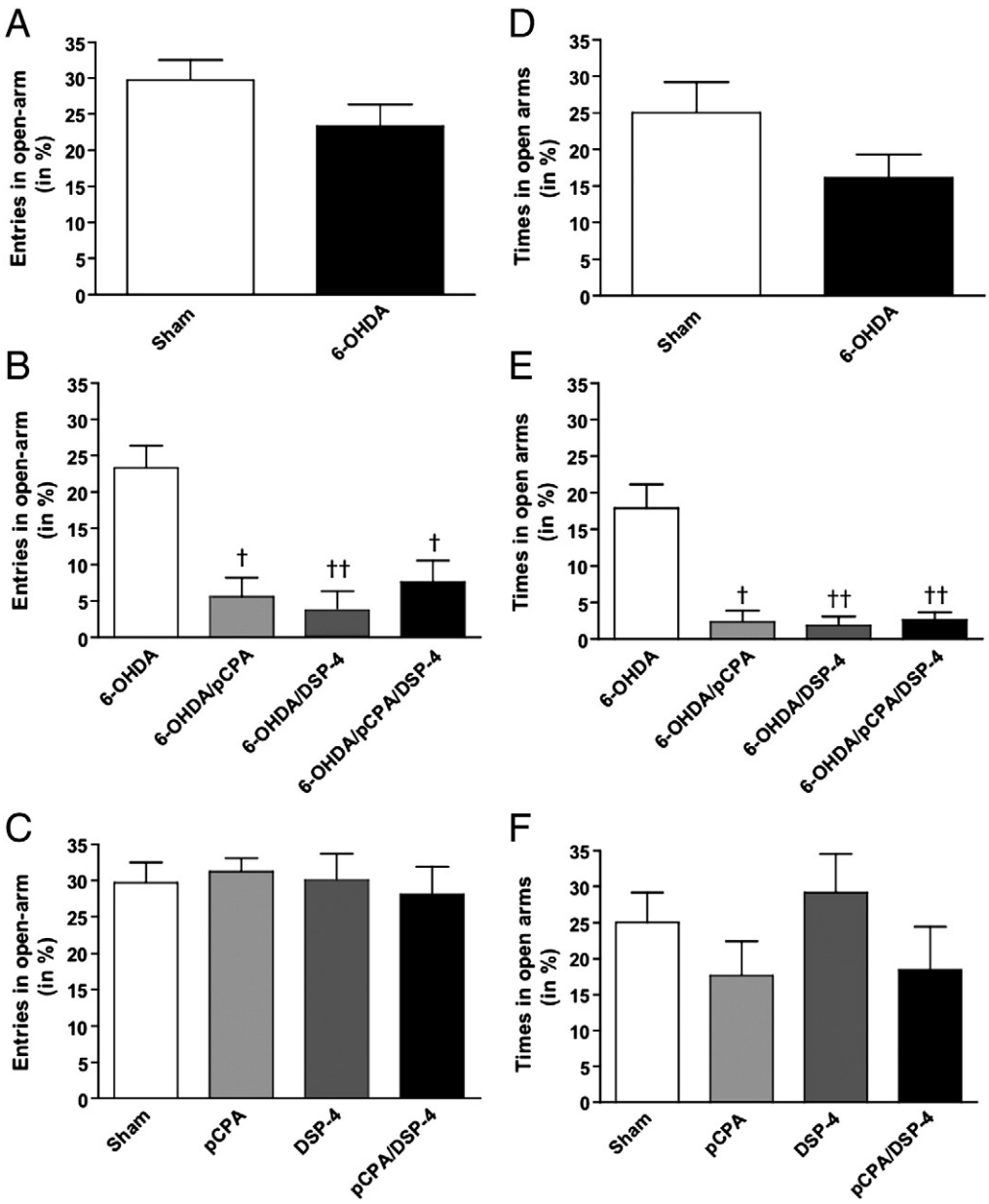

Fig. 3. DA depletion is necessary to induce anxiety behavior but only when it is combined with NA depletion and/or that of 5-HT. Histograms showing the percentage of entries into the open arms of elevated plus maze relative to the total entries into the four arms (A–C). D–F: time spent in the open arms is in percentage of total time spent in the four arms. Values are the mean ± SEM in sham and 6-OHDA rats (A); in 6-OHDA-lesioned groups (B) and in sham-lesioned groups (C). †: $p<0.05$, ††: $p<0.01$ in comparison with 6-OHDA group (Dunn's multiple comparison test). n = 9 rats in sham group, n = 21 rats in 6-OHDA group, n = 6 rats in pCPA group, n = 7 rats in DSP-4 group, n = 8 rats in pCPA/DSP-4 group, n = 6 rats in 6-OHDA/pCPA group, n = 7 rats in 6-OHDA/DSP-4 group and n = 8 rats in 6-OHDA/pCPA/DSP-4 group.

Fig. 6A). Interestingly, when 6-OHDA-lesion was combined with both NA and 5-HT depletions, the firing rate of STN neurons was markedly and significantly increased (one-way ANOVA, $F = 13.14$, $p<0.0001$, followed by Newman–Keuls multiple comparison post-hoc test, $p<0.001$; Fig. 6B). However, in 6-OHDA-lesioned rats, additional 5-HT or NA depletion singly did not induce any change in the firing rate (Newman–Keuls test, $p>0.05$; Fig. 6B).

In sham-lesioned animals, NA depletion alone or combined with 5-HT depletion significantly decreased the firing rate of STN neurons (Newman–Keuls test, $p<0.001$ after significant one way ANOVA, $F = 5.63$, $p = 0.001$, Fig. 6C). However, 5-HT depletion alone did not induce any effect by itself (Newman–Keuls post-hoc test, $p>0.05$; Fig. 6C).

Concerning the firing pattern, 6-OHDA-lesion significantly increased the proportion of STN neurons discharging with bursts compared to sham-lesioned animals (Chi^2 test, $X^2 = 15.7$, $df = 2$, $p<0.001$, Fig. 6D). The impact of additional depletions of NA and 5-HT differed between 6-OHDA-lesioned and sham-lesioned animals. In 6-OHDA-lesioned rats, the increase in the population of STN bursty neurons was not affected by 5-HT depletion alone or combined with NA depletion ($X^2 = 34.4$, $df = 6$, $p<0.001$ followed by $X^2 = 0.4$, $df = 2$ for 6-OHDA/pCPA group, $p>0.05$ and $X^2 = 0.3$, $df = 2$ for 6-OHDA/pCPA/DSP-4 group, $p>0.05$). However, NA depletion, when combined with 6-OHDA-lesion increased the proportion of irregular neurons ($X^2 = 19.1$, $df = 2$, $p<0.001$; Fig. 6E). Furthermore, NA depletion alone increased the proportion of bursty and irregular neurons compared to sham-lesioned rats ($X^2 = 7.9$, $df = 2$, $p<0.01$; Fig. 6F). 5-HT depletion, which did not induce any effect by itself ($X^2 = 4.4$, $df = 2$, $p>0.05$; Fig. 6F), when added to NA depletion did not affect the increase in the proportion of bursty and irregular neurons

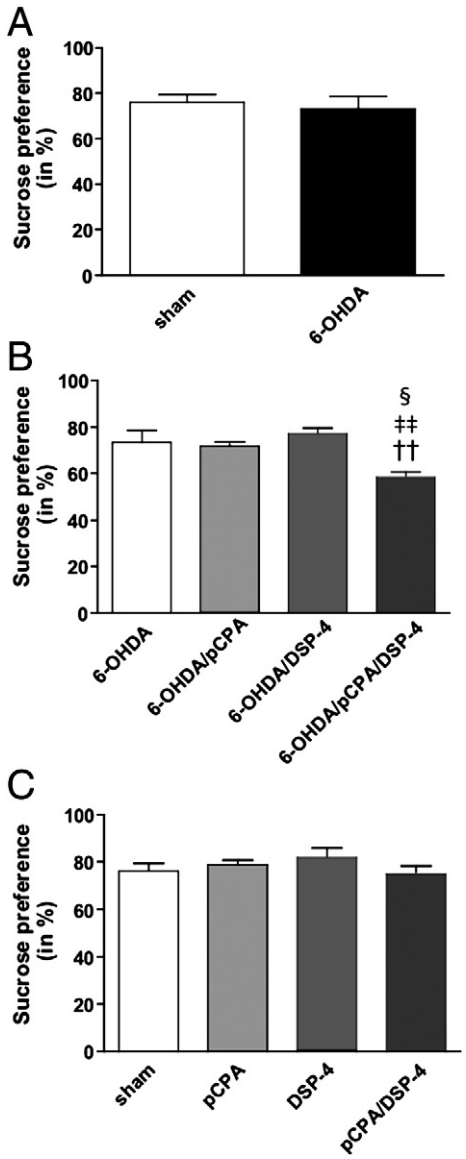

Fig. 4. Only combined depletion of the three monoamines induces anhedonia behavior. Histograms represent the percentage of sucrose consumption relative to the total consumption. Values are the mean ± SEM in sham and 6-OHDA rats (A); in 6-OHDA-lesioned groups (B) and (C) in sham-lesioned groups (B). ††: $p<0.01$ in comparison with 6-OHDA group; ‡‡: $p<0.01$ in comparison with 6-OHDA/pCPA group; §: $p<0.05$ in comparison with 6-OHDA/DSP-4 group (Dunn's multiple comparison test). n = 11 rats in sham group, n = 10 rats in 6-OHDA group, n = 8 rats in pCPA group, n = 9 rats in DSP-4 group, n = 6 rats in pCPA/DSP-4 group, n = 12 rats in 6-OHDA/pCPA group, n = 6 rats in 6-OHDA/DSP-4 group and n = 10 rats in 6-OHDA/pCPA/DSP-4 group.

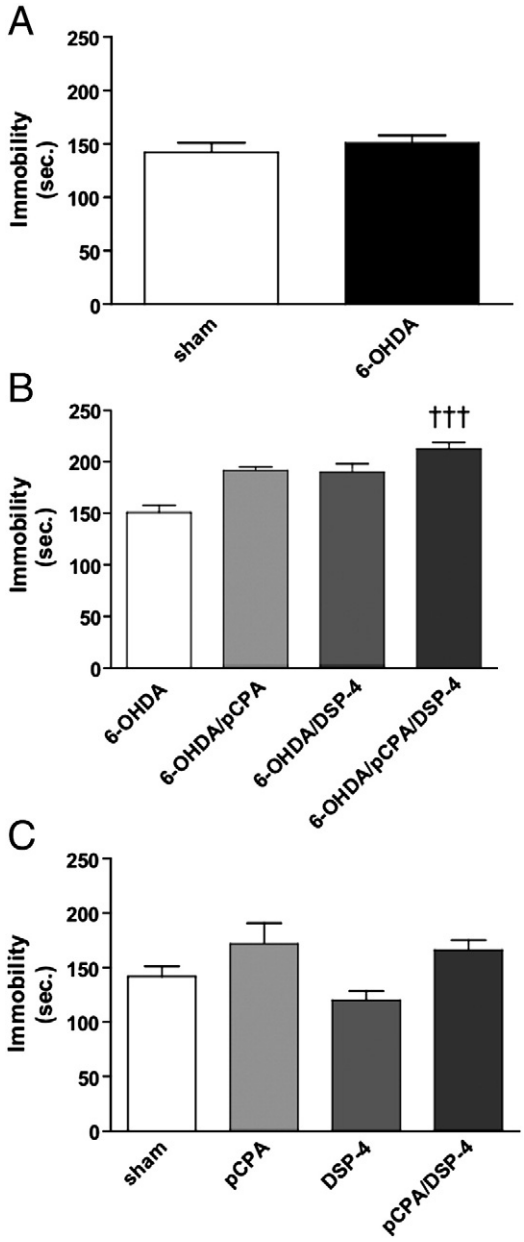

Fig. 5. Only lesion of all three monoaminergic system induces "depressive-like" behavior. Histograms show the duration of immobility during the five minutes test. Values are the mean ± SEM in sham and 6-OHDA rats (A); in 6-OHDA-lesioned groups (B) and in sham-lesioned groups (C). †††: $p<0.001$ in comparison with 6-OHDA group (Dunn's multiple comparison test). n = 10 rats in sham group, n = 8 rats in 6-OHDA group, n = 8 rats in pCPA group, n = 8 rats in DSP-4 group, n = 9 rats in pCPA/DSP-4 group, n = 6 rats in 6-OHDA/pCPA group, n = 6 rats in 6-OHDA/DSP-4 group and n = 8 rats in 6-OHDA/pCPA/DSP-4 group.

observed in DSP-4 group of animals ($X^2 = 11.49$, df = 6, $p > 0.05$ followed by $X^2 = 2$, df = 2, $p > 0.05$; Fig. 6F).

Discussion

The identification of specific neurochemical substrates that mediate the motor and non-motor disorders is important because it provides insight into strategies for developing more effective treatments of different symptoms of PD. The results of the present study provide evidence that (1) NA, like DA, is essential in the control of motor behavior and that its depletion results in hypokinesia, (2) the manifestation of anhedonia and "depressive-like" behavior is due to the combined depletion of the three monoamines DA, NA and 5-HT, (3) anxiety behavior is a consequence of DA depletion, only when combined with that of NA and/or 5-HT.

NA depletion, like DA depletion, reduced locomotor activity

One of the main finding showed that NA depletion induced severe motor deficits that resemble to those reported after 6-OHDA-lesion. The extent of NA depletion we obtained after DSP-4 treatment corresponds closely to that reported in advanced stages of Parkinson's disease (≥80%) (Chan-Palay and Asan, 1989; Gaspar et al., 1991; Jenner et al., 1983; Taquet et al., 1982). In contrast to 6-OHDA lesion (Deumens et al., 2002), the motor impairments consequent to NA depletion were not related to DA depletion. Indeed, as previously reported (Dailly et al., 2006; Fritschy and Grzanna, 1991), DSP-4 provoked a specific degeneration of NA terminals from the LC in mice and rats without affecting 5-HT or DA systems. Furthermore, the

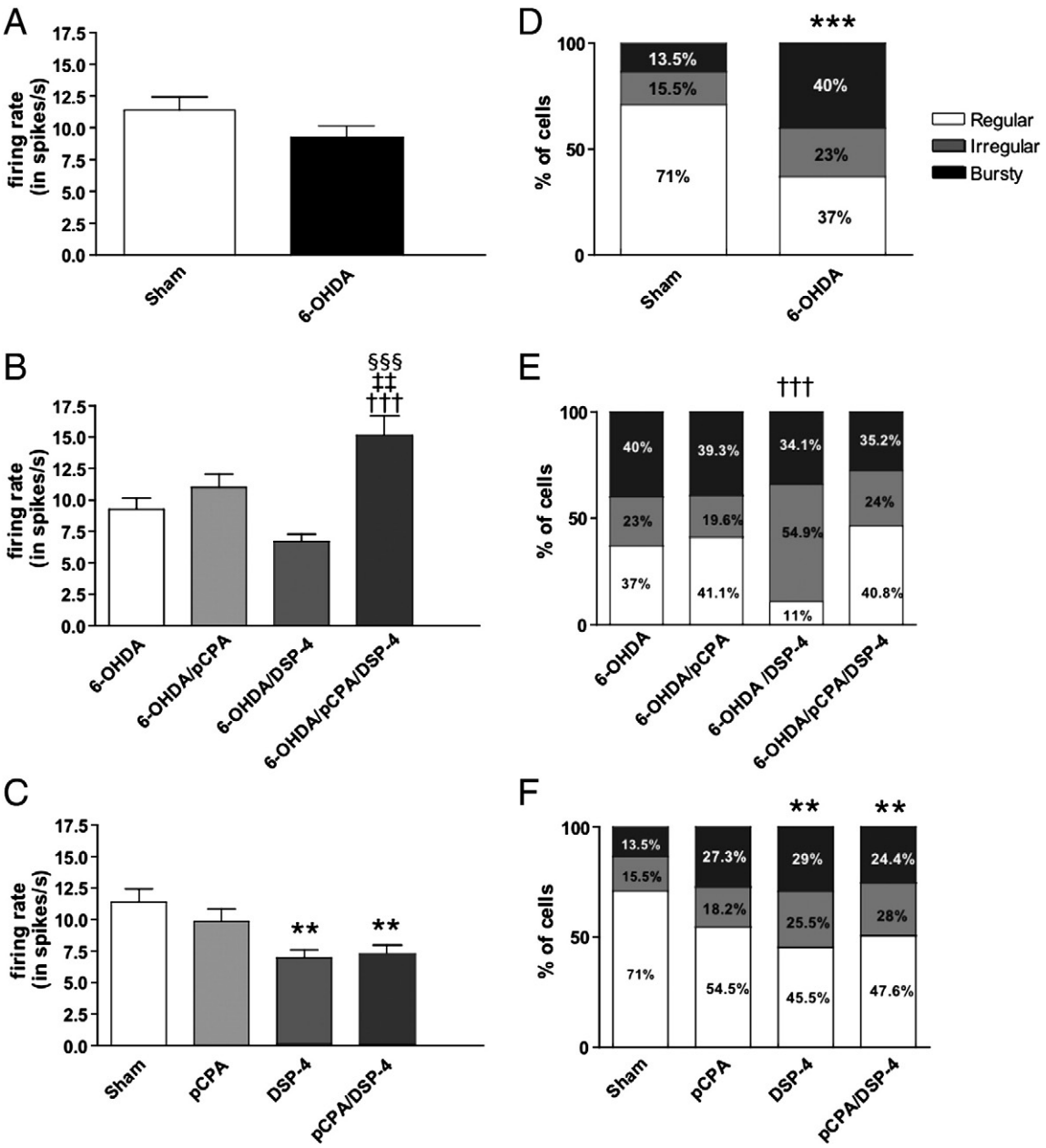

Fig. 6. DSP-4 lesion alone, or combined with that of 6-OHDA or pCPA, decreases the firing rate and increases the proportion of bursty and irregular neurons. Combined depletion of the three monoamines increases the proportion of bursty and irregular neurons as well as the firing rate of STN neurons. A–C: Firing rate histograms, values are the mean ± SEM. D–F: Firing pattern histograms showing the proportion (in percent) of STN cells discharging regularly, irregularly or with bursts. Values are the mean ± SEM in sham and 6-OHDA rats (A and D); in 6-OHDA-lesioned groups (B and E) and in sham-lesioned groups (C and F). **: $p<0.01$, ***: $p<0.001$, in comparison with sham. †††: $p<0.001$ in comparison with 6-OHDA group. ‡‡: $p<0.01$ in comparison with 6-OHDA/pCPA group; §§§: $p<0.001$ in comparison with 6-OHDA/DSP-4 group (Newmann–Keuls test for firing rate (A–C) and Chi^2 test for firing pattern(D–E)). n = 59 neurons in sham group (n = 12 rats), n = 63 neurons in 6-OHDA group (n = 10 rats), n = 68 neurons in pCPA group (n = 13 rats), n = 55 neurons in DSP-4 group (n = 5 rats), n = 67 neurons in pCPA/DSP-4 group (n = 6 rats), n = 56 neurons in 6-OHDA/pCPA group (n = 5 rats), n = 82 neurons in 6-OHDA/DSP-4 group (n = 5 rats) and n = 58 neurons in 6-OHDA/pCPA/DSP-4 group (n = 5 rats).

hypolocomotor activity observed after NA depletion is not due to a lack of motivation as we show that this depletion alone or combined with that of DA did not induce any "depressive-like" behavior and anhedonia (see below).

The motor deficits noticed in 6-OHDA-lesioned animals were not aggravated by the additional NA depletion, as evidenced by hypolocomotor activity and the lack of catalepsy. This fits with the study of Srinivasan and Schmidt (2003), which reported that denervation of LC NAergic terminals potentiated the 6-OHDA-induced partial DAergic neurodegeneration and akinesia only in rats treated with a D2 receptor antagonist, haloperidol. However, other studies carried out in MPTP-treated mice have reported that NA depletion by DSP-4 increased parkinsonism (Archer and Fredriksson, 2006). These discrepancies can be explained by the fact that, although MPTP effectively mimics the DAergic neuropathology of PD, NA loss produces more profound motor deficits than MPTP treatment in mice (Rommelfanger et al., 2007).

From a pathophysiological point of view, the lack of additional deficits in motor behavior produced by DA or NA depletion suggests a convergence of these monoamines on the same circuit involved in motor behavior. This hypothesis is compatible with our electrophysiological data showing that both DA and NA depletions induced similar increases in the number of STN neurons discharging with burst and irregular patterns compared to control animals. Additionally,

we cannot totally exclude that the decrease in STN neuronal firing rate may participate in alterations of motor behavior in the case of NA depletion alone. Nevertheless, it should be noted that the firing rate has been reported as an unstable parameter in animal models of parkinsonism (Belujon et al., 2007; Bergman et al., 1994; Hassani et al., 1996; Meissner et al., 2005; Ni et al., 2001; Tai et al., 2003). Indeed, the same studies have reported that the most relevant pathological parameter is the firing pattern. The reasons for the profound behavioral and electrophysiological alterations induced by NA depletion are unclear presently. It could result from the removal of a direct action of NA in the STN (Belujon et al., 2007) as this nucleus receives NAergic innervations from the LC and expresses alpha 1 and 2 receptors (Belujon et al., 2007; Delaville et al., 2011). Alternatively, it could be related to the loss of a NA control on the firing activity of SNc DA neurons and striatal DA release. Indeed, it has been shown that central NA facilitates nigro-striatal dopamine transmission *in vivo* (Lategan et al., 1992; Schank et al., 2006). These authors have shown that selective reduction in the level of NA decreased the concentration of endogenous DA in the striatum. NA depletion decreased the neuronal activity of SNc DA neurons, which mimicked the consequences of DA depletion (Belujon et al., 2007; Delaville et al., 2011).

Only combined depletion of the three monoamines induced anhedonia and "depressive-like" behavior

We found that 5-HT, NA or DA depletion alone did not induce anhedonia or "depressive-like" behavior, evaluated by the sucrose preference test and the forced swim test, respectively (Detke et al., 1995; Tadaiesky et al., 2008). These findings fit with a study using 5-HT or NA depletions with various neurotoxins that showed no difference in baseline immobility in the forced swim test (Lucki and O'Leary, 2004). The lack of effect of DA depletion alone on anhedonia and "depressive-like" behavior could be due to the DA depletion being only unilateral. We did not perform bilateral injection of 6-OHDA because it is known to induce severe aphagia and high death rate, which would bias the outcomes of the study. For this reason we have used the classical rat model of PD. This unilateral depletion model is commonly used and it is therefore necessary to characterize its behavioral consequences. In addition, as some behavioral symptoms, such as motor deficits, were found with unilateral DA depletion, this type of lesion could be considered to be sufficient to reveal other symptoms, such as anhedonia and "depressive-like" behavior if they are caused by DA depletion. Our results challenge those of Tadaiesky et al. (2008). However, the discrepancy could be related to the different time frame for the experiments in both studies. Tadaiesky et al. tested their animals one week after 6-OHDA injection, while our tests were carried out three to four weeks after DA depletion. Previous studies have reported that the stable stage of behavioral deficits, as well as the pathological activity in basal ganglia nuclei, appeared 2 to 3 weeks after the injection of 6-OHDA (Neve et al., 1982; Ni et al., 2001; Orieux et al., 2000).

Together, these observations suggest that NA and 5-HT are not necessary for the tonic regulation of baseline performance in these non-motor tasks, although it is possible that compensatory mechanisms from other neurotransmitters, such as DA, stabilize the behavior. Interestingly, only combined depletion of the three monoamines induced anhedonia and "depressive-like" behavior in rats as revealed by the sucrose preference and forced swim tests. It is unlikely that the decrease in sucrose consumption can be a consequence of olfactory or gustatory dysfunctions observed in PD (Ponsen et al., 2004) because the animals still had a preference for sucrose solution rather than water. Previous anatomical and electrophysiological studies have shown the existence of reciprocal and functional relationships between the three monoaminergic systems (Aston-Jones et al., 1991; Guiard et al., 2008). In addition, *in vivo* microdialysis studies reported that the improvement of "depressive-like" behavior induced by antidepressants, such as selective serotonin reuptake inhibitors, is accompanied by an increase in the level of all three monoamines, 5-HT, NA and DA (Damsa et al., 2004; Hajos-Korcsok et al., 2000). Consequently, the non-motor abnormalities reported in our study could result from the loss of the interaction of the three monoaminergic systems.

Supporting this possibility, we found that only the combination of all monoamine depletions enhanced the firing rate of STN neurons, in addition to producing burst activity and irregular pattern of discharge due to the loss of DA and/or NA systems. It is possible that this additional alteration in the STN neuronal activity participates in associative and limbic dysfunctions (Gubellini et al., 2009; Temel et al., 2005). Indeed, implication of the STN in non-motor disorders is argued for by a number of studies in patients reporting that STN high frequency stimulation (HFS), which improves motor disabilities (Limousin et al., 1995), may induce behavioral changes such as depression (Gubellini et al., 2009; Temel et al., 2005). Similar abnormalities were also observed in the 6-OHDA rat model of parkinsonism submitted to STN HFS (Temel et al., 2007). "Depressive-like" behavior in this animal model was paralleled by a decrease in the firing activity of 5-HT dorsal raphe neurons (Temel et al., 2007) and 5-HT release in several brain regions (Navailles et al., 2010).

DA depletion is necessary, but not sufficient alone, to induce anxiety behavior

A single depletion of DA, NA or 5-HT did not change the time spent in the open-arms or the number of entries into open-arms in the elevated plus maze, whereas NA and/or 5-HT depletions combined with that of DA induced anxiety behavior. The performance in the test is not affected by the motor deficiency because DA or NA depletion, both inducing motor deficits, did not induce anxiety behavior alone. Thus, our study is the first to show that DA depletion is necessary, but not sufficient alone to induce an anxious phenotype. Little is known about the interaction between the three monoamines in anxiety behavior. Taylor et al. (2009) reported anxiety behavior in a genetic deficient mice for vesicular monoamine transporter (VMAT2)-, a situation associated with the severe depletion of the three monoamines. This model is the equivalent of our group with a depletion of the three monoamines. However, it was not possible with their transgenic model to determine whether DA, NA or 5-HT was predominant in the observed effect. Although STN HFS has been shown to improve anxiety compared with the conventional and best medical treatment in a controlled clinical study (Witt et al., 2008), our electrophysiological results did not show any specific change in the firing activity of STN neurons related to anxiety behavior.

Clinical relevance

Our data offer an interesting echo to the hypothesis of Braak et al. (2003) that proposed a progressive caudo-rostral alteration of monoaminergic centers in the symptomatology of PD. In particular, NA neurons would be the first system altered and we show that NA depletion is as detrimental as DA lesion with regard to motor deficits and the pathological activity of STN neurons. Fundamentally, PD symptomatology does not result solely from lesion of DA neurons and we show that deleterious and non-motor symptoms emerge from the combined and cumulative loss of the other monoaminergic systems. Anxiety and depression frequently occur, and often coexist, in PD patients with a similar prevalence of 40–45% across studies (Menza et al., 1993; Walsh and Bennett, 2001). Clinically, anxiety can occur as a DA-dependent event and would therefore respond to DAergic treatments. Our results in rats show that anxiety could reflect at least the existence of two depletions including that of DA. Of note, it can also remain a constant underlying problem that is independent of DAergic state in non-parkinsonian patients (Richard et al., 1996). Depression

in PD could also bear witness to the cell loss in the NA (Chan-Palay and Asan, 1989) and 5-HT (Kish, 2003; Kish et al., 2008) systems in addition to that of DA system. Additional systematic analyses of all the monoaminergic systems are needed to confirm this hypothesis. Taken together, our data suggest that motor deficits are associated with a loss of DA and/or NA function and non-motor symptoms are a consequence of DA dysfunction concomitant with NA and/or 5-HT depletion.

In conclusion, the present study provides new insights into the respective roles of monoamines in the manifestation of parkinsonian-like motor and non-motor symptoms. We propose that PD should be modeled as a monoaminergic pathology that is the result of more than the loss of the DA system.

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