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RESEARCH PAPER

The acute and long-term L-DOPA effects are independent from changes in the activity of dorsal raphe serotonergic neurons in 6-OHDA lesioned rats

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BACKGROUND AND PURPOSE

L-DOPA is still the most efficacious pharmacological treatment for Parkinson's disease. However, in the majority of patients receiving long-term therapy with L-DOPA, its efficacy is compromised by motor complications, notably L-DOPA-induced dyskinesia. Evidence suggests that the serotonergic system is involved in the therapeutic and the side effects of L-DOPA. Here, we investigate if long-term L-DOPA treatment alters the activity of the dorsal raphe nucleus (DRN) and its responses to serotonergic drugs.

EXPERIMENTAL APPROACH

We measured the responses of serotonergic neurons to acute and chronic L-DOPA treatment using *in vivo* electrophysiological single unit-extracellular recordings in the 6-OHDA-lesion rat model of Parkinson's disease.

KEY RESULTS

The results showed that neither acute nor chronic L-DOPA administration (6 mg·kg^{-1} s.c.) altered the properties of serotonergic-like neurons. Furthermore, no correlation was found between the activity of these neurons and the magnitude of L-DOPA-induced dyskinesia. In dyskinetic rats, the inhibitory response induced by the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT (0.0625–16 μg·kg^{-1}, i.v.) was preserved. Nonetheless, L-DOPA impaired the ability of the serotonin reuptake inhibitor fluoxetine (0.125–8 mg·kg^{-1}, i.v) to inhibit DRN neuron firing rate in dyskinetic animals.

CONCLUSIONS AND IMPLICATIONS

Although serotonergic neurons are involved in the dopaminergic effects of L-DOPA, we provide evidence that the effect of L-DOPA is not related to changes of the activity of DRN neurons. Rather, L-DOPA might reduce the efficacy of drugs that normally enhance the extracellular levels of serotonin.

LINKED ARTICLES

This article is part of a themed section on Updating Neuropathology and Neuropharmacology of Monoaminergic Systems. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v173.13/issuetoc

Abbreviations

8-OH-DPAT, 8-hydroxy-2-(dipropylamino)tetralin; AIM, abnormal involuntary movements; DRN, dorsal raphe nucleus; L-DOPA, L-3,4-dihydroxyphenylalanine; LID, L-DOPA-induced dyskinesia; 6-OHDA, 6-hydroxydopamine; PD, Parkinson's disease; SSRI, selective serotonin reuptake inhibitor

DOI:10.1111/bph.13447

Tables of Links

TARGETS
GPCRs[a]
5-HT_{1A} receptors
Transporters[b]
SERT, SLC6A4

LIGANDS
8-OH DPAT, 8-hydroxy-2-(dipropylamino)tetralin
Amphetamine
L-DOPA
Fluoxetine

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

Introduction

Parkinson's disease (PD) is characterized by the degeneration of dopaminergic neurons in the substantia nigra *pars compacta* and subsequent striatal dopamine depletion. Pharmacological replacement with L-3,4-dihydroxyphenylalanine (L-DOPA) sets the basis of the standard treatment for PD. However, long-term administration of L-DOPA induces a range of abnormal involuntary movements (AIM) known as L-DOPA-induced dyskinesia (LID), which affects up to 40% of PD patients within 5 years of treatment (Manson *et al.*, 2012). The mechanisms underlying LID are still not well understood, but evidence suggests that the serotonergic system plays a crucial role both in the therapeutic and side effects of L-DOPA (see Navailles *et al.* (2013)).

The dorsal raphe nucleus (DRN) contains the main population of serotonergic neurons in the brain and provides extensive innervation to the basal ganglia nuclei (Di Matteo *et al.*, 2008). A growing amount of preclinical data indicates that serotonergic neurons mediate some of the behavioural effects of L-DOPA attributed to an enhancement of dopaminergic tone such as L-DOPA-induced rotating behaviour or LID (Hollister *et al.*, 1979; Carta *et al.*, 2007; Eskow *et al.*, 2009). These findings agree with neurochemical data reporting that serotonergic neurons are involved in the release of the L-DOPA-derived dopamine in the brain (Tanaka *et al.*, 1999; Navailles *et al.*, 2010). Acute administration of 5-HT_{1A} receptor agonists or selective serotonin reuptake inhibitors (SSRI) reduces the expression of LID (Carta *et al.*, 2007; Dupre *et al.*, 2007; Munoz *et al.*, 2008; Bishop *et al.*, 2012; Bezard *et al.*, 2013; Fidalgo *et al.*, 2015), dopamine release (Kannari *et al.*, 2001; Yamato *et al.*, 2001; Lindgren *et al.*, 2010; Navailles *et al.*, 2010) and neuronal activity in the DRN (Martin-Ruiz and Ugedo, 2001; Miguelez *et al.*, 2011b). Additionally, pharmacological lesion of the DRN ameliorates LID (Carta *et al.*, 2007; Eskow *et al.*, 2009), while transplantation of serotonergic neuron grafts exacerbates them (Carlsson *et al.*, 2007). The activity of serotonergic neurons thus appears to be a key component of the effects of L-DOPA.

The serotonergic system undergoes various changes as a consequence of dopamine depletion or to the chronic administration of L-DOPA (Miguelez *et al.*, 2011b; Miguelez *et al.*, 2014a). In case of LID, discriminative and anatomical changes in the serotonergic innervation or serotonergic receptors have been reported in the striatum of dyskinetic animals or patients (Zhang *et al.*, 2007 , 2008; Rylander *et al.*, 2010; Riahi *et al.*, 2011), but not in the DRN (Rylander *et al.*, 2010; Bishop *et al.*, 2012; Inden *et al.*, 2012). Nevertheless, prolonged treatment with L-DOPA reduces the number of serotonergic cells in the DRN leading to a decrease in serotonin content (Stansley and Yamamoto, 2014) and basal serotonin release (Navailles *et al.*, 2011) in various brain areas. In addition, chronic L-DOPA treatment lowered and strengthened the ability of L-DOPA to enhance dopamine release and inhibit serotonin release, respectively (Navailles *et al.*, 2011; Nevalainen *et al.*, 2011). Although acute L-DOPA does not alter the firing rate of serotonergic neurons (Miguelez *et al.*, 2013), these findings would be compatible with an altered electrophysiological responsiveness of serotonergic neurons consequent to its chronic administration.

In the present study, we sought to clarify if the expression of LID is associated with changes of the responsiveness of serotonergic neurons to L-DOPA. We investigated, using extracellular single-cell recordings *in vivo*, the electrophysiological characteristics of serotonergic neurons in the DRN of 6-hydroxydopamine (6-OHDA) lesioned rats, rendered dyskinetic by prolonged L-DOPA administration. The electrophysiological properties of serotonergic neurons were further studied with the 5-HT_{1A} receptor agonist 8-hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) and the SSRI fluoxetine.

Methods

Animals

All animal care and experimental protocols conformed to the European Economic Community (86–6091 EEC) and Spanish Law (RD 1201/2005) for the care and use of laboratory animals, and were approved by the Local Ethical Committee for Animal Research at the University of the Basque Country (approved protocols: 17-P07-02/2009, 17-P07-05/2009 and 185/2011). Experiments involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath & Lilley, 2015). All procedures were as humane as possible, and efforts were made to reduce the number of animals used. Experiments were performed with Sprague–Dawley rats (Harlan, Barcelona, Spain) weighing 220–225 g on arrival and housed under a 12:12 h light: dark cycle with food and water *ad libitum*. A total of 72 animals were used in this study.

Experimental design and groups

As illustrated in Figure 1A, at the beginning of the experiment, animals received 6-OHDA or its vehicle in the medial forebrain bundle. Two weeks after, all animals received d-amphetamine and were tested for rotational screening. Four weeks from the beginning of the experiment, animals were divided into four groups according to the type of lesion and chronic treatment received (saline or L-DOPA), and AIMs were evaluated for at least 3 weeks. Twenty-four hours after the last dose of L-DOPA or saline, electrophysiological experiments were performed in all animals, and after transcardial perfusion, brain tissue was processed for histological verifications. The groups included in this study are referred to as sham/saline (n = 18), sham/L-DOPA (n = 16), 6-OHDA/saline (n = 15) and 6-OHDA/L-DOPA (n = 23). Animals were assigned to groups following a randomized block design. Before the surgery, animals were labelled and assigned to either sham-lesioned or 6-OHDA-lesioned group using a table of random numbers to select the labelled animals. Then, animals within each group were randomly assigned to treatment conditions, saline or L-DOPA. The experimental procedure was unblinded due to the lack of experimenters involved.

6-OHDA lesion and rotational screening

The 6-OHDA lesion procedure was performed according to previous protocols (Miguelez *et al.*, 2011a; Aristieta *et al.*, 2012). To protect noradrenergic neurons, desipramine (25 mg·kg^{-1}, i.p.) was administered 30 min before the infusion of 6-OHDA. Then, rats were anaesthetized with isoflurane and mounted on a Kopf stereotaxic frame. Two injections of 2.5 and 2 μL of 6-OHDA (free-base, 3.5 μg μL^{-1}) were then given at two coordinates into the right medial forebrain bundle (relative to bregma, toothbar set at −2.4; AP: −4.4 mm; MD: +1.2 mm, DV: −7.8 mm; toothbar set at +3.4 mm; AP: −4. mm; MD: +0.8 mm, DV: −8 mm) at a rate of 1 μL min^{-1}. Sham animals were equally treated, but received the vehicle of 6-OHDA instead of the toxin.

Two weeks after 6-OHDA lesion, animals were given 2.5 mg·kg^{-1} of d-amphetamine to screen for the severity of the dopaminergic loss. Ipsilateral rotations were recorded for 90 min. Only rats rotating more than five full turns per minute, corresponding to a >90% striatal dopamine depletion, were selected for receiving chronic treatment with saline or L-DOPA.

Abnormal involuntary movement (AIM) rating

The development of L-DOPA-induced AIM was monitored and scored according to a rat dyskinesia scale described previously (Miguelez *et al.*, 2011a; Aristieta *et al.*, 2012). AIM were induced by daily s.c. injections of L-DOPA (6 mg kg^{-1} in combination with the peripheral decarboxylase inhibitor benserazide, 12 mg·kg^{-1}, s.c.) over 3 weeks. Afterwards, the dosage was changed to a maintenance dose (i.e. a twice-weekly administration regimen). On the testing days, rats were placed individually in transparent empty plastic cages prior to drug administration and observed for 1 min every 20 min for 3 h following L-DOPA injection. Three subtypes of dyskinetic movements (axial, limb and orolingual AIM) and asymmetric locomotive behaviour (locomotive AIM) were scored on a basic severity scale in which each item is rated on a scale from 0 to 4 depending on its duration. In addition, axial and limb AIM were rated on an amplitude scale from 0 to 4. Axial, limb and orolingual AIM were analysed and expressed as follows: sum AIM, sum of axial, limb and orolingual AIM scores (basic severity scale) for an entire testing session; Global AIM: for each AIM subtype, the basic severity score was multiplied by the amplitude score on each monitoring period, and all of these scores were summed for each testing session. In this study, 20 out of 23 6-OHDA-lesioned animals developed stable dyskinesia, while three of them remained non-dyskinetic. Because no difference was found between dyskinetic and non-dyskinetic animals, both groups were pooled together. Sham animals and 6-OHDA-lesioned animals treated with saline did not develop dyskinetic movements.

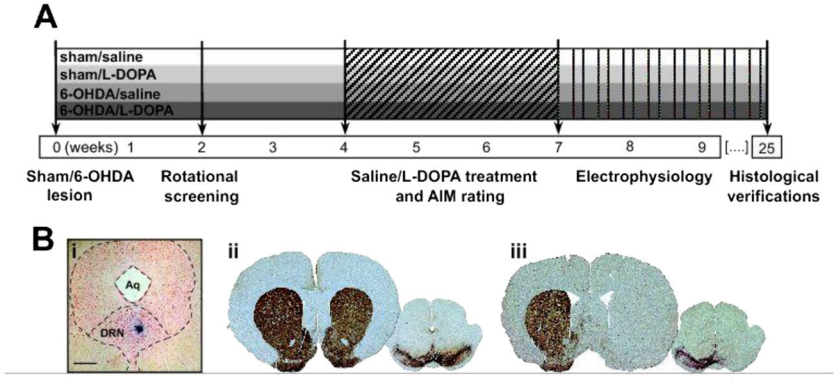

Figure 1

Experimental design and histological verifications. (A) At the beginning of the study, animals were injected with 6-OHDA or saline into the right medial forebrain bundle. Two weeks later, the severity of the 6-OHDA lesion was checked by amphetamine-induced rotations. To develop stable AIM, rats were injected daily with 6 mg·kg^{-1} L-DOPA (plus 12 mg·kg^{-1} benserazide) or saline for 21 days, and AIM were rated 2–3 days per week. At the end of the chronic treatment, L-DOPA was administered twice per week, and electrophysiological experiments were performed. At the end of the study, all animals were transcardially perfused, and the brains were removed, sectioned and stored for subsequent histological procedures. (B) Histological verification of the recording site in the DRN (Bi), dopaminergic denervation in the striatum and substantia nigra compacta from a control (Bii) or 6-OHDA-lesioned rat (Biii). DRN, dorsal raphe nucleus; Aq, aqueduct. The number of animals included was 18, 16, 15 and 23 for sham/saline, sham/L-DOPA, 6-OHDA/saline, 6-OHDA/L-DOPA respectively. Scale bar 400 μm.

Extracellular single unit recordings of dorsal raphe neurons

Single-unit extracellular recordings of DRN neurons were performed as described previously (Miguelez *et al.*, 2011b; 2013). Animals were anaesthetized with chloral hydrate (400-mg·kg^{-1}, i.p.) and placed on a stereotaxic frame where the body temperature was maintained at ~37°C for the entire experiment. After ligature and division of the sagittal sinus, the recording electrode consisting of an Omegadot single-barrel glass micropipette (filled with a 2% solution of Pontamine Sky Blue in 0.5% sodium acetate) was lowered into the DRN (in millimetre relative to λ: AP: +1.0, ML: 0, DV: −4.5 to −6.0). Serotonergic neurons were identified by the following criteria: (1) wide-duration action potentials that occurred between 1 and 2 ms positive–negative spikes, (2) a regular rhythm and (3) a slow firing rate (0.5–3 Hz). Burst-firing DRN serotonergic neurons were identified as described in Hajos *et al.* (1995a), by criteria including fired-spike doublets or triplets with an intraburst time-interval <20 ms. Firing pattern, firing rate and the coefficient variation of pattern (ratio of SD to the mean interval value of an interspike time-interval histogram, as percent) were analysed offline using the computer software Spike2 (Cambridge, England). Only one cell was studied per animal when any drug was administered. At the end of each experiment, a Chicago Sky Blue 6B (Sigma-Aldrich, Spain) mark was deposited at the recording site by passing a 5 μA cathodic current for 10 min through the recording electrode (Figure 1B).

After electrophysiological recordings, animals were deeply anaesthetized and transcardially perfused with 4% ice-cold buffered paraformaldehyde prepared in 0.1 M phosphate buffer. Brains were removed and transferred to a 25% sucrose solution until they sank. The brains were cut in coronal 40 μm sections using a freezing microtome (HM 430, Microm, Germany). Slices containing the DRN were stained using Neutral Red (Miguelez *et al.*, 2011b) to verify the recording site. All cells included in the study were confirmed to belong to the DRN. Slices containing the striatum were processed for tyrosine hydroxylase (TH) immunostaining to qualitatively confirm the alterations of dopaminergic fibres expected after induction of the 6-OHDA lesions.

Pharmacological treatments

Electrophysiological recordings were performed in sham and 6-OHDA-lesioned animals chronically treated with L-DOPA or saline (6-OHDA/L-DOPA, 6-OHDA/saline, sham/L-DOPA and sham/saline groups). To match behavioural experiments, L-DOPA was used at a dose of 6 mg·kg^{-1} (plus 12 mg·kg^{-1} of benserazide, s.c.). Basal DRN firing rate and coefficient of variation were measured every 20 for 60 min in the four groups (at least 180 s of stable discharge activity) 24 h after the last dose of L-DOPA or saline and after an acute L-DOPA challenge. Sixty minutes after L-DOPA challenge, increasing doses of fluoxetine (0.125–16 mg kg^{-1}, i.v.) were tested. After complete inhibition of serotonergic neuron activity, the 5-HT$_{1A}$ antagonist WAY 100635 (50 μg·kg^{-1}, i.v.) was administered for recovery of cell firing. In another set of the four experimental groups, the inhibitory influence of the 5-HT$_{1A}$ agonist 8-OH-DPAT (0.0625–16 mg·kg^{-1}, i.v.) was assessed on DRN neuron activity 24 h after the last L-DOPA or saline administration and subsequently blocked by WAY 100635 (50 μg kg^{-1}, i.v.).

Immunohistochemistry for TH and dopaminergic loss verification

After inactivation of endogenous peroxidases [3% H_2O_2 and 10% methanol in potassium-PBS (KPBS) for 30 min], sections were pre-incubated for 1 h in 5% normal goat serum +0.25% Triton X-100 in KPBS. The sections were incubated for 36 h at 4°C with the primary antibody (rabbit anti-TH, Pel-Freez Biologicals, P40101-O) diluted at 1:1000. Subsequently, the sections were treated with a biotinylated goat antibody against rabbit IgG (BA 1000, Vector Laboratories, Burlingame, CA, USA) diluted at 1:200 for 2 h. The primary and secondary antibodies were diluted respectively in 5% or 2.5% normal goat serum +0.5% Triton X-100. Thereafter, sections were incubated with an avidin–biotin–peroxidase complex (ABC kit, PK-6100, Vector Laboratories, 1 h). Finally, the peroxidase reaction was developed with 3,3′-diaminobenzidine and 0.03% H_2O_2. Sections were rinsed, mounted onto gelatin-coated slides, dehydrated, cleared with xylene and coverslipped.

OD was measured in three sections containing the striatum. First, capture was performed using a digital camera, and mean OD was visualized using image analysis NIH-produced software, ImageJ (http://rsb.info.nih.gov/nih.image/default.html). The whole striatum was delineated, and its OD was expressed as a percentage of that on the contralateral intact side after background subtraction (background was measured in the cortex on the side ipsilateral to the lesion). All the animals included in the study had a severe dopaminergic loss and showed >90% reduction in TH-fibre density in the striatum on the side ipsilateral to the lesion.

Statistical analysis of data

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.*, 2015). Experimental data were analysed using the computer programme GraphPad Prism (v. 5.01, GraphPad Software, Inc, La Jolla, CA, USA). The data were expressed as the mean values ± SEM, and the level of probability that constitutes the threshold for statistical significance was $P < 0.05$. To analyse the basic electrophysiological characteristics, the effect of L-DOPA on DRN cell activity or firing reversion induced by WAY 100635, a two-way ANOVA was used. *F* values provided correspond to the factor interaction (lesion*treatment). When ANOVA was significant, Bonferroni *post hoc* test was subsequently used. The proportion of cell type was analysed by χ^2. Linear regression analyses were used to assess correlations between electrophysiological and behavioural data by using Pearson's test. DRN basal electrophysiological parameters were calculated as the average of four to seven neurons per animal monitored 24 h after the last injection of the chronic L-DOPA treatment. DRN electrophysiological parameters after acute L-DOPA challenge were taken from 1 neuron per animal monitored for 60 min after L-DOPA injection in 6-OHDA/L-DOPA rats. Data compiled from dose–response curves of fluoxetine or 8-OH-DPAT were analysed for the best simple nonlinear fit to the three-parameter logistic equation (Parker and Waud, 1971). The following equation was used:

$$E = E_{max}[A]^n/(ED_{50}^n + [A]^n),$$

where [A] is the dose of the drug, E is the effect on the firing rate induced by A, E_{max} is the maximal effect, ED_{50} is the effective

dose for eliciting 50% of the Emax and *n* is the slope factor of the dose–response curve. Extra sum-of-squares *F*-test was used for statistical comparison of ED_{50} values among groups. In the dose–response curves, normalized data was used to avoid distorted results due to the variation in firing among the different cells and to better evaluate the drug effect.

Materials

L-DOPA methyl ester hydrochloride, 6-OHDA hydrochloride, benserazide hydrochloride, desipramine hydrochloride, chloral hydrate, d-amphetamine sulphate, WAY 100635 maleate salt, (±)-8-OH-DPAT hydrobromide were purchased from Sigma (Sigma-Aldrich, St Louis, MO, USA) and (±)-fluoxetine hydrochloride was purchased from Tocris (Tocris Bioscience, UK). Chloral hydrate, desipramine, L-DOPA, benserazide, amphetamine, WAY-100635 and (±)-8-OH-DPAT were prepared in 0.9% saline, (±)-fluoxetine in distilled water and 6-OHDA in deionized water containing 0.2 mg·mL^{-1} ascorbate. Except for chloral hydrate, drugs were prepared on the day of the experiment.

Results

Basal DRN neuronal activity and correlation with dyskinetic behaviour

Chronic L-DOPA administration (6 mg·kg^{-1} plus benserazide 12 mg·kg^{-1} for 21 days) led to a rapid onset of AIM in the majority of the 6-OHDA-lesioned rats. As detailed previously (Miguelez *et al.*, 2011a; Aristieta *et al.*, 2012), animals developed axial, limb, orolingual and locomotive AIM that increased during the first week of treatment and reached a plateau by the second week. All 6-OHDA-lesioned animals included in the study showed a marked loss of TH-positive fibre density in the ipsilateral striatum, corresponding to the ipsilateral side turning behaviour recorded after amphetamine (data not shown, Figure 1B).

A total of 596 serotonergic neurons were recorded in the DRN: 160 neurons from the sham/saline group ($n = 18$ animals), 122 neurons from the sham/L-DOPA group ($n = 16$), 133 neurons from the 6-OHDA/saline group ($n = 15$) and 181 neurons from the 6-OHDA/L-DOPA group ($n = 23$). DRN neurons were recorded 24 h after the last saline or L-DOPA injection. The basal firing rate and coefficient of variation were determined for single spiking and bursting cells (Table 1). In the first set of experiments, only single-spiking cells were recorded, but in the last group of experiments, both types of cells were registered and analysed. Single-spiking cells from both sets were pooled together because no differences were observed. In those experiments where both types of cells were recorded, the percentage of bursting cells was as follows: sham/saline, 46 ± 2%; sham/L-DOPA, 34 ± 3%: 6-OHDA/saline, 42 ± 4%; and 6-OHDA/L-DOPA, 41 ± 6% (obtained from 7, 9, 10 and 11 animals, respectively). The respective proportion of each cell type did not vary among the four groups ($\chi^2 = 3.14$, df = 3, n.s.). No difference was observed in the basal firing rate of single spiking ($F_{(1,432)} = 0.54$, n.s.) and bursting cells ($F_{(1,163)} = 0.01$, n.s.) in the DRN. Similarly, no differences were detected as for the coefficient of variation of DRN cells ($F_{(1,404)} = 3.10$, n.s. for single-spiking cells and $F_{(1,162)} = 0.47$, n.s. for bursting cells). In the bursting cells, mean spikes per burst was always two, regardless of the experimental group. These results confirmed that firing frequency and pattern remained unchanged among the groups.

No correlation was found between either the basal firing rate or pattern of DRN neurons and the AIM score in the 6-OHDA/L-DOPA group (statistical values are detailed in Table 2) (Figure 2E).

Effect of L-DOPA challenge on DRN neuronal activity and correlation with dyskinetic behaviour

In the 6-OHDA/L-DOPA group, AIM expression reached a peak 60 min after the administration of L-DOPA (Figure 2A). Therefore, the reactivity of DRN serotonergic neurons to a subsequent challenge of L-DOPA was investigated every 20 min for 1h after the acute administration of L-DOPA (6 plus 12 mg·kg^{-1} benserazide, s.c.) (Figure 2B).

Acute L-DOPA did not alter the firing frequency or pattern of any of the studied groups measured 60 min after the administration ($F_{(1,29)} = 0.03$ for frequency and $F_{(1,29)} = 0.02$ for pattern, n.s. for both) (Figure 2C–D). Although exogenous L-DOPA is transformed in dopamine into serotonergic neurons, L-DOPA failed to alter firing properties in any of the

Table 1

Basal electrophysiological properties of DRN neurons in control and 6-OHDA-lesioned rats that received chronic saline or L-DOPA treatment

		Groups			
Cell type	**Parameter**	**Sham/saline**	**Sham/L-DOPA**	**6-OHDA/saline**	**6-OHDA/L-DOPA**
Single spiking	Basal firing rate (Hz)	1.65 ± 0.07	1.76 ± 0.09	1.49 ± 0.09	1.50 ± 0.07
	Coefficient of variation (%)	28.90 ± 0.92	29.07 ± 0.97	29.35 ± 1.04	26.19 ± 0.94
Bursting	Basal firing rate (Hz)	1.43 ± 0.11	1.41 ± 0.13	1.47 ± 0.11	1.46 ± 0.12
	Coefficient of variation (%)	83.74 ± 7.77	86.67 ± 15.62	83.57 ± 5.60	105.2 ± 11.11
	% bursting cells	46 ± 2	34 ± 3	42 ± 4	41 ± 6

Rats were treated daily with saline or L-DOPA (6 mg·kg^{-1}in combination with benserazide 12 mg·kg^{-1}, s.c.) for at least 21 days. All neurons were recorded 24 h after the last administration of saline or L-DOPA. Each value represents the mean ± SEM basal firing rate or coefficient of variation. Each cell was recorded for 180 s. The number of single-spiking and bursting cells analysed were, respectively, 109 and 51 in sham/saline, 92 and 30 in sham/L-dopa, 99 and 34 in 6-OHDA/saline, 129 and 51 in 6-OHDA/L-DOPA. The number of animals included was 18, 16, 15 and 23 for sham/saline, sham/L-DOPA, 6-OHDA/saline, 6-OHDA/L-DOPA respectively.

Table 2

Summary of Pearson's *r* values for correlation between the severity of dyskinetic movements and the electrophysiological properties of DRN neurons in dyskinetic animals before and after L-DOPA administration.

Abnormal involuntary movements		Firing rate		Coefficient of variation	
		Basal	60 min after L-DOPA	Basal	60 min after L-DOPA
Sum AIM score	Ax + Li + Or	$r = 0.19$	$r = -0.09$	$r = 0.03$	$r = 0.25$
	Axial	$r = 0.32$	$r = 0.02$	$r = 0.03$	$r = 0.31$
	Limb	$r = 0.16$	$r = 0.14$	$r = 0.08$	$r = 0.39$
	Orolingual	$r = -0.15$	$r = -0.36$	$r = -0.01$	$r = 0.07$
Global AIM score	Ax + Li + Or	$r = 0.07$	$r = -0.27$	$r = -0.15$	$r = 0.05$
	Axial	$r = 0.32$	$r = -0.08$	$r = -0.14$	$r = 0.17$
	Limb	$r = 0.25$	$r = -0.09$	$r = -0.01$	$r = 0.23$
	Orolingual	$r = -0.38$	$r = -053$	$r = -0.14$	$r = -0.15$
Locomotive AIM		$r = 0.23$	$r = 0.24$	$r = 0.18$	$r = 0.20$

Regression analysis showed no correlation between DRN electrophysiological parameters (firing rate and coefficient of variation) and AIM scores, neither in basal conditions or 60 min after L-DOPA administration (6 mg·kg^{-1}, plus benserazide 12 mg·kg^{-1}, s.c., $n = 10$ animals). For regression analysis performed in basal conditions (24 h after the last dose), basal electrophysiological parameters (expressed as the average of four to seven neurons recorded from the same animal) were correlated with AIM scores monitored during the last testing session in the same animal. For regression analysis performed 60 min after L-DOPA administration, electrophysiological parameters obtained at 60 min after L-DOPA (one neuron per animal) were correlated with dyskinetic score monitored during the 60 min after L-DOPA administration. The sum AIM score is based on the frequency of the abnormal movement, whereas the global AIM score is based on the frequency and the amplitude of the abnormal movements. Ax + Li + Or refers to the AIM scores of axial, limb and orolingual dyskinesia. DRN, dorsal raphe nucleus; Ax, axial; Li, limb; Or, orolingual; AIM, abnormal involuntary movements.

groups for the following 60 min after the administration. For instance, the basal firing rate of DRN neurons before or 60 min after L-DOPA administration varied from 1.24 ± 0.07 to 1.32 ± 0.99 Hz in the 6-OHDA/saline group (n = 9) and from 1.56 ± 0.6 to 1.51 ± 1.03 Hz in the 6-OHDA/L-DOPA group (n = 11).

Additionally, no correlation was found between the DRN firing rate or pattern and AIM scores calculated 60 min after L-DOPA administration for the 6-OHDA/L-DOPA group (statistical values are detailed in Table 2) (Figure 2F).

Influence of fluoxetine and 8-OH-DPAT on DRN neuronal activity

The sensitivity of serotonergic neurons to the inhibitory effect of fluoxetine on DRN firing properties involves an increase in the extracellular levels of serotonin that, subsequently, stimulates 5-HT$_{1A}$ receptors. The inhibitory effect of fluoxetine should be affected if acute and/or chronic L-DOPA modifies the extracellular levels of serotonin.

The effect of fluoxetine was tested on DRN cells 60 min after an acute challenge of L-DOPA (6 mg·kg^{-1}). In agreement with previous work from our laboratory (Miguelez *et al.*, 2011b; Miguelez *et al.*, 2013), cumulative doses of fluoxetine (0.125–8 mg·kg^{-1}, i.v.) dose-dependently decreased DRN firing rate in all studied groups (Figure 3A–B). The dose–response curve of fluoxetine was shifted to the right in rats chronically treated with L-DOPA, so that the mean ED50 value in this group was significantly higher than that obtained in sham/saline, sham/L-DOPA or 6-OHDA/saline (ED50: 0.75 ± 0.16 mg·kg^{-1}, n = 5 for sham/saline; ED50: 0.63 ± 0.14 mg·kg^{-1}, n = 6 for sham/L-DOPA; ED50: 0.51 ± 0.09 mg·kg^{-1}, n = 5 for 6-OHDA/saline; ED50: 1.67 ± 0.64 mg·kg^{-1} n = 7 for 6-OHDA/L-DOPA, respectively, $P < 0.05$) (Figure 3C). This indicates that in the 6-OHDA/L-DOPA group, higher doses of fluoxetine were required to obtain the same inhibitory effect compared with the other groups. This inhibitory effect was reversed by a final injection of the 5-HT$_{1A}$ antagonist, WAY 100635 (50 µg·kg^{-1}, i.v.) (sham/saline: 91 ± 21%, n = 4; sham/L-DOPA: 124 ± 21%, n = 5; 6-OHDA/saline: 138 ± 48%, n = 4; 6-OHDA/L-DOPA: 43 ± 10, n = 5). Reversal was complete in all but the 6-OHDA/L-DOPA group ($F_{(1,14)}$ = 5.79, $P < 0.05$; Figure 3D).

To ascertain if the rightward shift in the dose–response curve of fluoxetine in 6-OHDA/L-DOPA was depended on changes in 5-HT$_{1A}$ autoreceptors, we tested the effect of the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT 24 h after the last injection of L-DOPA or saline. As described for naïve animals (Martin-Ruiz *et al.*, 2001), cumulative doses of 8-OH-DPAT (0.0625–16 µg·kg^{-1}, i.v.) induced a dose-dependent inhibition of DRN neuronal activity in all groups. Unlike fluoxetine, the inhibitory effect of 8-OH-DPAT was similar in all groups (ED50: 1.06 ± 0.12 mg·kg^{-1}, n = 9, for sham/saline; ED50: 1.02 ± 0.11 mg·kg^{-1}, n = 9, for sham/L-DOPA; ED50: 1.03 ± 0.57 mg·kg^{-1}, n = 8, for 6-OHDA/saline; ED50: 1.81 ± 0.79 mg·kg^{-1}, n = 11, for 6-OHDA/L-DOPA, n.s.) (Figure 4A). At the end of the experiment, the injection of WAY 100635 (50 µg·kg^{-1}, i.v.) totally reversed the basal firing frequency in all groups ($F_{(1,23)}$ = 0.08, n.s.; Figure 4B).

Discussion

Despite the central role of serotonergic neurons in the behavioural or biochemical responses to L-DOPA, our results

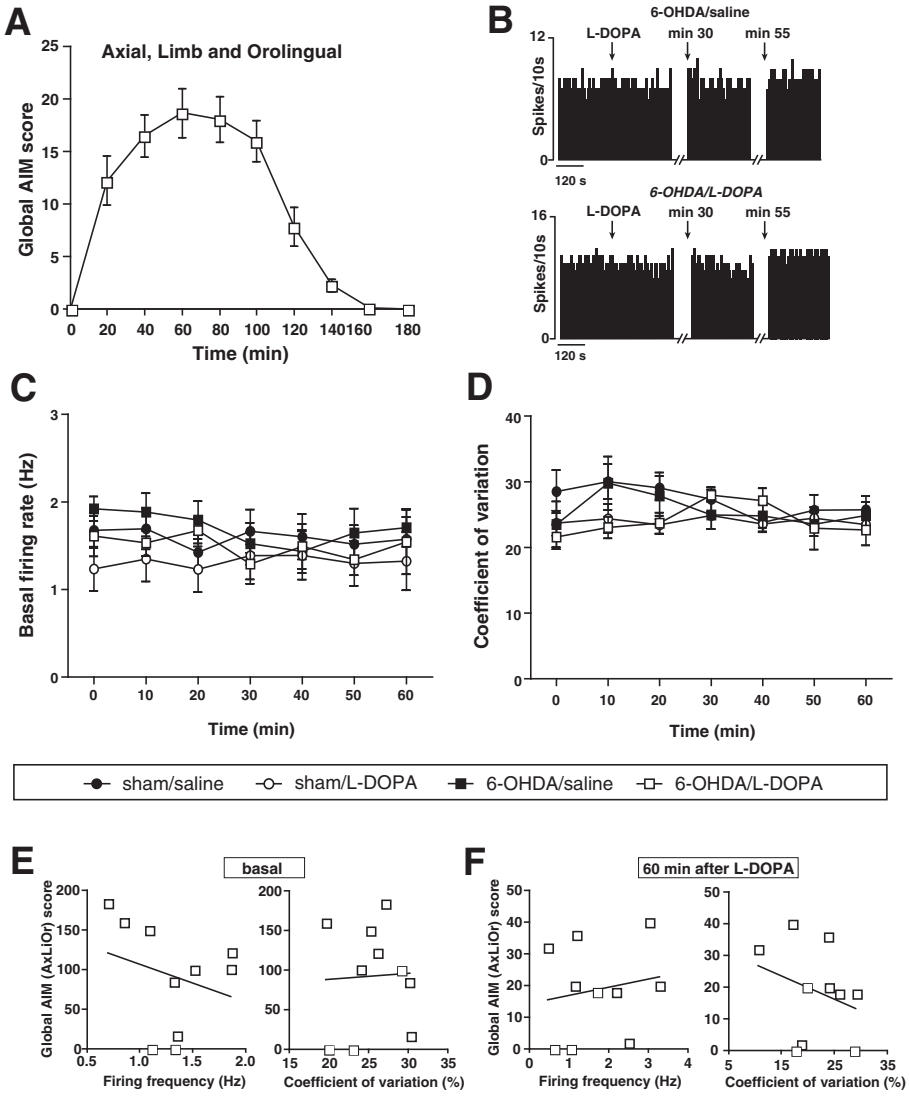

Figure 2

AIM expression and DRN responses to L-DOPA administration: correlation analysis. (A) Time course of global AIM scores (axial + limbic + orolingual) induced by L-DOPA on the last testing session of the chronic L-DOPA treatment (6 mg·kg^{-1} plus benserazide 12 mg·kg^{-1} for 21 days) in 6-OHDA rats (6-OHDA/L-DOPA group, $n = 23$). (B) Representative examples of DRN neuron firing frequency monitored for 60 min after L-DOPA administration in a 6-OHDA/saline rat and a 6-OHDA/L-DOPA rat. (C) Average basal firing rate monitored every 10 min for an hour after L-DOPA administration in the sham/saline ($n = 7$), sham/L-DOPA ($n = 7$), 6-OHDA/saline ($n = 9$) and 6-OHDA/L-DOPA ($n = 11$) groups. (D) Same as C for the coefficient of variation. (E) Regression analysis between Global AIM (AxLiOr) scores monitored on the last testing session of the chronic L-DOPA treatment, and basal electrophysiological parameters of DRN cells monitored 24 h after the last L-DOPA administration in 6-OHDA/L-DOPA rats ($n = 10$). (F) Regression analysis between Global AIM (AxLiOr) scores and electrophysiological parameters of DRN cells monitored on the 60th minute after L-DOPA administration in 6-OHDA/L-DOPA rats ($n = 10$). DRN basal electrophysiological parameters were calculated as the average of 4–7 neurons per animal monitored 24 h after the last injection of the chronic L-DOPA treatment. DRN electrophysiological parameters after acute L-DOPA challenge were taken from one neuron per animal monitored for 60 min after L-DOPA injection in 6-OHDA/L-DOPA rats. AIM, abnormal involuntary movements; Ax, axial; Li, limb; Or, orolingual.

indicate that these responses occur independently of changes in their electrical responsiveness to L-DOPA. The loss of efficacy of fluoxetine but preservation of 5-HT$_{1A}$ autoreceptors would suggest a better efficacy of 5-HT$_{1A}$ agonists in the treatment of L-DOPA-induced dyskinesia.

We observed that basal electrophysiological characteristics of DRN cells were identical in the four groups studied 24 h after the last L-DOPA administration, regardless the type of lesion or treatment followed. The effects of the selective loss of dopaminergic neurons on DRN cell activity has been a confounding factor, and different studies have revealed discrepant results with increased basal firing frequency (Zhang *et al.*, 2007; Kaya *et al.*, 2008; Wang *et al.*, 2009; Prinz *et al.*, 2013), decreased (Guiard *et al.*, 2008) or no change (Miguelez *et al.*, 2011b). Here, we found that the dopaminergic lesion did not alter the activity of serotonergic neurons, in agreement with the lack of changes observed on basal serotonin release in various brain areas (Navailles *et al.*, 2010; Navailles and De

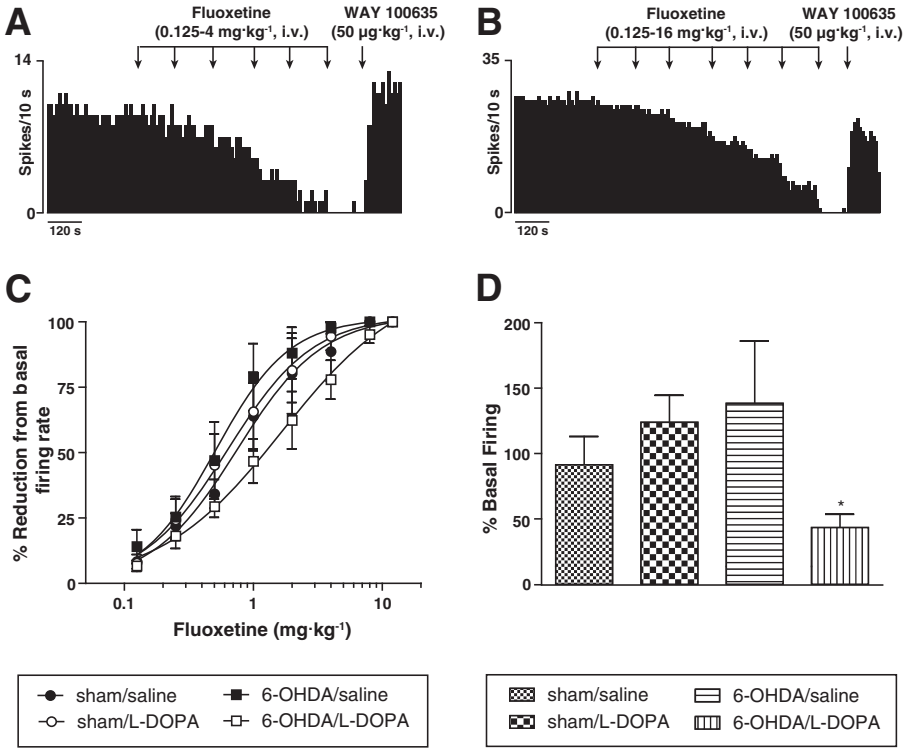

Figure 3

Effect of fluoxetine on DRN serotonergic neurons after L-DOPA administration. (A–B) The histograms summarise the inhibitory effect of fluoxetine (0.125–16 mg·kg^{-1}, i.v.) on the firing rate of serotonergic neurons in the DRN from 6-OHDA saline (A) and 6-OHDA L-DOPA groups (B) 60 min after L-DOPA (6 mg·kg^{-1} plus benserazide 12 mg·kg^{-1}, s.c.) administration. (C) Dose-effect curves illustrating the effect of fluoxetine on DRN cells in all studied groups. (D) Percentage of reversal from the basal firing rate produced by WAY 100635 (50 µg·kg^{-1}, i.v.) after complete inhibition induced by fluoxetine. *N* = 5, 6, 5 and 7 for sham/saline, sham/L-DOPA, 6-OHDA/saline, 6-OHDA/L-DOPA respectively. * $P < 0.05$ versus 6-OHDA/saline, Bonferroni *post hoc* test.

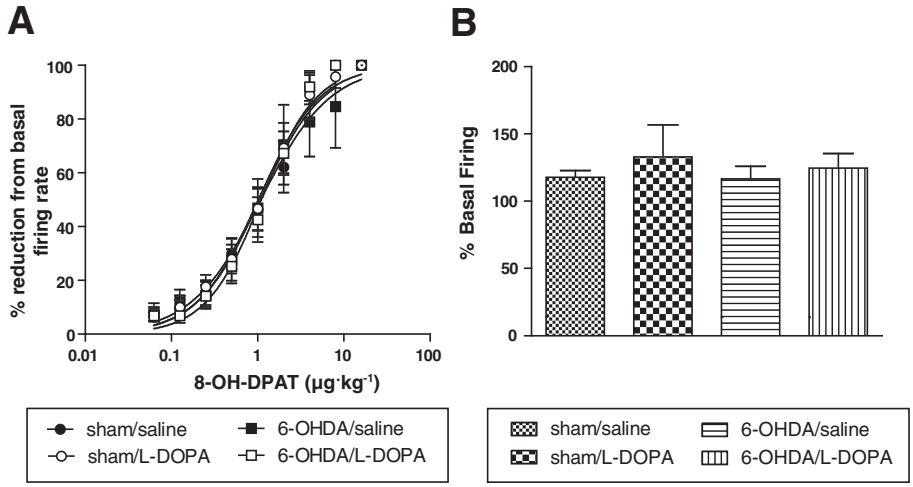

Figure 4

Effect of the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, on DRN serotonergic neuron activity. (A) Dose-effect curves depicting the inhibitory effect of cumulative doses of the 5-HT$_{1A}$ agonist, 8-OH-DPAT (0.0625–16 µg·kg^{-1}, i.v.), on DRN cell firing in sham/saline, sham/L-DOPA, 6-OHDA/saline and 6-OHDA/L-DOPA groups. (B) Subsequent administration of the 5-HT$_{1A}$ antagonist, WAY 100635 (50 µg·kg^{-1}, i.v.), totally restored the initial firing rate values. *N* = 9, 9, 8 and 11 for sham/saline, sham/L-DOPA, 6-OHDA/saline and 6-OHDA/L-DOPA groups respectively.

Deurwaerdere, 2012) and previous results from our laboratory (Miguelez *et al.*, 2011b). Regarding chronic treatment with L-DOPA, our data are in line with studies reporting that the number of serotonergic neurons (Rylander *et al.*, 2010; Inden *et al.*, 2012), the tissue levels of serotonin or dopamine in the DRN (Bishop *et al.*, 2012) or the serotonergic cell firing frequency (Prinz *et al.*, 2013) remain unchanged in dyskinetic animals.

The most striking result of our study is the complete lack of changes evoked by L-DOPA on the electrical activity of serotonergic-like neurons during the 60 min following drug injection in any of the analysed groups. Furthermore, the linear regression analysis to correlate the severity of dyskinesia triggered by L-DOPA with the electrophysiological features of serotonergic-like neurons failed to show any significant correlation. One explanation for these latter results is that the use of anaesthetics may blunt the activity of DRN cells, which can vary according to the sleep–awake or motor state of the animal. The use of chloral hydrate as the anaesthetic in our experiments is relevant because this anaesthetic does not alter the basal firing rate of DRN neurons (McCardle and Gartside, 2012). Moreover, the use of anaesthetics until now has been necessary to study potential changes of electrophysiological responsiveness of serotonergic neurons to pharmacological challenges (Hajos *et al.*, 1995b; Guiard *et al.*, 2008; Miguelez *et al.*, 2011b; Miguelez *et al.*, 2013). It allows one to address the reactivity of these cells and their electrophysiological properties using full cumulative dose–responses *in vivo*. These results are unexpected because they show that the inhibition of serotonin release following L-DOPA (Navailles *et al.*, 2010), magnified after a chronic L-DOPA treatment (Navailles *et al.*, 2011), was not related to changes of DRN serotonergic neuron activity.

In the last years, SSRIs have been assessed as possible antidyskinetic agents. Here, we tested the effect of fluoxetine on the activity of DRN neurons, 60 min after L-DOPA administration. We report that the dose–response and inhibitory curve induced by fluoxetine on the electrical activity of DRN neurons, not modified in 6-OHDA-lesioned or control animals (Miguelez *et al.*, 2011b; Miguelez *et al.*, 2013), was shifted to the right in dyskinetic animals. This result emphasizes a weaker effect of fluoxetine after chronic L-DOPA treatment and raises questions about the efficacy of SSRIs for treating L-DOPA-induced dyskinesia or depression associated with PD, because in those pathological situations, both drugs are chronically co-administered. Indeed, the efficacy of inhibitors of the serotonin transporter (SERT) relies on the extracellular levels of serotonin that indirectly stimulate somatic 5-HT_{1A} receptors. In this regard, modifications of 5-HT_{1A} autoreceptors in the DRN and basal ganglia in the different stages of Parkinsonism have been inconsistently reported. Parkinsonian patients on chronic L-DOPA treatment show decreased 5-HT_{1A} receptor binding in the raphe (Doder *et al.*, 2003), but fail to show major changes in several nuclei of the basal ganglia (Huot *et al.*, 2012). In L-DOPA-primed MPTP-lesioned macaques, both an increased expression and no modification have been found, among others, in the caudate nucleus (Huot *et al.*, 2012; Riahi *et al.*, 2012). In Parkinsonian rats treated with saline or L-DOPA, reduced expression of 5-HT_{1A} receptors has been reported in the striatum and prefrontal cortex (Mo *et al.*, 2010; Zhang *et al.*, 2014) together with a weaker inhibitory effect of 5-HT_{1A} receptor agonists on DRN neurons and cortical interneurons from Parkinsonian animals (Wang *et al.*, 2009; Zhang *et al.*, 2014). Because the inhibitory effect of SSRIs in the DRN has been attributed, in part, to the activation of 5-HT_{1A} receptors on cortical neurons (Hajos *et al.*, 1999), one could explain the weaker effect of fluoxetine as a consequence of the loss of cortical control on the serotonergic neurons. However, in our hands, fluoxetine produced the same inhibitory effect in 6-OHDA and sham animals, confirming that the dopaminergic lesion does not modify the electrical response to the drug administration. In view of our results, the dyskinetic state was not related to an altered control exerted by 5-HT_{1A} receptor agonists upon the electrical activity of DRN serotonergic-like neurons. Indeed, dose–effect curves performed under systemic administration of 8-OH-DPAT in control, Parkinsonian and dyskinetic animals revealed that the 5-HT_{1A} receptor agonist equally inhibited the firing frequency of DRN neurons. The changes proposed by other authors are, therefore, unlikely to interfere in our model. The loss of efficacy of fluoxetine in dyskinetic animals unrelated to altered 5-HT_{1A} receptors could also not be related to changes of SERT expression. Indeed, in contrast to the divergent results regarding the striatal expression of the SERT in humans or rats (Calon *et al.*, 2003; Kish *et al.*, 2008; Politis *et al.*, 2010; Rylander *et al.*, 2010; Nevalainen *et al.*, 2011; Prinz *et al.*, 2013), the data concerning SERT expression in the DRN show unaltered levels in dyskinetic animals or humans (Chinaglia *et al.*, 1993; Rylander, 2012). Considering that the SERT in the DRN is mainly involved in the inhibitory response to fluoxetine, these results discount the possibility that alterated expression of SERT contributed to the weaker efficacy of fluoxetine in L-DOPA-treated dyskinetic animals. We cannot rule out the possibility that the changes reported in dyskinetic animals regarding the expression of 5-HT_{2C} or 5-HT_{2A} receptors (Zhang *et al.*, 2007), known to bind fluoxetine (Palvimaki *et al.*, 1996; Koch *et al.*, 2002), contributed to the weaker effect of fluoxetine in dyskinetic animals. Nonetheless, the lower efficacy of fluoxetine parallels recent findings showing that high doses of the SSRI citalopram are needed to obtain a reduction of LID, presumably by indirectly activating 5-HT_{1A} autoreceptors (Fidalgo *et al.*, 2015). Rather, the loss of inhibitory responses to fluoxetine or other SSRIs could highlight the loss of endogenous serotonergic tone after chronic treatment with L-DOPA (Navailles *et al.*, 2011).

Our results are important in the context of the therapeutic use of SSRIs in the treatment of depression and dyskinesia in patients treated with L-DOPA. Regarding depression, it has been reported that L-DOPA worsens the antidepressant-like profile of fluoxetine in rats and that SSRIs display a weak efficacy in humans (Aarsland *et al.*, 2009; Skapinakis *et al.*, 2010; Miguelez *et al.*, 2013). The efficacy of these compounds relies on their inhibitory action on DRN neuron firing rate, and considering also that moderate loss of serotonergic innervation has been reported in Parkinsonian patients (Kish *et al.*, 2008), our data offer a good explanation for the lower efficacy of SSRIs against depression in patients treated with L-DOPA. The antidyskinetic profile is much more complicated to predict, because it involves the concomitant dopaminergic tone stimulated by L-DOPA from serotonergic terminals. The inhibition of serotonergic neurons by the stimulation of 5-HT_{1A} autoreceptors, would reduce L-DOPA-stimulated dopamine release from serotonergic terminals (Tanaka *et al.*, 1999; Navailles *et al.*, 2010), thereby limiting the occurrence of dyskinesia. Our finding that the 5-HT_{1A} receptor-dependent control of serotonergic neurons is still fully operational in dyskinetic animals agrees with the efficacy of 5-

HT_{1A} agonists in reducing both dopamine release and dyskinesia (Kannari *et al.*, 2001; Carta *et al.*, 2007; Iderberg *et al.*, 2015b), particularly when targeting 5-HT_{1A} autoreceptors (Iderberg *et al.*, 2015a). The SSRIs would be less efficacious than 5-HT_{1A} receptor agonists against dyskinesia due to their loss of efficacy to modulate serotonergic neurons. Clinical data show that the antidyskinetic effects of 5-HT_{1A} receptor agonism would be more robust compared with those of SSRIs (Mazzucchi *et al.*, 2014; Svenningsson *et al.*, 2015). Indeed, fluoxetine has been proposed to alleviate (Kuan *et al.*, 2008; Bishop *et al.*, 2012) or not (Dekundy *et al.*, 2007) dyskinesia while a recent clinical data report a modest effect with SSRI (Mazzucchi *et al.*, 2014). The extent to which SSRIs reduce LID via the decrease in the impulse-dependent/flow-dependent release of dopamine is unclear. Alternatively, one may consider the non-vesicular and SERT-dependent dopamine release triggered by L-DOPA (Miguelez *et al.*, 2014b). This phenomenon occurs at doses comparable with those used in our study particularly in the hippocampus, and it might be a general phenomenon at higher doses of L-DOPA (Navailles *et al.*, 2010). After long-term treatment with L-DOPA, it is possible that the proportion of the non-vesicular release of dopamine through the SERT increases with respect to the vesicular release. Additional data are warranted to further explore this possibility.

In this work, we conclude that the biochemical and behavioural effects of L-DOPA are independent from changes of electrical serotonergic neurons responsiveness. This implies that the changes of dopamine and serotonin release represent biochemical events at serotonergic axon terminals and dendrites. Nevertheless, the 5-HT_{1A} receptor-dependent control of serotonergic neurons is unaltered in dyskinetic rats, confirming that its stimulation may convey a decrease in the impulse-dependent release of L-DOPA-stimulated dopamine release and ultimately LID. The finding that fluoxetine loses its efficacy cautions the use of SSRI in the treatment of depression or dyskinesia in L-DOPA-treated Parkinsonian patients.

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Author contributions

C.M. performed the research, L.U. and C.M. designed the research study; C.M. analysed the data; C.M., L.U., S.N. and P. D.D. wrote the paper. All authors approved the final version of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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