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### ► To cite this version:

Demetra Ballardin, Leila Makrini-Maleville, Alexander Seper, Emmanuel Valjent, Heike Rebholz. 5-HT<sub>4</sub>R agonism reduces L-DOPA-induced dyskinesia via striatopallidal neurons in unilaterally 6-OHDA lesioned mice. *Neurobiology of Disease*, 2024, 198, pp.106559. 10.1016/j.nbd.2024.106559 . hal-04609896

**HAL Id: hal-04609896**

**<https://hal.science/hal-04609896>**

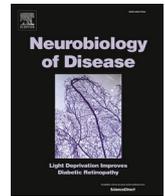
Submitted on 19 Jun 2024

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## 5-HT<sub>4</sub>R agonism reduces L-DOPA-induced dyskinesia via striatopallidal neurons in unilaterally 6-OHDA lesioned mice

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### ARTICLE INFO

#### Keywords:

Parkinson's disease  
Dopamine  
Serotonin  
5-HT<sub>4</sub> receptor  
L-Dopa  
Dyskinesia  
Striatum  
Akinesia  
Signaling

### ABSTRACT

Parkinson's disease is caused by a selective vulnerability and cell loss of dopaminergic neurons of the Substantia Nigra pars compacta and, consequently, striatal dopamine depletion. In Parkinson's disease therapy, dopamine loss is counteracted by the administration of L-DOPA, which is initially effective in ameliorating motor symptoms, but over time leads to a burdening side effect of uncontrollable jerky movements, termed L-DOPA-induced dyskinesia. To date, no efficient treatment for dyskinesia exists. The dopaminergic and serotonergic systems are intrinsically linked, and in recent years, a role has been established for pre-synaptic 5-HT<sub>1a/b</sub> receptors in L-DOPA-induced dyskinesia. We hypothesized that post-synaptic serotonin receptors may have a role and investigated the effect of modulation of 5-HT<sub>4</sub> receptor on motor symptoms and L-DOPA-induced dyskinesia in the unilateral 6-OHDA mouse model of Parkinson's disease. Administration of RS 67333, a 5-HT<sub>4</sub> receptor partial agonist, reduces L-DOPA-induced dyskinesia without altering L-DOPA's pro-kinetic effect. In the dorsolateral striatum, we find 5-HT<sub>4</sub> receptor to be predominantly expressed in D2R-containing medium spiny neurons, and its expression is altered by dopamine depletion and L-DOPA treatment. We further show that 5-HT<sub>4</sub> receptor agonism not only reduces L-DOPA-induced dyskinesia, but also enhances the activation of the cAMP-PKA pathway in striatopallidal medium spiny neurons. Taken together, our findings suggest that agonism of the post-synaptic serotonin receptor 5-HT<sub>4</sub> may be a novel therapeutic approach to reduce L-DOPA-induced dyskinesia.

### 1. Introduction

Parkinson's disease (PD), the second most common neurodegenerative disease, is characterized by the death of dopaminergic (DA) neurons of the Substantia Nigra pars compacta (SNpc). This loss results in reduced activation of striatal DA receptors, causing the cardinal PD motor symptoms: resting tremors, akinesia, bradykinesia and postural instability (Graybiel, 2000). To date, the DA precursor Levodopa (L-DOPA) is the most used and effective drug to alleviate these motor symptoms. While beneficial at the beginning, its ability to relieve motor impairments declines over time and chronic oral administration induces pulsatile stimulation of striatal DA receptors, resulting in a serious

adverse side effect termed L-DOPA-induced dyskinesia (LID) in up to 80% of patients and in 30% of patients after just 3 years (Ahlskog and Muentner, 2001; Fahn, 2004; Hely et al., 2005). The pathogenesis and mechanisms underlying LID are still poorly understood. It is thought that LID might occur due to the functional imbalance in the striatal medium spiny neurons (MSNs), with a simultaneous over-activation of D1R (striatonigral)- and over-inhibition of D2R (striatopallidal)-expressing cells, from now on termed D1R- and D2R-MSNs, respectively (Gerfen, 2000; Bateup et al., 2010; Fieblinger et al., 2014; Alcacer et al., 2017; F Hernández et al., 2017). Other PD drugs, such as ergot-derived and non-ergot-derived DA agonists like Cabergoline and Ropinirole are effective in reducing LID in PD models (Espa et al., 2023) and patients,

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<https://doi.org/10.1016/j.nbd.2024.106559>

Received 25 March 2024; Received in revised form 28 May 2024; Accepted 6 June 2024

Available online 7 June 2024

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but are associated with side effects and high risk of cardiac, pulmonary and peritoneal disorders (Choi and Horner, 2023). In 2017, the FDA approved Amantadine, a non-competitive NMDA antagonist, which is to date the only available treatment for LID. However, along with side effects such as confusion and hallucinations (Snow et al., 2000; Sharma et al., 2018; Hauser et al., 2021), Amantadine's efficacy is limited and its long-term use leads to tolerance and rebounds in dyskinesia (Thomas et al., 2004; Wolf et al., 2010).

Non-dopaminergic systems are also involved in LID pathogenesis because, as disease progresses and DA neurons are continuously lost, the DA outflow from the remaining DA neurons alone cannot explain the development of dyskinesia (Melamed et al., 1980; Mura et al., 1995). Serotonin (5-HT) nerve terminals densely innervate the striatum and 5-HT neurons possess the aromatic L-aminoacidic decarboxylase (AADC) enzyme, necessary for L-DOPA conversion to DA. Accordingly, L-DOPA was found in 5-HT striatal terminals, and lesion of the striatal 5-HT system reduced the L-DOPA-derived DA in the synaptic cleft by 80% in hemiparkinsonian rats (Arai et al., 1995; Tanaka et al., 1999). Thus, 5-HT neurons are capable to take up extracellular L-DOPA, convert it to DA and release it, however, these neurons lack autoregulatory feedback and are unable to buffer high extracellular DA levels, leading to excessive synaptic DA peaks which contribute to LID (Carta et al., 2007). This raised the possibility that removing striatal 5-HT afferents or blocking the 5-HT neurons activity could counteract LID. Indeed, after L-DOPA administration, higher extracellular DA levels were found in dyskinetic patients, and this increase could be normalized by HT1aR agonist, indicating that striatal 5-HT hyperinnervation likely contributes to dysregulated release of L-DOPA-derived DA (Politis et al., 2014).

Among the various postsynaptic 5-HT receptors, we propose the 5-HT<sub>4</sub> receptor (5-HT<sub>4</sub>R) as a new target to reduce LID. It is highly expressed in the limbic system, in structures including the basal ganglia (striatum and substantia nigra, especially in the SN lateralis), hypothalamus, hippocampus, amygdala and olfactory tubercle (Waeber et al., 1996; Bonaventure et al., 2000; Bockaert et al., 2008). Most cortical regions exhibit low expression (Waeber et al., 1996). It is furthermore expressed in the gastrointestinal system (esophagus, ileum, and colon), bladder, heart and adrenal glands. Interestingly, two independent clinical studies using 5-HT<sub>4</sub>R agonists Cisapride and Mosapride to increase gastric motility in PD patients, reported reduced incidence and severity of L-DOPA response fluctuations and improved motor functions (Djaldetti et al., 1995; Asai et al., 2005). While Cisapride was discontinued due to fatal arrhythmias, Mosapride and Prucalopride, another 5-HT<sub>4</sub>R agonist, are used in the clinic for gastroparesis in PD (Berardi, 2004; Bhidayasiri et al., 2022).

5-HT<sub>4</sub>R is a G $\alpha$ s/Golf coupled receptor that acts through the canonical cAMP/PKA pathway (Barthet et al., 2005) and non-canonically through Src-ERK 1/2 (Norum et al., 2003; Barthet et al., 2007), and has a strong potential to modulate MSN activity and plasticity. To assess the involvement of 5-HT<sub>4</sub>R in PD and LID, we unilaterally 6-OHDA lesioned mice in the dorsolateral (DL) striatum and chronically treated them with escalating doses of L-DOPA to induce the expression of abnormal involuntary movements (AIMs), an established readout for LID in rodents (Lundblad et al., 2005). We co-injected the 5-HT<sub>4</sub>R partial agonist RS 67333 to assess changes to dyskinetic and pro-kinetic effects of L-DOPA. Our findings show that 5-HT<sub>4</sub>R agonism reduces LID, while not affecting the anti-kinetic properties of L-DOPA, and point towards an involvement of D2R-MSNs mediating the effect. Our findings highlight the relevance of this post-synaptic receptor in LID alleviation.

## 2. Materials and methods

### 2.1. Animals

C57BL/6 male mice were crossed with Drd2a-GFP BAC transgenic mice (kindly provided by Dr. JA Girault, Institut du Fer à Moulin, Paris). Animals were maintained in a 12 h light/dark cycle, with *ad libitum*

access to food and water. They underwent 6-OHDA lesion at 4–6 months old, weighing 25–30 g, and were let to rest for three weeks after surgery before starting behavioral analysis.

All animals' procedures were in accordance with the Regional Ethics Committee (CEEA.34) of the University Paris Cité and approved by the Ministère de l'Enseignement Supérieur de la Recherche et de l'Innovation (APAFIS#29888-2020112511539929v7, approved on February 22, 2021).

### 2.2. 6-OHDA unilateral lesion

Unilateral 6-Hydroxydopamine (6-OHDA) injection into DL striatum was performed according to (He et al., 2001; Cortés et al., 2017). Briefly, mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (12 mg/kg) injected intraperitoneally (i.p.); the local anesthetic Bupivacaine was subcutaneously injected near the surgery site. Desipramine was also injected (25 mg/kg, i.p.) to minimize noradrenergic/serotonergic damage. 6-OHDA-HCl (4.4 mg/ml; Sigma-Aldrich) was dissolved in 0.2 g/l ascorbic acid solution and injected into left striatum at the following coordinates: anteroposterior (AP), +1.0 mm; lateral (L), -2.1 mm; dorsoventral (DV), -3.4 mm; and AP, +0.3 mm; L, -2.3 mm; DV, -3.4 mm (He et al., 2001; Cortés et al., 2017). Each injection of 2  $\mu$ l was performed with a Hamilton needle (33 gauge) connected to a syringe micropump (WPI) by a polyethylene catheter, at a rate of 0.5  $\mu$ l/min to minimize tissue damage. After the injection, the needle was left in place for an additional 4 mins before being slowly retracted. After surgery, animals were kept warm with a heated mat and, in the following days, injected with sterile 4% sucrose in 0.9% NaCl (10 ml/kg, s.c.) as well as given hydrogel pouches to avoid dehydration and high-fat chow to reduce weight loss. Mice were allowed to recover for 3 weeks before behavioral evaluation and drug treatment.

### 2.3. Behavioral experiments

L-DOPA (L-3,4-Dihydroxyphenylalanine methyl ester hydrochloride) and Benserazide-HCl were purchased from Sigma Aldrich. Starting three weeks after surgery, mice were divided in two experimental groups ( $n = 8$  per group), based on the outcome of the cylinder test (see below). Both groups were chronically injected i.p. with escalating doses of freshly prepared L-DOPA, combined with a fixed dose of Benserazide (10 mg/kg), to induce dyskinesia in the intrastriatal 6-OHDA-lesioned mice: subthreshold (5 mg/kg), low (10 mg/kg) and high (20 mg/kg) L-DOPA (Lundblad et al., 2004). The 5-HT<sub>4</sub>R partial agonist RS 67333 (Abcam) was dissolved in physiological saline solution and administered (1.5 mg/kg and 3 mg/kg, i.p.) on test days to one group. The selected doses of RS 67333 were shown to yield antidepressant and anxiolytic effects in rodent models of depression, the prime research focus of 5-HT<sub>4</sub>R action in the central nervous system (Licht et al., 2010; Pascual-Brazo et al., 2012; Mendez-David et al., 2014; Chen et al., 2020).

*Cylinder test of asymmetric limb use.* The lesion-induced motor deficit, the anti-kinetic effect of L-DOPA and the possible effect of RS 67333 were assessed using the cylinder test of forelimb paw placement 15 mins after drug administration. Briefly, mice were placed in a glass cylinder (10 cm wide  $\times$  14 cm high) and recorded for 4 mins. The number of supporting paw placements performed independently with the left and right paw was counted. The limb use asymmetry score was calculated by expressing the number of wall contacts performed with the forelimb contralateral to lesion (right paw) as a percentage of total wall contacts (Lundblad et al., 2002). The limb asymmetry score was used to divide animals into experimental groups, paying attention to balance animals with similar paw use reduction across groups and ensuring similar average in each group. Treatments were then randomly assigned to the groups.

*Open field test.* Locomotion was measured using the open field analysis in a new environment (50  $\times$  50 cm open-field arena) equipped with infrared photocells. Mice were tested 30 mins after drug injections. Data

were collected for 10 mins intervals. Open field data analysis was carried out using Videotrack v2.6 Automated Behavioral Analysis (ViewPoint) software, which records the large (LM) movements of each animal, defined as the movement detected when the animal's center of gravity moves over at least 10 cm/s between two acquired images. Clockwise (in our case, contralateral) and counter clockwise (ipsilateral) rotations, including asymmetric motor action as well as axial twisted rotations, were manually counted during 10 mins.

**Abnormal Involuntary Movements (AIMs) test.** Abnormal involuntary movements (AIMs) were evaluated starting 20 mins after L-DOPA/benserazide +/- RS 67333; every mouse was observed for 1 min every 20 mins for 2 h after drug injection. Abnormal movements were classified into four different subtypes as described in (Lundblad et al., 2005): contralateral rotations (asymmetric locomotor action), axial (contralateral rotated posture of the body), limb (jerky movements of the limb contralateral to the lesion), and orofacial (empty jaw movements, tongue protrusions). Each subtype was scored on a severity scale from 0 to 4 (0: absent; 1: occasional; 2: frequent; 3: continuous; 4: continuous and not interruptible by external stimuli) by an experimenter unaware of the treatment. The total AIMs score corresponded to the sum of individual scores for each AIM subtype. A composite score was obtained by the addition of scores for axial, limb, and orofacial AIMs (ALO score), and one only for rotations (LOC score).

#### 2.4. Immunohistochemistry (IHC)

Thirty minutes after the last i.p. injection of either L-DOPA or L-DOPA with RS 67333, animals were anesthetized with Euthasol (Pentobarbital, DECHRA Veterinary Products, 150 mg/kg, i.p.) and transcardially perfused with 40 ml of 4% paraformaldehyde (PFA) in PBS. After a night in PFA, brains were incubated in 30% sucrose/PBS, embedded in OCT embedding medium (ThermoScientific) and coronally sliced at a thickness of 30  $\mu$ m with cryostat (Leica). Labeling was performed as described (Castello et al., 2020). Briefly, after PBS washes to remove the cryoprotectant buffer, slices were blocked for 1 h in blocking solution (2% Goat serum in PBST), followed by overnight incubation at 4 °C with primary antibodies. The antibodies used were: anti-Tyrosine Hydroxylase (Rabbit, Santa Cruz Technologies sc-14,007), anti-GFP (Chicken, SySy 132,006), anti-pPKA substrate (Rabbit, Cell Signaling 9621 L), anti-pERK (Rabbit, Cell Signaling 9101S), anti-FosB (Rabbit, SantaCruz sc-48), and anti-Choline Acetyltransferase (ChAT, Goat, Millipore ab144P). Slices were then incubated with secondary antibodies (Goat  $\alpha$ -Rabbit 594, Life Technologies A11037; Camel  $\alpha$ -Chicken 488, SySy N0702-At488-S) for 1 h at room temperature (RT). Images were acquired with Zeiss Widefield Scanner or with Leica TCS SP8 Confocal microscope with 20 $\times$  objective. Intensity analysis and automated cell counting were performed on ImageJ. Anti-TH quantification (using Image J) in the DL striatum was used to determine lesion efficiency: (signal minus background signal of lesioned striatal section)/(signal minus background signal of unlesioned striatal section)\*100.

#### 2.5. RNA scope

Hemi-parkinsonian mice were divided in three groups: lesioned basal, lesioned + L-DOPA 10 mg/kg, and lesioned + L-DOPA 10 mg/kg + RS 67333 1.5 mg/kg ( $n = 4$  per group). RS 67333 was administered every other day. The correct development of dyskinesia was assessed with AIM test after 10 days of L-DOPA administration. After 2 weeks of treatment, mice were decapitated 30 mins after the last injection and the brain quickly extracted and immediately frozen in dry ice. Brains were coronally sliced at 12  $\mu$ m with cryostat (Leica) and directly mounted onto room temperature Superfrost Slides (Fisher Scientific); after 1 h at RT, slices were stored at -80 °C until processed. Analysis of *Htr4*, *Drd1*, *Drd2*, *Pvalb* and *Sst* mRNAs expression were performed as described (Puighermanal et al., 2020). Probes for *Htr4* (ACDBio; Mm-htr4-C1, Cat# 408241), *Drd2* (ACDBio; Mm-drd2-C3, Cat# 406501-C3), *Drd1*

(ACDBio; Mm-drd1-C2, Cat# 406491-C2), *Pvalb* (ACDBio; Mm-pvalb-C2, Cat# 421931-C2) and *Sst* (ACDBio; Mm-sst-C1, Cat# 404631-C2) were used with the RNAscope Fluorescent Multiplex Kit (ACDBio; Cat# 323110) according to manufacturer's recommendations. After incubation with fluorescent-labeled probes, slides were counterstained with DAPI. Slices were mounted with ProLong Diamond Antifade mounting medium (Thermo Fisher scientific P36961). Confocal microscopy was carried out at the Montpellier RIO imaging facility. Double- and triple-labeled images of the DL striatum of each hemisphere (2 images per hemisphere and 3–4 slices per mouse) were captured using sequential laser scanning confocal microscopy (Leica SP8) using a 40 $\times$  objective. Manual cell counting was performed with ImageJ software.

#### 2.6. RT-qPCR

Hemi-parkinsonian mice were divided in two groups: lesioned basal and lesioned chronically treated with L-DOPA 10 mg/kg ( $n = 10$  per group). The correct development of dyskinesia was assessed with AIM test after 10 days of L-DOPA administration. After 2 weeks of treatment with either saline or L-DOPA, mice were decapitated 30 mins after the last injection and the brain quickly extracted. DL striata were collected and immediately frozen in liquid nitrogen. RNA was extracted using TRIzol (INVITROGEN) and retrotranscribed to cDNA using the High-Capacity Reverse Transcriptase Kit with random primers (Applied Biosystems). Relative mRNA levels were quantified on a 7900HT Fast Real-Time PCR system (ThermoFisher) using Taqman gene expression assays for *htr4* (FAM Mm00434129\_m1) and *GAPDH* (FAM Mm99999915\_g1) as housekeeping gene. *Htr4* relative mRNA expression was determined with the  $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001).

#### 2.7. Haloperidol-induced catalepsy

Haloperidol-induced catalepsy was tested in unlesioned animals ( $n = 7-8$  per group). Haloperidol was freshly dissolved in 3% acetic acid in 0.9% NaCl, pH was adjusted to 5.2 with 1 M NaOH and the solution was sonicated for 10 mins. Mice were injected i.p. with 0.75 mg/kg haloperidol and tested 30, 60, 90 and 120 mins after administration. Haloperidol-induced catalepsy was assessed by positioning the mouse paws on a 4.5 cm high plastic bar of 3 mm diameter. The latency of catalepsy was measured, considering it to end when both forelimbs were removed by the bar or the mouse displayed exploratory behaviors, such as strong head movements. To assess the involvement of 5-HT<sub>4</sub>R, mice were co-injected with Haloperidol and either 5-HT<sub>4</sub>R partial agonist RS 67333 (1.5 mg/kg, dissolved in 0.9% NaCl) or antagonist GR 113808 (0.1 mg/kg, dissolved in 10% DMSO in 0.9% NaCl).

#### 2.8. Statistical analysis

Data are shown as mean  $\pm$  standard error of mean (SEM). Statistical differences were assessed by nonparametric unpaired Student's *t*-tests, Mann-Whitney test and one-way or two-way ANOVA with Bonferroni's post-hoc analysis. A *p*-value under 0.05 was considered statistically significant, with \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 and \*\*\*\**p* < 0.0001. A table with information on *p*- and *t*-values for individual experiments analyzed by *t*-test and Mann-Whitney as well as group interactions for ANOVA analysis, is supplied in supplemental information.

### 3. Results

#### 3.1. 5-HT<sub>4</sub>R expression is altered in response to DA depletion

We first wanted to determine whether 5-HT<sub>4</sub>R levels are affected by DA depletion and replenishment via L-DOPA in hemiparkinsonian mice. Lesion efficiency was assessed by quantifying the tyrosine hydroxylase (TH) signal in coronal slices (Fig. S1 A). Animals with lesions of <70%

reduction in TH signal in the dorsolateral striatum were excluded from analysis. Using RT-qPCR, we found that 5 weeks post-lesion (Fig. 1A), the relative *Htr4* mRNA expression was significantly higher in the lesioned compared to the unlesioned hemisphere (Fig. 1B). This increase was not significantly altered by the chronic administration of L-DOPA 10 mg/kg. Because of the dichotomy of signaling downstream of D1R or D2R-, with D1R-MSNs particularly linked to LID (Gerfen et al., 2002), we sought to assess the cell-specific expression profile of *Htr4* transcript and whether DA depletion has differential impact on one MSNs type. We employed single molecule fluorescent in situ hybridization (smFISH, RNAscope®) on striatal slices from hemiparkinsonian mice at 5 weeks post-lesion (Fig. 1A) and probed for *Drd1*, *Drd2* and *Htr4* transcripts in lesioned and unlesioned dorsolateral striata. *Htr4* appears to be broadly expressed in the brain (Suppl Fig. S2A), with particular abundance in the ventral striatum where it is expressed in both *Drd1*- and *Drd2*-expressing cells (Suppl Fig. S2A).

In the DL striatum the number of *Htr4*-expressing cells is significantly increased in DA-depleted as well as the L-DOPA treated striata compared to the intact ones, confirming the result obtained with the RT-qPCR (Fig. 1D). When normalized to the total number of either *Drd1*- and *Drd2*-positive cells, we found that *Htr4* transcripts are expressed in 11% of D1R- and 62% of D2R-MSNs cells in the intact dorsolateral striatum. Under DA depletion, 18% and 79% of D1R and D2R-MSNs, respectively, express *Htr4* (Fig. 1E). DA replenishment by chronic administration of L-DOPA 10 mg/kg does not significantly alter the expression in D1R-MSNs, while it increases it in D2R-MSNs to 91% in the lesioned hemisphere (Fig. 1E). Importantly, although low *Htr4* expression is detected in about 33% of somatostatin (*Sst*) and 32% of cholinergic (identified here by *Drd2*) interneurons (CINs), and in 51% of parvalbumin (*Pvalb*) cells in the dorsolateral striatum, *Htr4* mRNA levels within these populations are not altered in the DA-depleted compared to intact striatum (Suppl Fig. S2B-E). The quantification of *Htr4* expression in CINs was performed based on *Drd2* expression in combination with larger cell size (Matamales et al., 2009). Altogether, these data indicate that, following DA depletion, *Htr4* expression increases in D2R-MSNs and that L-DOPA administration further enhances its expression in D2R-MSNs.

### 3.2. RS 67333 reduces L-DOPA-induced dyskinesia

We next wanted to assess the effect of RS 67333, a 5-HT4R partial agonist, on LID expression. We chronically injected hemiparkinsonian mice with escalating doses of L-DOPA (5, 10 and 20 mg/kg) to induce dyskinesia and assessed abnormal involuntary movements with the AIMs test. RS 67333 was injected every 2–3 days, when behavioral tests were performed (Fig. 2A).

Chronic administration of L-DOPA 10 and 20 mg/kg leads to the development of abnormal involuntary movements, a well-established equivalent of LID (Cenci and Crossman, 2018), with the induction of axial dystonia of the trunk towards the contralateral side, limb spasms, orofacial spasms (ALO score) and contralateral rotations (asymmetric locomotive action or LOC score) (Fig. 2B-H).

The expression of L-DOPA-induced dyskinesia was significantly reduced in mice treated with L-DOPA 10 mg/kg with RS 67333 1.5 mg/kg (interaction by treatment group:  $F_{(1, 84)} = 27.36$ ;  $p < 0.0001$ ) and 3 mg/kg (interaction by treatment group:  $F_{(1, 84)} = 5.544$ ;  $p = 0.0209$ ), as well as for L-DOPA 20 mg/kg with RS 67333 1.5 mg/kg (interaction by treatment group:  $F_{(1, 84)} = 9.417$ ;  $p = 0.0029$ ) and 3 mg/kg (interaction by treatment group:  $F_{(1, 84)} = 20.87$ ;  $p < 0.0001$ ). Post-hoc analysis shows a significant difference for L-DOPA 10/RS 67333 1.5 mg/kg at 60 mins after administration (Fig. 2B). When the total AIM score is compounded over the 2 h test period, RS 67333 treatment has a significant effect on both L-DOPA treatment doses (10 and 20 mg/kg) (Fig. 2F).

We assessed the effect of RS 67333 specifically in the ALO and LOC components of the AIM score. Interestingly, RS 67333 significantly reduces the total and ALO score when co-administered with both L-DOPA 10 and 20 mg/kg (Fig. 2G) without affecting the cumulative LOC score

over 2 h (Fig. 2H). Together, our results show that activation of 5-HT4R reduces LID in DA-depleted mice treated by chronic L-DOPA administration.

### 3.3. L-DOPA anti-akinetic effect is not altered by RS 67333

Since it has been established that the LOC score is not part of the dyskinetic, but rather of the motor activation induced by L-DOPA (Fasano et al., 2010) and since our results showed a trend towards LOC reduction in the presence of RS 67333, it was important to interrogate whether modulation of 5-HT4R impacts on L-DOPA's pro-kinetic effect, by assessing the induction of contralateral paw use and locomotion in the open field environment. As previously reported, administration of L-DOPA induces a dose-dependent rescue of the contralateral paw use, with an overcompensation at high doses in the cylinder test (interaction by treatment group:  $F_{(11, 132)} = 33.52$ ;  $p < 0.0001$ ) (Fig. 3B) (Cortés et al., 2017). Administration of RS 67333, both at 1.5 and 3 mg/kg, does not alter the rescue of contralateral paw use induced by L-DOPA alone. Interestingly, RS 67333 alone induces a significant rescue of the contralateral paw use compared to lesioned basal in the cylinder test at 1.5 mg/kg ( $p = 0.0016$ ). This effect is not as strong as the one induced by L-DOPA (Fig. 3A).

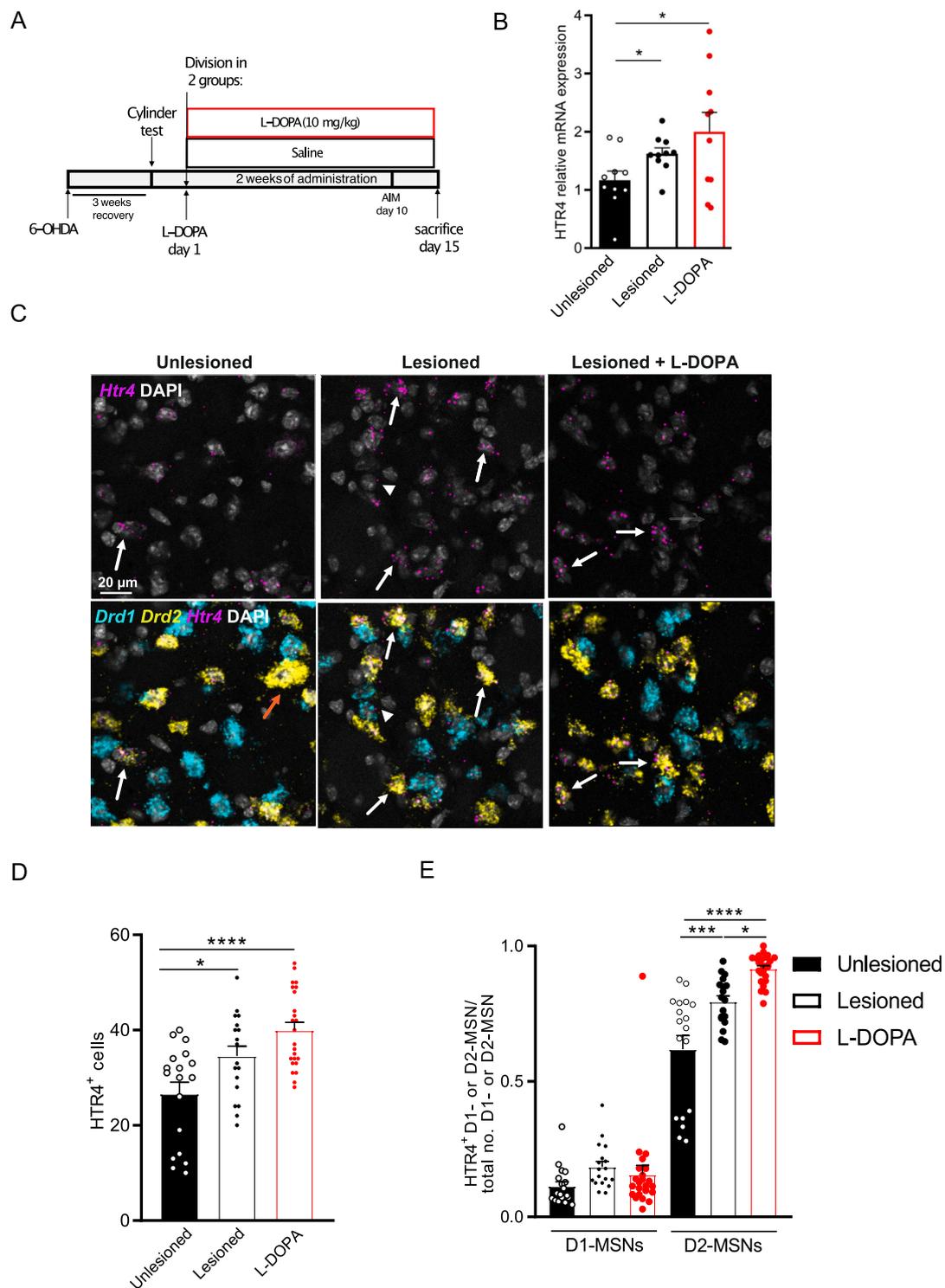
In the open field setup, lesioned mice turn ipsilaterally to the injection site, and L-DOPA administration induces a dose-dependent induction of contralateral rotations (interaction by treatment group:  $F_{(11, 132)} = 15.45$ ;  $p < 0.0001$ ) (Fig. 3D). Administration of RS 67333 (both 1.5 and 3 mg/kg) with L-DOPA does not significantly alter the number of contralateral rotations (Fig. 3B). Here, all contralateral rotations, including a certain percentage of axially distorted (dyskinetic) rotations were counted over 10 mins. In contrast, in Fig. 2G, H during the AIM scoring test on another test day, the axial dyskinesia and locomotor components were separately assessed. L-DOPA dose-dependently reduces the total distance traveled (interaction by treatment group:  $F_{(11, 132)} = 15.09$ ;  $p < 0.0001$ ) (Fig. 3F), which may seem in contrast to the pro-kinetic effect of L-DOPA, but can be explained by the induction of contralateral rotations. Again, co-administration of RS 67333 with L-DOPA had no significant effect on distance traveled. However, RS 67333 3 mg/kg alone reduced the total distance traveled ( $p = 0.0317$ , Fig. 3E). We believe that this finding may correspond to the trend towards a reduction in ipsilateral rotations (Fig. 3C) which may represent a tendency towards normalization of the laterality phenotype, similar to the rescue in paw use laterality seen in Fig. 3A. Taken together, these experiments confirm that the pro-kinetic effect of L-DOPA is not impacted by 5-HT4R agonism.

To exclude an overall effect of RS 67333 on locomotion, we also tested unlesioned mice in the open field environment. We found that RS 67333 did neither alter total distance traveled, nor for small and large movements (Suppl. Fig. S3), indicating that the effects observed in Fig. 3 may be specific to the DA depleted state.

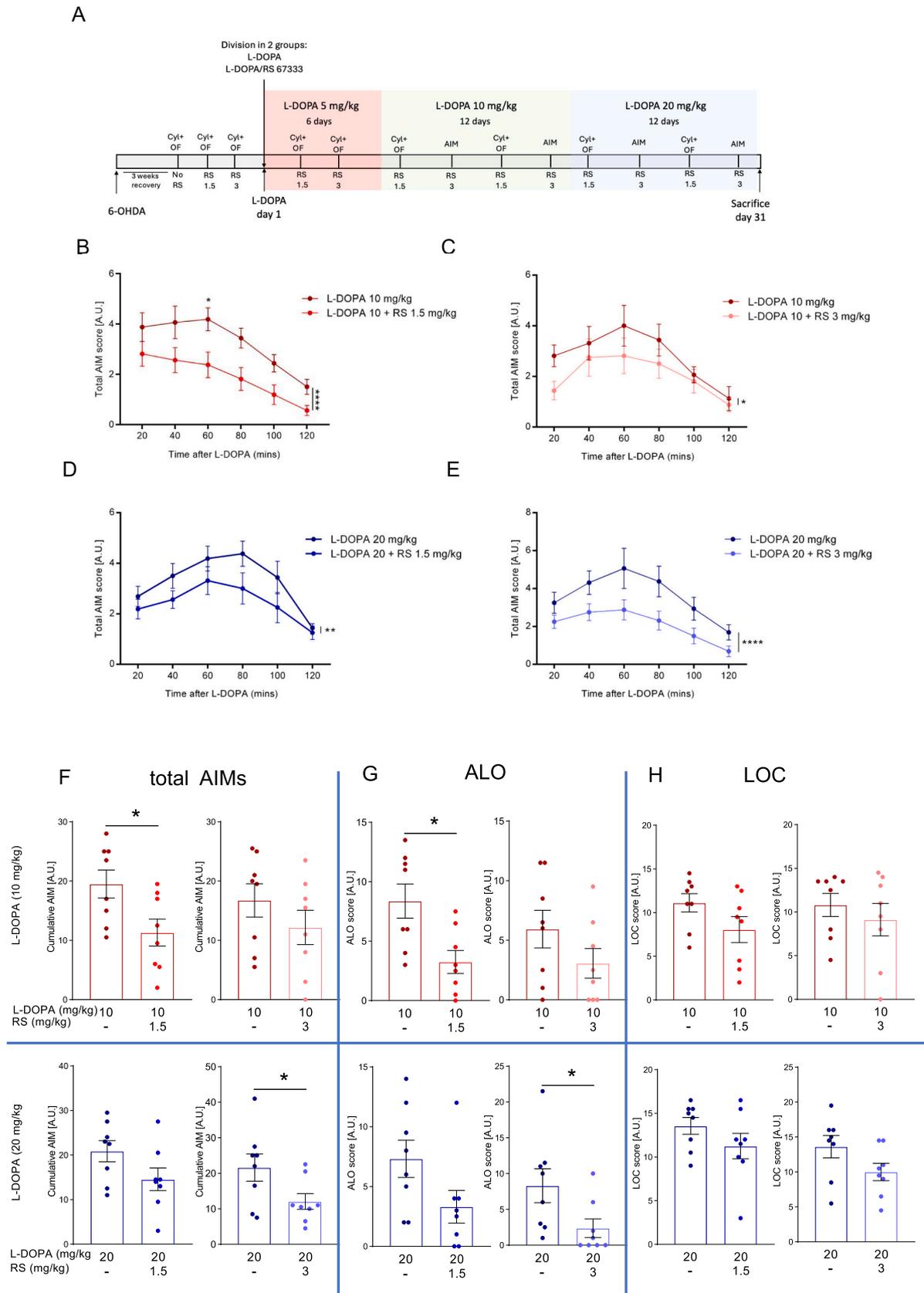
### 3.4. 5-HT4R agonism alters activation markers in D2R-MSNs

Dyskinesia has been linked to alterations in signaling events in D1R-MSNs, most likely due to the hypersensitivity of these cells. Transcription factors such as c-FOS and ΔFOSB are induced by acute and chronic L-DOPA administration (Gerfen et al., 2002; Santini et al., 2009; Alcacer et al., 2012). Similarly, PKA and ERK activation are specifically induced in D1R-MSNs (Gerfen et al., 2002; Santini et al., 2009; Alcacer et al., 2012). pERK in D1R-MSNs is considered a marker of LID, as its expression is strongly induced in D1R-MSNs after acute and subchronic L-DOPA administration (Pavón et al., 2006; Santini et al., 2007; Westin et al., 2007). After long-term chronic administration of L-DOPA over 7 weeks, pERK expression becomes gradually reduced in D1R-MSNs, and concomitantly induced in CINs in the dorsolateral striatum (Ding et al., 2011; Castello et al., 2017).

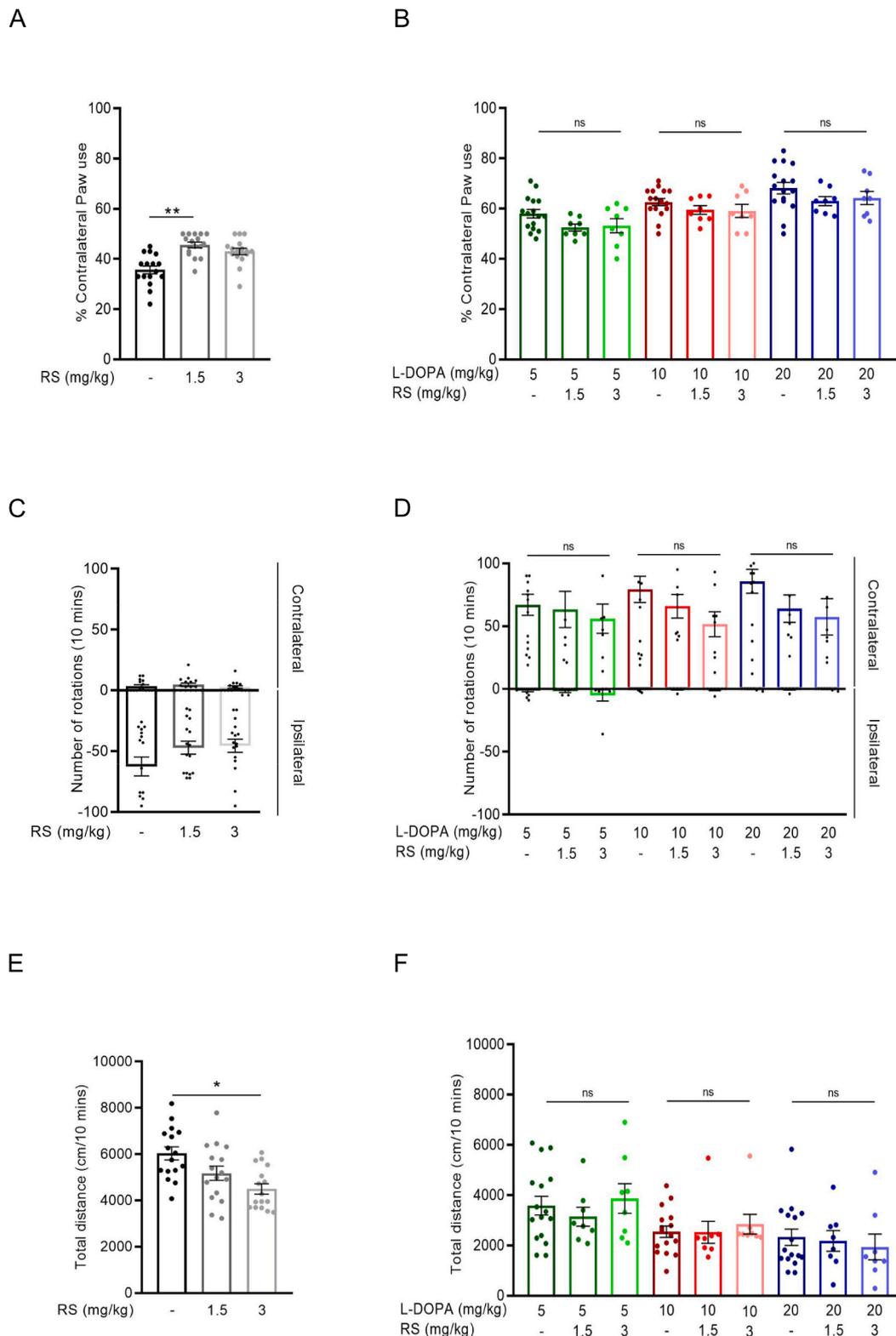
We therefore investigated the status of these signaling proteins in



**Fig. 1.** Expression of *Htr4* in DA-depleted and replenished states. Timeline of RT-qPCR and RNAscope experiments (A). Two separate cohorts of mice were treated for the two experiments. RT-qPCR of *Htr4* mRNA in unlesioned, lesioned and lesioned/L-DOPA-treated (10 mg/kg) dorsolateral striatum ( $n = 10$  mice/condition). Normalization was performed with GAPDH and results from  $\Delta\Delta$ CT quantification method were plotted (B). Representative images for smFISH staining (RNAscope) using probes for D1R (*Drd1*, cyan), D2R (*Drd2*, yellow) and 5-HT4R (*Htr4*, magenta), nuclei are stained with DAPI (white). Arrows indicate D2R-MSNs, arrowheads D1R-MSNs and orange arrow depicts CIN (C). Counts of *Htr4*-positive D1R- and D2R-MSNs in the unlesioned, lesioned and lesioned/L-DOPA (10 mg/kg) dorsolateral striatum (D). Percentage of *Htr4*-positive D1R- or D2R-MSNs normalized by total number of the respective MSN type (E).  $n = 4$  mice/condition, 12 images/mouse. Graphs represent mean values  $\pm$  SEM. Statistical analysis was performed using unpaired Student *t*-test (B) or One-way ANOVA with Bonferroni post-test (D, E). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** 5-HT4R partial agonist RS 67333 reduces L-DOPA-induced dyskinesia. Timeline of the experiment (A). Time-course of abnormal involuntary movements (AIM) after administration of L-DOPA 10 mg/kg ± RS 67333 1.5 mg/kg (B); L-DOPA 10 mg/kg ± RS 67333 3 mg/kg (C); L-DOPA 20 mg/kg ± RS 67333 1.5 mg/kg (D); L-DOPA 20 mg/kg ± RS 67333 3 mg/kg (E). Cumulative AIM score over the 2 h observation time (F). Cumulative axial, limb and orofacial dyskinesia (ALO) score (G). Cumulative locomotive (LOC) score (H) ( $n = 8$  mice/condition). Graphs represent mean values ± SEM. Statistical analysis was performed using Two-way ANOVA with Bonferroni post-test (B-E) or Mann Whitney test (F–H).

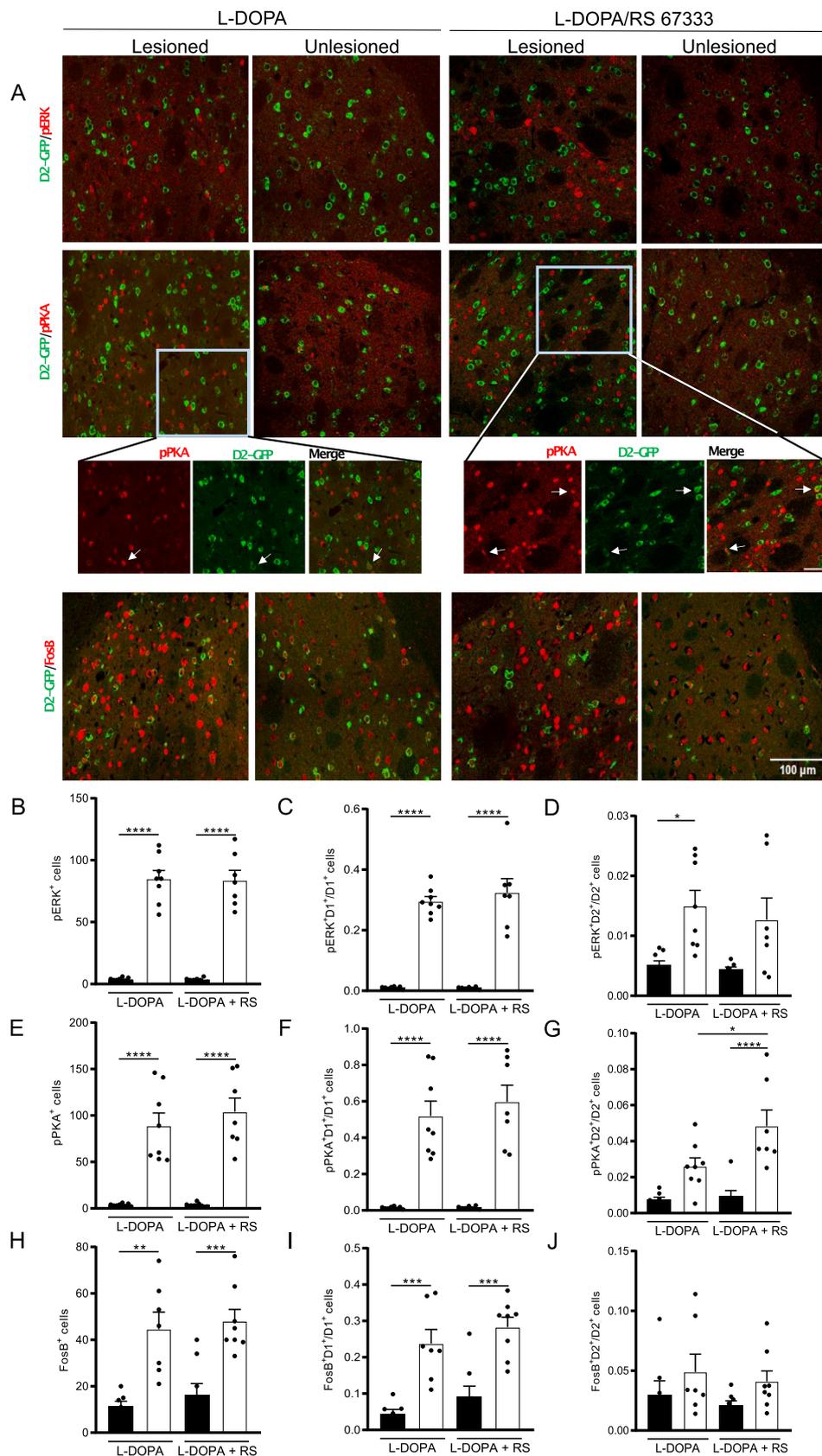


**Fig. 3.** 5-HT4R partial agonist RS 67333 does not alter L-DOPA’s pro-kinetic effect. Quantification of the effect of L-DOPA and RS 67333 in contralateral paw use in cylinder test (A, B), number of rotations (C, D) and distance traveled in open field (E, F) ( $n = 8$  mice/condition). Graphs represent mean values  $\pm$  SEM. Statistical analysis was performed using One-Way ANOVA test with Bonferroni post-test.

D1R- and D2R-MSNs using hemiparkinsonian *Drd2*-GFP mice. The immunohistochemical analysis was carried out after mice were treated with increasing doses of L-DOPA over 4 weeks (Fig. 2A). D2R-MSNs and CINs were visualized by GFP expression, while D1R-MSNs and

GABAergic interneurons were identified as being GFP-negative (Mata-males et al., 2009; Rallapalle et al., 2021) (Fig. 4A).

As reported by others, we observe an overall increase in pERK (Fig. 4B), phospho-PKA substrate- (pPKA substrate, Fig. 4E) and FosB-



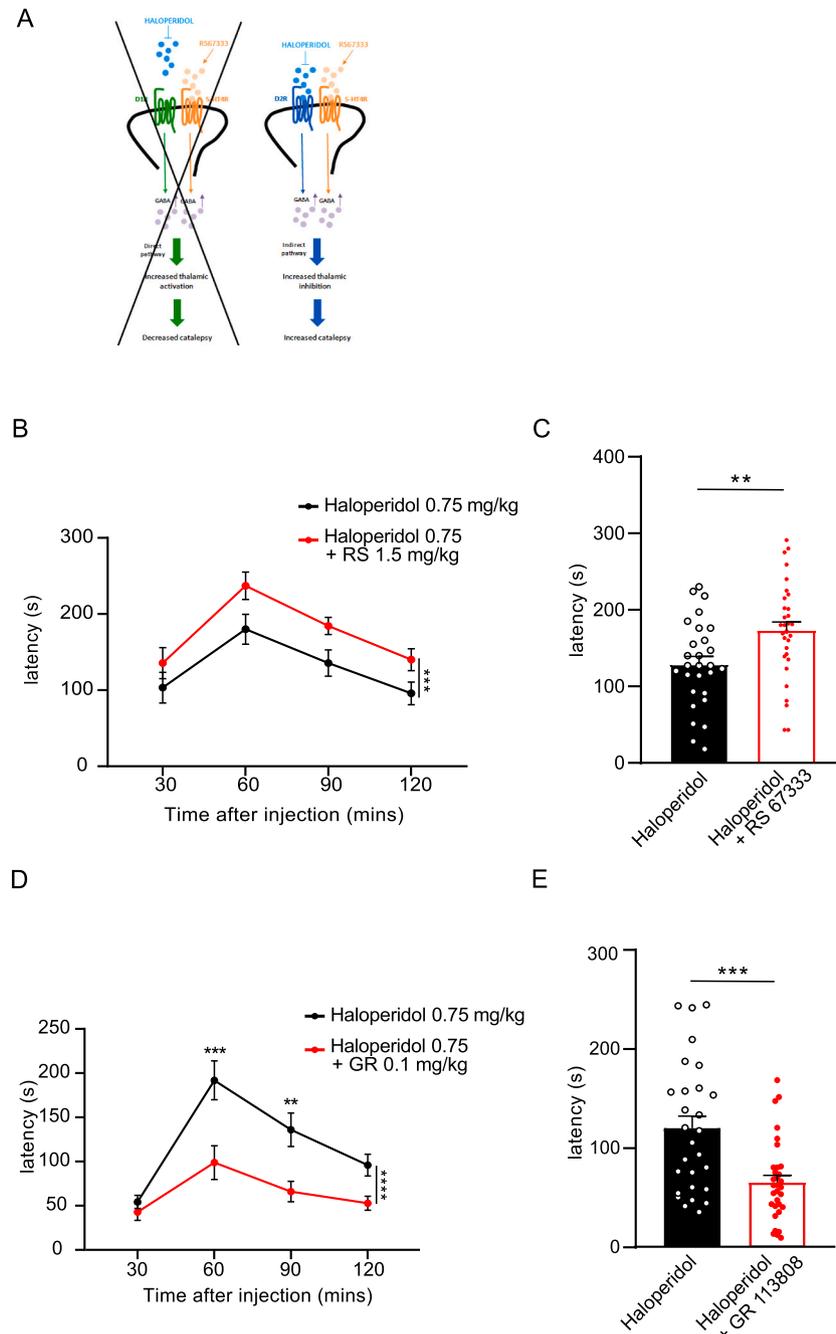
**Fig. 4.** 5-HT4R partial agonist RS 67333 significantly increases L-DOPA-induced PKA-dependent phosphorylation in D2R-MSNs. Representative images (A) and quantification of immunohistochemical analysis of coronal striatal slices from unilaterally lesioned mice after chronic L-DOPA administration in all striatal cells, and separately, in D1R- and D2R-MSNs, using antibodies against pERK (4B–D), pPKA substrate (4E–G) and FosB (4H–J). Arrows indicate pPKA substrate + D2R-MSNs. (n = 8 mice/condition). Graphs represent mean values  $\pm$  SEM. Statistical analysis was performed using One-Way ANOVA test with Bonferroni post-test.

positive cells (Fig. 4H) in the lesioned DL striatum compared to the unlesioned side of L-DOPA-treated mice. This increased number of immunoreactive cells preferentially occurs in D1R-MSNs (Fig. 4C, F, I) compared to D2R-MSNs (Fig. 4D, G, J). Expression of pERK, pPKA substrate and FosB were induced by L-DOPA by factors of 34, 371 and 5, respectively in D1-MSNs, while the induction was 3-fold for pERK, with no significant changes for pPKA substrate and FosB, in D2R-MSNs. Co-administration of RS 67333 with L-DOPA does not change the level of the three activation markers in D1-MSNs, but interestingly, it almost doubles pPKA substrate-positive D2R-MSNs ( $p = 0.0227$ ), but does not alter pERK and FosB levels, in D2R-MSNs compared to L-DOPA alone.

Since repeated L-DOPA administration induces ERK phosphorylation in CINs, we assessed pERK in CINs, marked by anti-ChAT antibody. Consistently, pERK expression was increased after chronic L-DOPA in the lesioned striatum, but RS 67333 did not change the level of pERK in CINs (Suppl. Fig. S4).

### 3.5. RS 67333 enhances haloperidol-induced catalepsy

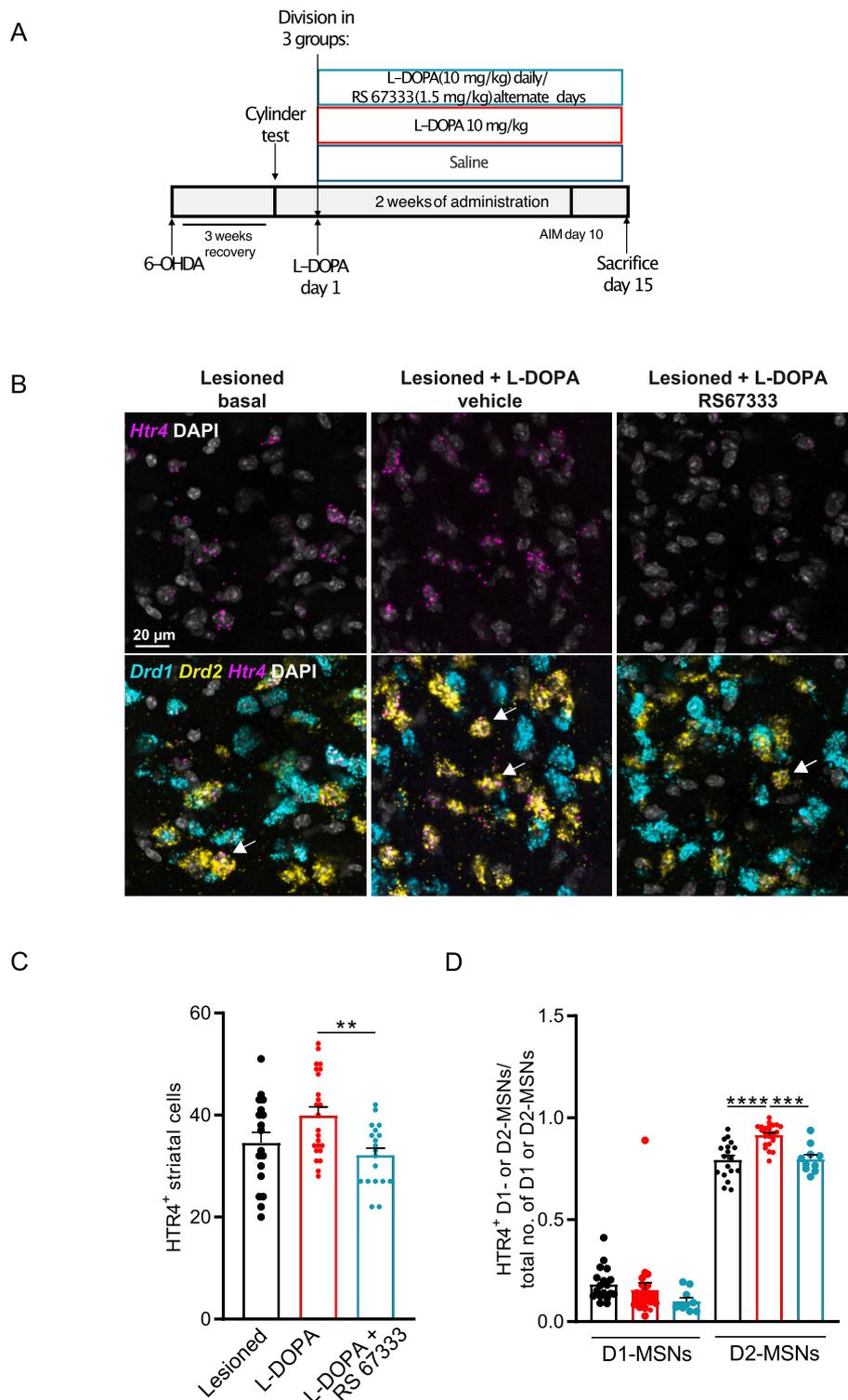
Our data show that RS 67333 primarily leads to signaling changes in D2R-MSNs. To further probe for the cells mediating the effect of 5-HT4R modulation, we administered mice with haloperidol, a D2R antagonist,



**Fig. 5.** Haloperidol induced-catalepsy is altered by 5-HT4R modulation. Scheme depicting behavioral outcome in dependency of 5-HT4R action in either D1R- or D2R-MSNs (A). Duration of cataleptic status induced by haloperidol (0.75 mg/kg) ± RS 67333 (1.5 mg/kg) measured at 4 time points after injection (B) and compounded plot thereof (C). Duration of cataleptic status induced by haloperidol (0.75 mg/kg) ± antagonist GR 113808 (0.1 mg/kg) measured at 4 time points after injection (D) and compounded plot thereof (E) ( $n = 7-8$  mice/condition). Graphs represent mean values ± SEM. Statistical analysis was performed using Two-way ANOVA with Bonferroni post-test (B, D) or Mann Whitney test (C, E).

known to induce catalepsy (Boulay et al., 2000). If RS 67333 were to act predominantly via activation of D1R-MSNs a reduction in catalepsy would be expected, if it were to act via D2R-MSN, enhanced catalepsy should be observed, while the inverse should be true in the case of 5-

HT4R antagonism (Fig. 5A). We administered haloperidol (0.75 mg/kg) alone or together with RS 67333 (1.5 mg/kg) or the 5-HT4R antagonist GR 113808 (0.1 mg/kg) and observed the duration of catalepsy at various times points up to 2 h post injection. RS 67333



**Fig. 6.** 5-HT4R partial agonist RS 67333 alters *Htr4* expression in D2R-MSNs. Timeline of the experiment (A). Representative images for smFISH staining (RNAscope) using probes for D1R (*Drd1*, cyan), D2R (*Drd2*, yellow) and 5-HT4R (*Htr4*, magenta); nuclei are stained with DAPI (white). Arrows point to *Htr4*<sup>+</sup> D2R-MSNs. (B). Counts of *Htr4*-positive D1R- and D2R-MSNs in the unlesioned, lesioned and lesioned/L-DOPA (10 mg/kg) dorsolateral striatum (C). Percentage of *Htr4*-positive D1R- or D2R-MSNs normalized by total number of the respective MSN type (D). *n* = 4 mice/condition, 12 images/mouse. Graphs represent mean values  $\pm$  SEM. Statistical analysis was performed using One-Way ANOVA test with Bonferroni post-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significantly enhanced catalepsy over 2 h (interaction by treatment group:  $F_{(1, 52)} = 14.06$ ;  $p = 0.0004$ ) (Fig. 5B, C), while GR 113808 reduced it (interaction by treatment group:  $F_{(1, 52)} = 28.67$ ;  $p < 0.0001$ ) (Fig. 5D, E). Our results clearly indicate that both modulators of 5-HT4R activity act through a D2R-MSN-driven effect.

We next analyzed brain slices from L-DOPA  $\pm$  RS 67333 treated mice by smFISH analysis, in order to determine if the agonist also affects *Htr4* expression. Interestingly, administration of RS 67333 induces an overall reduction in *Htr4* expression compared to L-DOPA alone. In D2R-MSNs, RS 67333 normalizes the L-DOPA mediated upregulation of *Htr4*, while in it had no effect in D1R-MSNs. (Fig. 6 C, D).

Taken together, our data highlight a novel potential role of the 5-HT4R in mediating LID via its selective expression in and activation of D2R-MSNs, and re-equilibration of activities of direct and indirect striatal output pathways.

#### 4. Discussion

The mechanisms underlying LID are complex and in particular the role of D2R-MSNs is not very well understood. Along with multiple changes in the DA system, the 5-HT system has been implicated in LID pathogenesis. The DA and 5-HT systems are, indeed, intrinsically linked, as various 5-HT receptors were shown to influence DA release.

5-HT neurons, which also express enzymes for L-DOPA conversion and DA storage, uncontrollably release DA upon L-DOPA administration, thereby exacerbating LID (Arai et al., 1995). Pharmacological silencing of the 5-HT1a/b autoreceptors reduces AIMs in 6-OHDA-lesioned rats and MPTP-treated macaques (Carta et al., 2007; Munoz et al., 2008). However, in clinical studies they showed only moderate efficacy and affected the anti-akinetic effect of L-DOPA (Bonifati et al., 1994; Goetz et al., 2007; Svenningsson et al., 2015).

During L-DOPA treatment, exogenously derived DA is thought to compete and displace 5-HT inside exocytotic vesicles, leading to reduced 5-HT release in the striatum in rodent and non-human primate PD models (Lohoff, 2010; Tronci et al., 2013; De Deurwaerdère et al., 2017). As a result of reduced 5-HT concentration in the cleft, post-synaptic 5-HT receptors are poised to be affected by L-DOPA treatment. We therefore hypothesize that targeting specific post-synaptic 5-HT receptors may be effective in treating LID. We focused on the 5-HT4R based on its high striatal expression and prior clinical studies aimed at testing the effect of 5-HT4R agonists on gastroparesis and constipation, well-established and early symptoms of PD. These studies described an unexpected beneficial reduction in L-DOPA response fluctuations, with more “on time” responses and improved motor functions upon agonists treatment (Djaldetti et al., 1995; Berardi, 2004; Asai et al., 2005).

##### 4.1. Cell-specific 5-HT4 receptor expression in the striatum

Importantly, 5-HT4R expression was found to be preserved in PD patients' brains, and to have increased binding of [3H]GR113808 by 93% in the caudate-putamen and globus pallidus of 6-OHDA rodent models (Compan et al., 1996). Recently, elevated 5-HT4R protein levels were also shown in rat and primate post-mortem brains, an effect that was exacerbated by L-DOPA treatment (Cirillo et al., 2024). *Htr4* mRNA levels were found 2-fold increased after L-DOPA administration in rodents (Heiman et al., 2014). This is in line with our RT-qPCR and smFISH findings showing increased *Htr4* mRNA expression in the lesioned striatum. We found that chronic L-DOPA administration leads to an even higher *Htr4* expression, and that RS 67333 administration normalizes this increase, specifically in the lesioned striatum. This effect could be attributed to an upregulation of receptor expression due to lowered striatal 5-HT tone during degeneration and L-DOPA treatment.

We show that in the intact DL striatum the expression of *Htr4* is biased towards the D2R-MSNs (~62%) compared to D1R-MSNs (~11%). In the lesioned striatum, *Htr4* levels increase in D2R-MSNs

and L-DOPA further enhances this increase in D2R-MSNs, while it does not significantly affect *Htr4* in D1R-MSNs. Such a homeostatic adaptation in response to L-DOPA could represent a mechanism by which upregulation of 5-HT4R enables enhanced D2R-MSN activity to counteract the overactivation of D1R-MSNs.

##### 4.2. Arguments for D2R-MSN mediated reduction in LID

On the behavioral level, co-administration of L-DOPA with RS 67333 reduces the AIM score. This reduction is found in axial, limb and orofacial dyskinesia (ALO score), but not in the asymmetric locomotor action (LOC score). Importantly, administration of RS 67333 alone induces a significant rescue of the contralateral paw use, while in combination with L-DOPA does not hinder nor enhance its anti-akinetic effect.

Thus far, the induction of cellular activation markers previously linked to LID, such as pERK,  $\Delta$ FOSB/FOSB or activation of the PKA pathway was mainly described in D1R-MSNs (Andersson et al., 1999; Gerfen et al., 2002; Fasano et al., 2010; Lebel et al., 2010; Santini et al., 2012). In recent years, evidence in support of D2R-MSN activity as mediator of LID expression has been accumulating: In dyskinetic mice, the majority of MSNs that were activated and FosTRAP-labeled during a single L-DOPA dosage were D1R-MSNs, yet, 10% of D2R-MSNs were also labeled (Girasole et al., 2018). It was further shown that selective chemogenetic and optogenetic stimulation of D2R-MSNs, lead to suppression of LIDs (Alcacer et al., 2017; Castela et al., 2023), while D2R agonist quinpirole had an LID enhancing effect on optogenetically induced LID (Sáez et al., 2023). This is in congruence with our findings of 5-HT4R-mediated D2R-MSN cell activation in response to RS 67333 and a correlative LID reduction.

We wanted to determine if we can detect any marker of activation after RS 67333 treatment in either D1R-MSNs, D2R-MSNs or CINs. As expected, we found a consistent and robust response at the level of all three activation markers to L-DOPA in the DL striatum. Our findings clearly recapitulate that D1R-MSNs are by numbers the predominant expressors of known LID markers, in our hands for all three markers at least 10 $\times$  higher expression was detected in D1R- versus D2R-MSNs. pERK and FOSB expression ranges in D2R-MSNs are low, ranging from 0.4 to 1.5% of D2R-MSNs for pERK and from 2.1–4.9% of D2R-MSNs for FOSB. For both markers, no change in the presence of RS 67333 was observed. It was shown that 5-HT4R can activate ERK signaling, in a non-canonical manner, however, such signaling was so far described only in cellular models (Barthet et al., 2007).

However, we found that 5-HT4R agonism with L-DOPA enhances pPKA substrate antibody-positive D2R-MSNs by a factor of 1.9, compared to L-DOPA alone, and leading to labeling of 4.8% of D2R-MSNs with pPKA substrate antibody. Thus, a reduction in LID expression correlates with an upregulation of the PKA pathway activity in these cells which could be a direct result of the G $\alpha$ olf-coupled 5-HT4R activation by its agonist. We cannot state at this point if such a relatively small number of cells is capable of leveraging the LID reduction. Further studies are needed to ascertain for example activation kinetics, in terms of days of treatment and time after RS 67333 injection. Another consideration is that RS 67333 led to downregulation of 5-HT4R expression which will reduce the responsiveness of D2R-MSNs to agonism. An acute treatment scheme could provide further indications to address this question.

Recently, it was shown that optogenetic activation of GABAergic neurons in the substantia nigra pars reticulata, a basal ganglia output structure, reduces LID in a mouse model (Hu et al., 2023) and importantly, 5-HT4R is expressed in the SNpr (Waeber et al., 1996). This suggests the possibility that RS 67333 may target other neurons that could contribute to the outcome of LID reduction.

The 5-HT4R is not the only non-dopaminergic postsynaptic G $\alpha$ s-coupled GPCR expressed in D2R-MSNs that affects motor responses to L-DOPA: Adenosine A2a receptor (A2aR) antagonists have been discussed as neuroprotectants and as clinically beneficial agents during L-

DOPA treatment, based on epidemiological observations of caffeine consumption, an A2aR antagonist (Ascherio and Schwarzschild, 2016; Cenci et al., 2022). LID in A2aR KO mice is reduced (Xiao et al., 2011), however caffeine treatment in unilaterally lesioned mice did not have a beneficial effect on LID reduction (Xiao et al., 2011; Cortés et al., 2017). Interestingly, in a rat LID model, the A2aR antagonist KW-6002 together with low dose L-DOPA yields therapeutic-like effects comparable to full dose L-DOPA, without worsening LID (Lundblad et al., 2003), indicating an effect on sensitization to L-DOPA's prokinetic efficacy rather than LID. This was confirmed in MPTP-treated primates (Grondin et al., 1999; Chase et al., 2003). The signaling mechanisms by which these two receptors mediate their effects are presumably not identical and go beyond "linear" G $\alpha$ s coupling. Like the D2R, A2aR is highly expressed in D2R-MSNs, and the notion of A2aR-D2R heterodimerization is well established (Ferré et al., 2018). Through antagonizing downstream signaling molecules, G $\alpha$ s versus G $\alpha$ i, the interplay of these two receptors enables the fine-tuning of behavioral outputs via a network of downstream components including PLC, Adenyl cyclase 5, DARPP-32 and many more (Ferré et al., 2018). No interaction between 5-HT4R and D2R has been described thus far, and clearly the stoichiometry of such receptor heterodimers would be low, given that D2R by far outnumbers the 5-HT4R (Puighermanal et al., 2020). A more complex mechanism of action of the 5-HT4R in the context of LID reduction, besides a G $\alpha$ s-mediated PKA pathway stimulation and concomitant D2R-MSNs activation, may well be envisioned.

## 5. Conclusion

In Parkinson's disease, DA loss leads to an imbalance of the direct and indirect DA pathways, with hyper-sensibilization of D1R-MSNs. L-DOPA treatment leads to hyper-activation of the D1 pathway through increased pERK levels and PKA activity. We propose that agonism of the post-synaptic serotonin 5-HT4R leads to increased D2R-MSNs activity and PKA substrate phosphorylation in D2R-MSNs, thereby partially rebalancing direct and indirect basal ganglia pathways and reducing LID without affecting L-DOPA's pro-kinetic effect. Our findings show that in the lesioned dyskinetic striatum, activation of D2R-MSNs can correlate with changes in LID expression. Our data highlight the critical role of D2R-MSNs in the regulation of motor responses to L-DOPA and identify the 5-HT4R as a pharmacological target to treat LID. This is particularly relevant when taking into consideration that 5-HT4R agonists are already in use for alleviation of gastroparesis in PD, making this receptor a target to address multiple PD symptoms.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.nbd.2024.106559>.

## CRedit authorship contribution statement

**Demetra Ballardin:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis. **Leila Makrini-Maleville:** Investigation. **Alexander Seper:** Investigation. **Emmanuel Valjent:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Heike Reibholz:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare no conflicts of interest.

## Data availability

No data was used for the research described in the article.

## Acknowledgements

We thank JM Cruz-Gamero, I. Matei, J. Forget, N. Ramos and N. Lebrun for help, reagents and technical support. We would like to thank the IPNP animal facility, Phenobrain and NeurImag platforms for animal care, technical and scientific support. NeurImag is part of IPNP, Inserm U1266 and Université Paris Cité and member of the national infrastructure France-BioImaging supported by the French National Research Agency (ANR-10-INBS-04). We thank Leducq establishment for funding the Leica SP8 confocal/STED3Dx system. This work was supported by a MSCA-fellowship (HT4PD-DLV-894207, HR), Doctoral school fellowship Bio Sorbonne Paris Cité (DB), French National Research Agency (DISCOMMODE, ANR-21-CE37-0013, EV) and Fondation pour la Recherche Médicale (EQU202203014705, EV).

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