

Striatal and nigral muscarinic type 1 and type 4 receptors modulate levodopa-induced dyskinesia and striato-nigral pathway activation

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May 5, 2020

Abstract

Background and purpose: Muscarinic receptors contribute to both the facilitation and inhibition of levodopa-induced dyskinesia operated by striatal cholinergic interneurons, although the receptor subtypes and the mechanisms involved have not been clearly identified. Also muscarinic receptors in substantia nigra reticulata, activated by cholinergic midbrain afferents, regulate striatal functions although their role in levodopa-induced dyskinesia remains to be proven. Here, we investigate whether striatal and nigral muscarinic M1 and/or M4 receptors modulate dyskinesia expression and the underlying striato-nigral GABAergic pathway activation in 6-hydroxydopamine hemilesioned rats. Experimental approach: Reverse microdialysis allowed to deliver the M1 and M4 preferential antagonists telenzepine, PD-102807, tropicamide and the selective M4 positive allosteric modulator VU0152100 in striatum or substantia nigra, while levodopa was administered systemically. Dyskinetic movements were monitored along with nigral GABA (and glutamate) and striatal glutamate levels, taken as neurochemical markers of striato-nigral pathway activation. Key results: Intrastratial telenzepine, PD-102807 and tropicamide alleviated dyskinesia and inhibited nigral GABA and striatal glutamate release. This was partially replicated by intrastratial VU0152100 that, however, failed to inhibit striatal glutamate. The M2 preferential antagonist AFDX-116, used to elevate striatal acetylcholine levels, blocked the behavioral and neurochemical effects of PD-102807. Intranigral VU0152100 prevented levodopa-induced dyskinesia and its neurochemical correlates whereas PD-102807 was ineffective. Conclusions and Implications: Striatal M1 and M4, likely postsynaptic, receptors facilitate dyskinesia and striato-nigral pathway activation. Striatal M4 receptors, possibly located presynaptically, also inhibit dyskinesia. Potentiation of striatal and nigral M4 transmission leads to powerful multilevel inhibition of striato-nigral pathway providing a new strategy to tackle dyskinesia.

Non standard abbreviations

LID, levodopa-induced dyskinesia; AUC, area-under-the-curve; AP, antero-posterior; BG, basal ganglia; ChIs, cholinergic interneurons; DA, dopamine; DLS, dorsolateral striatum; PPN, pedunclopontine nucleus; DV, dorso-ventral; ML, medio-lateral; PAM, positive allosteric modulator; Glu, glutamate; 6-OHDA, 6-hydroxydopamine; levodopa, L-DOPA; PD, Parkinson's disease; SNr, substantia nigra pars reticulata; TH, tyrosine hydroxylase.

Bulleted point summary

What is already known

- Non-selective muscarinic antagonists and ablation or functional inhibition of striatal cholinergic interneurons inhibit levodopa-induced dyskinesia
- Striatal M4 receptor activation reduces levodopa-induced dyskinesia in mice and non human primates

What this study adds

- Both striatal M1 and M4 muscarinic receptors mediate levodopa-induced dyskinesia and striato-nigral pathway activation

- Striatal and nigral M4 muscarinic receptor stimulation attenuate levodopa-induced dyskinesia and striato-nigral pathway activation

Clinical significance

M4 muscarinic receptor agonists might prove effective in palliating levodopa-induced dyskinesia

Introduction

The contribution of striatal cholinergic interneurons (ChIs) to striatal functions and parkinsonism has long been recognized (Calabresi *et al.*, 2000; Pisani *et al.*, 2007). In the last decade, the role of striatal ChIs in levodopa (L-DOPA) induced dyskinesia (LID) has also emerged (Bordia *et al.*, 2019; Moehle *et al.*, 2019). Dyskinesia is a major side-effect of L-DOPA pharmacotherapy of Parkinson’s disease (PD), and is clinically characterized by abnormal involuntary movements (AIMs) of dystonic and choreic nature (Bastide *et al.*, 2015). LID has a complex and partially unknown etiology, but the final neurobiological event is the sensitization of D1 expressing striato-nigral GABAergic medium-sized spiny neurons (MSNs) to L-DOPA (Bastide *et al.*, 2015). Striatal ChIs profusely innervate both striato-pallidal and striato-nigral MSNs and influence their activity either directly or indirectly, via modulation of dopamine, serotonin, glutamate release from striatal afferents, and GABA release from striatal interneurons. It was originally found that ablation or functional inhibition of ChIs attenuate LID (Ding *et al.*, 2011; Won *et al.*, 2014), although a more recent optogenetic study revealed that ChIs dually modulate LID, facilitating it at low firing rates and attenuating it at higher ones (Bordia *et al.*, 2016). Pharmacological studies showed that both muscarinic (Bordia *et al.*, 2016; Chambers *et al.*, 2019; Ding *et al.*, 2011; Shen *et al.*, 2015) and nicotinic (Quik *et al.*, 2015) receptors are involved in LID. However, while it has been convincingly demonstrated that nicotinic receptor blockade (with receptor antagonists) or desensitization (with receptor agonists) prevents LID (Quik *et al.*, 2015), the role of muscarinic receptors is far from clear (Bordia *et al.*, 2019). This might be due to the different neuronal distribution and functional roles of muscarinic receptor subtypes. In fact, muscarinic receptors are GPCR belonging to two major families which couple to $G_{i/o}$ (M2 and M4 subtypes) or $G_{q/11}$ (M1, M3 and M5 subtypes), and are unevenly distributed on the different populations of striatal neurons and nerve terminals. In particular, striato-pallidal MSNs predominantly express M1 receptors, while striato-nigral MSNs express both M1 and M4 receptors (Bernard *et al.*, 1992; Hersch *et al.*, 1994). The M4 receptor is considered the main autoreceptor type at striatal ChIs (Zhang *et al.*, 2002), although also M2 autoreceptors contribute to negative auto feed-back (Bonsi *et al.*, 2008). Another major problem in dissecting out the role of endogenous ACh and muscarinic receptor subtypes in LID is the poor selectivity of muscarinic antagonists available. Thus, the unselective muscarinic antagonists dicyclomine and atropine prevented LID in 6-hydroxydopamine (6-OHDA) hemilesioned mice (Bordia *et al.*, 2016; Ding *et al.*, 2011) whereas atropine also prevented LID inhibition induced by optogenetic elevation of ChIs firing rates (Bordia *et al.*, 2016). M4 selective positive allosteric modulators (PAMs) reduced LID in murine and non-human primate models of LID, pointing out a crucial role for M4 receptors in this movement disorder (Shen *et al.*, 2015). In partial agreement, the putative M4 “preferential” antagonist tropicamide (Lazareno *et al.*, 1993; Lazareno *et al.*, 1990) worsened AIMs induced by L-DOPA but attenuated those induced by a D1 receptor agonist in 6-OHDA hemilesioned rats (Chambers *et al.*, 2019), suggesting the involvement of different sets of M4 receptors. However, the M4 “selectivity” of tropicamide, if any (Croy *et al.*, 2016), is negligible which calls for a re-evaluation of its effects with relatively more selective compounds. Finally, the role of ACh in LID might extend beyond striatal ChIs. In fact, cholinergic inputs from the pedunculopontine tegmental nucleus (PPN) modulate basal ganglia function (Xiao *et al.*, 2016), and D1 signaling at striato-nigral MSNs terminals (Moehle *et al.*, 2017). Although a preliminary report showed that injection of tropicamide in PPN did not modulate LID expression (Chambers *et al.*, 2019), whether M4 receptor stimulation or blockade in SNr shape LID remains to be established.

In the present study, *in vivo* dual probe microdialysis was used to clarify the role of striatal and nigral M1 and M4 receptors in the modulation of LID and the underlying striato-nigral MSNs activation. A reverse microdialysis approach was adopted to deliver the M1 and/or M4 preferential antagonists telenzepine, PD-102807, tropicamide as well as the selective M4 PAM VU0152100 in the dorsolateral striatum or SNr, concurrently

with systemic L-DOPA administration. This approach had the two-fold advantage of i) targeting muscarinic receptors in specific brain areas, ii) minimizing the poor selectivity of muscarinic receptor antagonists by setting pharmacological selective concentrations in the perfusion Ringer (see Methods). Dyskinetic behavior was monitored concurrently with GABA release in SNr, a readout of striato-nigral MSNs activation (Marti *et al.*, 2012; Mela *et al.*, 2012; Mela *et al.*, 2007), and glutamate release in striatum, a possible index of cortico-basal ganglia-thalamo-cortical loop activation (Bastide *et al.*, 2015). The feasibility of this approach was previously demonstrated showing that reverse dialysis of the D1 antagonist SCH23390, but not the D2 antagonist raclopride, in striatum prevented LID and its neurochemical correlates (Mela *et al.*, 2012).

Methods

Animal subjects

Experimental procedures involving the use of animals complied with the ARRIVE and BJP guidelines. Male Sprague-Dawley rats (Charles River Lab, Calco, Lecco, Italy) were housed in a standard facility with free access to food (4RF21 standard diet; Mucedola, Settimo Milanese, Milan, Italy) and water, and kept under regular lighting conditions (12 hr dark/light cycle). Animals were housed in groups of two in an 850 cm² polysulfone cage (OptiRat; Animal Care Systems, Centennial, CO, USA) with a Scobis Uno bedding (Mucedola, Settimo Milanese, Milan, Italy) and environmental enrichments. Adequate measures were taken to minimize animal pain and discomfort. Experimental protocols were approved by the Ethical Committee of the University of Ferrara and the Italian Ministry of Health (license 714/2016-PR).

Unilateral 6-OHDA lesion

Rats (150 g) were unilaterally injected in the (right) medial forebrain bundle with 8 µg 6-OHDA hydrobromide (dissolved in 0.02% ascorbate-saline), according to the following stereotaxic coordinates from bregma and the dural surface (in mm): antero-posterior (AP) -4.4, medio-lateral (ML) 1.2, ventro-dorsal (VD) -7.8, tooth bar at -2.4 mm (Paxinos *et al.*, 1986), as previously described (Mela *et al.*, 2012; Morari *et al.*, 1996; Paolone *et al.*, 2015). Animals were pretreated with antibiotics (SynuloxTM, 50 µl kg⁻¹, i.p.). The wound was sutured and infiltrated with 2% lidocaine solution (EsteveTM). Two weeks later, rats were screened by assessing the motor asymmetry score in two different ethological tests (the bar and drag tests) (Pisanò *et al.*, 2020). Rats showing immobility time at the contralateral paw in the bar test >20 sec and a number of steps at the contralateral paw <3 (or alternatively a contralateral/ipsilateral paw ratio <50%) were enrolled in the study (Pisanò *et al.*, 2020).

Microdialysis experiments .

Dual probe microdialysis was performed as previously described (Mela *et al.*, 2012; Morari *et al.*, 1996; Paolone *et al.*, 2015). Concentric microdialysis probes were constructed using AN69 (Gambro Industries, Meyzieu, France) semipermeable hollow membranes (65 kDa molecular weight cut-off, 340 µm outer diameter). Ninety-six (96) 6-OHDA fully dyskinetic rats (AIMs > 100) were used in the study. One microdialysis probe was stereotactically implanted under isoflurane anesthesia in the DA-depleted dorsolateral striatum and another in the ipsilateral SNr. Implantation coordinates (in mm, from bregma and the dural surface) (Paxinos *et al.*, 1986), and dialysis membrane lengths were: dorsolateral striatum, AP +1.0, ML ±3.5, DV -6.0 (3 mm), SNr, AP -5.5, ML ±2.2, DV -8.3 (1 mm). Twenty-four hours after surgery, probes were perfused with a modified Ringer solution (CaCl₂ 1.2 mM; KCl 2.7 mM; NaCl 148 mM; MgCl₂ 0.85 mM) at a 3 µl min⁻¹ flow rate. Sample collection (every 20 min) started after 6 h rinsing. At least four baseline samples were collected, then treatments were administered in a randomized fashion. At the end of experiments, animals were sacrificed by an overdose of isoflurane, and the correct placement of the probes was verified histologically. Severe (93%) dopamine depletion was randomly confirmed by post-mortem analysis of tyrosine hydroxylase (TH) levels in Western blot assay (optical density of TH band normalized over α-tubulin: 0.22±0.04 vs 3.13±0.39 ipsilateral vs contralateral striatum, respectively, t=7.37 df=19, Student's t-test, two-tailed for unpaired data; n=20 rats; Supplementary Figure 1).

LID induction and AIMs ratings

6-OHDA hemilesioned rats received 6 mg kg⁻¹ L-DOPA (plus 12 mg kg⁻¹ benserazide, s.c.), once a day for 21 days as originally described by MA Cenci and collaborators (Cenci *et al.*, 1998; Cenci *et al.*, 2007) and reported by our group (Marti *et al.*, 2012; Mela *et al.*, 2012; Paolone *et al.*, 2015). Rats were observed individually for 1 min every 20 min during the 3 h that followed an L-DOPA injection. Dyskinetic movements were classified based on their topographic distribution into three subtypes: (i) axial AIMs, that is, twisted posture or choreiform twisting of the neck and upper body toward the side contralateral to the lesion; (ii) forelimb AIMs, that is, jerky or dystonic movements of the contralateral forelimb and/or purposeless grabbing movement of the contralateral paw; (iii) orolingual AIMs, that is, orofacial muscle twitching, empty masticatory movements and contralateral tongue protrusion. Each AIM subtype was rated on frequency and amplitude scales from 0 to 4 (Cenci *et al.*, 2007). Dyskinesia score was calculated either in the induction phase or during the microdialysis sessions, as the product of frequency x amplitude (Cenci *et al.*, 2007), and presented as cumulative AIMs score.

Endogenous Glu and GABA analysis

Glu and GABA in the dialysate were measured by HPLC coupled with fluorometric detection as previously described (Mela *et al.*, 2012; Paolone *et al.*, 2015). Thirty microliters of o-phthalaldehyde/mercaptoethanol reagent were added to 30 μ l aliquots of sample, and 50 μ l of the mixture were automatically injected (Triathlon autosampler; Spark Holland, Emmen, Netherlands) onto a 5-C18 Hypersil ODS analytical column (3 mm inner diameter, 10 cm length; Thermo-Fisher, USA) perfused at a flow rate of 0.48 ml min⁻¹ (Jasco PU-2089 Plus quaternary pump; Jasco, Tokyo, Japan) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glu and GABA were detected by means of a fluorescence spectrophotometer FP-2020 plus (Jasco, Tokyo, Japan) with the excitation and the emission wavelengths set at 370 and 450 nm respectively. Under these conditions, the limits of detection for Glu and GABA were \sim 1 nM and 0.5 nM, and their retention times \sim 3.5 min and \sim 18.0 min, respectively. Chromatogram analysis was made by a blinded experimenter using a dedicated ChromNav software (Jasco, Tokyo, Japan).

TH analysis

Rats were sacrificed by an overdose of isoflurane. Striata were solubilized and homogenized in lysis buffer (RIPA buffer, protease and phosphatase inhibitor cocktail) and centrifuged at 18,000 x g for 15 min at 4°C (Pisanò *et al.*, 2020). Supernatants were collected and total protein levels were quantified using the bicinchoninic acid protein assay kit (Thermo Scientific). Thirty micrograms of protein per sample were separated by SDS-PAGE, transferred onto polyvinylidene difluoride membrane and incubated overnight (4°C) with the rabbit anti-TH primary antibody (Merck Millipore, AB152, 1:1000). Membranes were then washed and incubated with horseradish peroxidase-linked secondary antibody (Merck Millipore, goat anti-rabbit IgG HRP-conjugate 12-348, 1:4000). Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) detection kit (Pierce BCA Protein Assay Kit, Thermo Scientific or ECL+, GE Healthcare). Images were acquired and quantified using the ChemiDoc MP System and the ImageLab Software (Bio-Rad). Membranes were then stripped and re-probed with rabbit anti- α -tubulin antibody (Merck-Millipore 04-1117, 1:25000). Data were analyzed by densitometry and the optical density of TH protein band was normalized to α -tubulin levels.

Experimental design

Dyskinetic rats were randomized to L-DOPA or L-DOPA in combination with a muscarinic ligand in the Day 1 microdialysis session, and treatments were crossed in the Day 2 session. Experimenters were blinded to treatments. Groups were balanced in terms of Day 1 or Day 2 treatments. The effect of muscarinic ligands OFF L-DOPA was investigated in separate groups of rats. In this case, one rat received two different ligands, randomized in Day 1 and Day 2 sessions in a balanced design.

Perfusing muscarinic antagonists through the microdialysis probe allowed to deliver constant amount of compounds in the selected brain areas so to set stable and pharmacologically selective levels, approximating the affinity values for the targeted receptor, in the extracellular space. This was accomplished by taking into account in vitro recovery, which in our microdialysis conditions, with the 3 mm probe implanted in

striatum, approximates 10%. We therefore estimated that roughly one tenth of the nominal concentration of the compound in the perfusion Ringer would be delivered to the extracellular space. Considering the poor selectivity of muscarinic receptor antagonists, this approach might represent an advantage over classical microinjection protocols. Telenzepine is an M1 receptor preferential antagonist, with an affinity of 1-3 nM for M1 receptors, a 2-5 fold selectivity for M1 over M4 receptors, and a 5-30 fold selectivity for M1 over M2, M3 and M5 receptors (Doods *et al.* , 1987; Lazareno *et al.* , 1990; Tanda *et al.* , 2007). Telenzepine was perfused through the probe at 100 nM, in order to generate extracellular concentrations of about 10 nM (i.e. 3-10 fold greater than the affinity for M1 receptors). PD-102807 is an M4 preferential receptor antagonist, with 7-28 nM affinity for M4 receptors, a 14-36 fold selectivity for M4 over M3 receptors, and 76-2600 fold selectivity for M4 over M1, M2 and M5 receptors (Bohme *et al.* , 2002; Croy *et al.* , 2016). PD102807 was perfused at 3 μ M to generate extracellular concentrations 10-30 greater than its affinity for M4 receptors and much lower than those for M1, M2 and M5 receptors. Tropicamide is considered an M4 preferential receptor antagonist, with an affinity of 15 nM for the M4 receptors (Lazareno *et al.* , 1993; Lazareno *et al.* , 1990). However, its selectivity for the M4 receptor over M1, M2 and M3 receptors is only 3-fold (Lazareno *et al.* , 1993; Lazareno *et al.* , 1990). In addition, a more recent study (Croy *et al.* , 2016) questioned even such small pharmacological preference. Thus, we decided to infuse 100 nM tropicamide to generate concentrations just below the affinity value for the M4 receptor. VU0152100 is an M4 PAM effective in increasing by 70-fold the potency of ACh to stimulate Ca²⁺ responses at M4 chimeric receptors (Brady *et al.* , 2008). It does not bind to the M4 orthosteric site nor it potentiates ACh binding to the other muscarinic receptor subtypes, thus appearing very selective for M4 receptors (Brady *et al.* , 2008). We perfused 100 μ M through the probe to generate extracellular levels about 30 times higher than its M4 activity (EC₅₀ 380 nM). AFDX-116 is a preferential antagonist of M2 receptors showing nanomolar affinity for M2 receptors (pKi 6.7-7.5), 3-4-fold selectivity over M4 receptors, 4-10 fold selectivity over M1 and >10-fold selectivity over M5 receptors (Billard *et al.* , 1995; Doods *et al.* , 1987; Stoll *et al.* , 2009). The concentration used for reverse dialysis (200 nM) was chosen based on a microdialysis study showing that perfusion of 100 nM AFDX-116 through the probe was capable of increasing by > 70% ACh levels in striatum (Billard *et al.* , 1995). Based on the affinity values reported above, such concentration is expected to generate extracellular levels slightly below the highest affinity value found in functional studies (pKi 7.5)(Billard *et al.* , 1995).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis *et al.* , 2018). Since in microdialysis studies, particularly when amino acid are measured, baseline values can differ greatly from animal to animal, to minimize experimental variability data were normalized as percent of baseline values, as in our previous publications. Statistical analysis was performed (Graphpad Prism 6.0) on area-under-the curve values (AUC; calculated in the 100-220 min range) followed by the Newman-Keuls test for multiple comparisons. AIMs were statistically analysed using the Mann-Whitney U-test or the Kruskal-Wallis test followed by the Dunn test for multiple comparisons when more than two groups were compared. Alpha was set at 0.05.

Materials

L-DOPA methyl ester, benserazide hydrochloride, tropicamide and VU-0152100 were purchased from Sigma-Aldrich (S. Louis MO, USA), 6-OHDA hydrobromide, telenzepine, PD-102807 and AFDX-116 from Tocris (Bristol, UK). Telenzepine, PD-102807, tropicamide, AFDX-116 and VU-0152100 were dissolved in Ringer for local perfusion through the microdialysis probe. 6-OHDA was dissolved in ascorbate-saline for intracerebral injection, whereas all the other drugs were dissolved in saline and administered systemically at the volume of 1.0 ml/Kg body weight.

Results

Striatal perfusion with telenzepine alleviated LID and inhibited nigral GABA and striatal Glu release

In order to evaluate whether M1 receptors influence LID and its neurochemical correlates, the M1 preferential antagonist telenzepine was perfused at the 100 nM concentration through the probe implanted in striatum,

starting from 40 minutes before systemic (s.c.) L-DOPA administration. Acute L-DOPA injection induced severe LID expression, which resulted ~40% reduced by telencephalon (U=10.50, p=0.022; Fig. 1A). Basal dialysate values were 16.26 ± 0.57 nM (n=208) and 63.09 ± 2.09 nM for GABA and Glu in SNr, respectively (n=208), and 57.79 ± 2.48 nM (n=184) for Glu in striatum. LID expression was accompanied by the rise of nigral GABA ($F_{2,21}=5.91$, p=0.0092; Fig. 1B) and Glu ($F_{2,21}=5.02$, p=0.0165; Fig. 1C), as well as striatal Glu ($F_{2,21}=6.06$, p=0.0084; Fig 1D). Intra-striatal perfusion with telencephalon prevented the L-DOPA-induced rise of nigral GABA (Fig. 1B) and striatal Glu (Fig.1D), but not nigral Glu (Fig. 1C).

Striatal perfusion with PD-102807 and tropicamide alleviated LID and inhibited nigral GABA and Glu along with striatal Glu release

In order to evaluate whether striatal M4 receptors influence LID and its neurochemical correlates, the M4 preferential antagonist PD-102807 and the putative M4 preferential antagonist tropicamide were perfused through the probe implanted in striatum. PD-102807 (3 μ M) reduced LID expression by ~60% (U=0.00, p=0.0009; Fig. 2A), and inhibited the L-DOPA-induced rise of GABA ($F_{2,21}=12.64$, p=0.0002; Fig. 2B) and Glu ($F_{2,21}=8.93$, p=0.0016; Fig. 2C) in SNr, and Glu in striatum ($F_{2,21}=14.04$, p=0.0001; Fig. 2D). Likewise, tropicamide (100 nM) attenuated LID expression by ~50% (U=24.50, p=0.0163; Fig 3A) and simultaneously prevented the rise of GABA ($F_{2,21}=8.04$, p=0.0025; Fig. 3B) and Glu ($F_{2,21}=7.91$; p=0.0027; Fig. 3C) in SNr and the elevation of Glu in striatum ($F_{2,21}=6.75$; p=0.0054; Fig. 3D) associated with LID appearance.

Striatal perfusion with VU0152100 alleviated LID and inhibited nigral GABA and striatal Glu release

Experimental evidence in mice and non-human primates showed that M4 receptor activation with M4 PAMs palliates LID (Shen *et al.* , 2015). In order to confirm this finding in rats and reveal the associated pathways, we perfused the M4 receptor PAM VU0152100 at 100 μ M through the probe implanted in striatum. Striatal perfusion of VU0152100 reduced LID by 40% (U=7.50, p=0.0076; Fig.4A), and prevented the LID-associated nigral GABA ($F_{2,21}=7.06$, p=0.0045; Fig. 4B) and Glu ($F_{2,21}=6.25$, p=0.0074; Fig. 4C) surge. Unlike M4 preferential antagonists, intra-striatal VU0152100 did not affect the LID-associated increase of striatal Glu release ($F_{2,21}=4.15$, p=0.0302; Fig. 4D).

Striatal perfusion with AFDX-116 blocked the antidyskinetic effect of PD-102807

Striatal M4 receptors are predominantly located presynaptically on ChIs (autoreceptors) or postsynaptically on glutamatergic cortico-striatal terminals and striato-nigral MSNs. To investigate whether the behavioral and neurochemical effects induced by striatal perfusion with PD-102807 were due to pre- or post-synaptic mechanisms, we intra-striatally perfused PD-102807 in combination with the M2 preferential antagonist, AFDX-116 (100 nM). Indeed, this concentration of AFDX-116 has been shown to elevate striatal ACh levels (Billard *et al.* , 1995; Galarraga *et al.* , 1999) and affect MSNs postsynaptically (Galarraga *et al.* , 1999). We reasoned that if the effects of PD-102807 were mediated by M4 autoreceptor blockade and elevation of striatal ACh levels, M2 autoreceptor blockade by AFDX-116 would further enhance or, at most, leave unchanged the effects of PD-102807. On the contrary, if the effects of PD-102807 were induced by postsynaptic M4 receptor blockade, an increase of ACh release would reverse them. Striatal perfusion with AFDX-116 did not affect LID expression but prevented the antidyskinetic effect of PD-102807. In fact, in the presence of AFDX-116, PD-102807 failed to attenuate LID severity (H=4.29 p= 0.11; Fig. 5A). Consistently, in the presence of AFDX-116, PD-102807 failed to inhibit the LID-associated rise of nigral GABA ($F_{3,30}=7.89$, p=0.0005; Fig 5B) or Glu ($F_{3,30}=5.54$, p=0.0038; Fig. 5C). Surprisingly enough, however, AFDX-116 failed to prevent the PD-102807 inhibition of the LID-associated increase of striatal Glu release (Fig. 5D).

Nigral perfusion with VU0152100 alleviated LID and its neurochemical correlates whereas nigral perfusion with PD-102807 was ineffective

Nigral M4 receptors activated by cholinergic afferents from the PPN exert a powerful modulation of D1 receptor signaling at striato-nigral MSNs terminals in SNr (Moehle *et al.* , 2017). To prove this modulation is relevant to LID, VU0152100 (100 μ M) or PD-102807 (3 μ M) were perfused through the probe

implanted in SNr. Nigral perfusion with VU0152100 reduced LID by ~60%, whereas PD-102807 was ineffective ($H=16.50$ $p=0.0003$ Fig. 6A). Intranigral VU0152100 also inhibited the LID-associated rise of nigral GABA ($F_{2,21}=18.34$, $p<0.0001$; Fig. 6B) and Glu ($F_{2,21}=4.99$, $p=0.0168$; Fig. 6C) and, differently from striatal perfusion, also the LID-associated rise of Glu in striatum ($F_{2,21}=6.31$, $p=0.0071$; Fig. 6D). Consistent with its failure in modulating LID, intranigral PD-102807 did not influence the LID-associated increase of nigral GABA ($F_{2,21}=5.52$, $p=0.0118$; Fig. 6E) and Glu ($F_{2,21}=4.860$, $p=0.0184$; Fig. 6F).

Discussion

The role of striatal and nigral M1 and M4 muscarinic receptors in LID was investigated using a dual probe microdialysis approach (Mela *et al.*, 2012) by which M1 and M4 receptor preferential antagonists and a M4 receptor selective PAM were delivered through the microdialysis probe into the dorsolateral striatum or SNr, simultaneously with systemic L-DOPA administration.

Telenzepine in striatum

Reverse dialysis of the M1 preferential antagonist telenzepine in striatum attenuated LID expression and the accompanying rise of GABA in SNr. Since previous microdialysis studies (Marti *et al.*, 2012; Mela *et al.*, 2012; Mela *et al.*, 2007) demonstrated that the rise of nigral GABA release which accompanies AIMs appearance reflects the activation of D1-expressing striato-nigral MSNs, it appears that M1 receptor stimulation contributes to LID expression and the underlying striato-nigral pathway activation. Indeed, M1 receptors increase striato-nigral and striato-pallidal MSNs excitability through different mechanisms: i) closure of Kir2 and KCNQ (Kv7) K^+ channels (Shen *et al.*, 2005), particularly in striato-pallidal MSNs (Shen *et al.*, 2007); ii) enhancement of Ca^{2+} -currents through Ca_v1 channels, particularly in striato-nigral MSNs (Hernandez-Flores *et al.*, 2015; Hernandez-Lopez *et al.*, 1997; Perez-Garci *et al.*, 2003); iii) facilitation of NMDA transmission (Calabresi *et al.*, 1998) through persistent sodium currents (Carrillo-Reid *et al.*, 2009); iv) inactivation of N- and P/Q Ca_v channels, leading to reduced GABA release from striatal interneurons (Perez-Rosello *et al.*, 2005). Telenzepine was proposed to improve motor symptoms in parkinsonian mice, acting at striato-pallidal M1 receptors (Ztaou *et al.*, 2016). Our study would point to an inhibitory action of telenzepine at M1 receptors on striato-nigral MSNs. To possibly confirm this view, telenzepine also reduced the LID-associated elevation of striatal Glu release (Brugnoli *et al.*, 2016; Gardoni *et al.*, 2018; Ostock *et al.*, 2011; Paolone *et al.*, 2015). In fact, elevation of striatal Glu release might result from striato-nigral MSNs stimulation leading to activation of cortico-basal ganglia-thalamo-cortical loop (Mark *et al.*, 2004; Marti *et al.*, 2005) and cortico-striatal terminals (Ostock *et al.*, 2011; Paolone *et al.*, 2015). However, Glu might be also released from striatal ChIs, which express the vesicular Glu transporter 3 (VGLUT3) (Kljakic *et al.*, 2017), thus contributing to PD symptoms and LID (Divito *et al.*, 2015; Gangarossa *et al.*, 2016). Finally, Glu released from thalamo-striatal terminals stimulates striato-pallidal MSNs, thereby sustaining akinesia in parkinsonian mice (Tanimura *et al.*, 2019). Therefore, the reduction of striatal Glu in our model might not only reflect cortico-basal ganglia-thalamo-cortical loop inhibition but also normalization of ChIs activity and cortico/thalamo-striatal transmission, which might impact behaviors beyond dyskinesia. Indeed, high frequency stimulation of the subthalamic nucleus normalized the elevation of cortico-striatal glutamatergic transmission in dyskinetic rats, which was associated with motor improvement but not LID attenuation (Gubellini *et al.*, 2006). Moreover, inhibition of Glu release from thalamo-striatal Glu terminals (via nicotinic receptor desensitization) reduced the firing of striato-pallidal MSNs and improved PD-related motor learning deficits (Tanimura *et al.*, 2019). This might explain why striatal Glu release can sometimes dissociate from AIMs appearance. In fact, in dyskinetic rats chronically treated with the MAO-B and Na_v channel inhibitor, safinamide (Gardoni *et al.*, 2018), or intrastriatally perfused with AFDX-116 and PD-102807 (present study) striatal Glu did not rise along with AIMs.

VU0152100, PD-102807 and tropicamide in striatum

Consistent with previous finding that M4 PAM VU0467154 attenuated LID and aberrant plasticity at striato-nigral MSNs in mice and non-human primates (Shen *et al.*, 2015), reverse dialysis of VU0152100 in striatum reduced LID and striato-nigral MSNs activation in vivo. Quite unexpectedly, this was also replicated by

reverse dialysis of the M4 preferential antagonist PD-102807 (and the putative M4 preferential antagonist tropicamide) in striatum, suggesting that both M4 receptor stimulation and blockade have the same outcome on LID and associated pathways. Since PD-102807 concentrations were chosen to generate extracellular levels (~300 nM) well below the affinity for the M1 receptor (1.3-1.6 μ M) (Bohme *et al.*, 2002; Croy *et al.*, 2016), it is unlikely that the effects of PD-102807 are due to an interaction with M1 receptors. Rather, the slight differences in the neurochemical patterns of VU0152100 with respect to M4 antagonists would point to an involvement of different sets of striatal M4 receptors. Striatal M4 receptors are expressed presynaptically on ChIs, where they operate as autoreceptors (Bonsi *et al.*, 2008; Zhang *et al.*, 2002), and postsynaptically, either on glutamatergic terminals where they inhibit Glu release (Pancani *et al.*, 2014), or striato-nigral MSNs where they modulate D1 signalling, Ca^{2+} channels, NMDA currents, and release endocannabinoids that retrogradely modulate DA release (Moehle *et al.*, 2019). Opposite actions have been attributed to postsynaptic M4 receptors at striato-nigral MSNs. In dissociated striato-nigral MSNs, M4 receptor stimulation enhanced excitability via opening of Ca_v1 channels, thus facilitating a subsequent stimulation induced by a D1 receptor agonist (Hernandez-Flores *et al.*, 2015). This is consistent with the increase of L-type Ca^{2+} currents mediated by M4 receptors in atrial cells (Pemberton *et al.*, 1995). Conversely, a negative interaction between M4 and D1 receptors on cAMP production, Ca^{2+} influx at dendritic NMDA receptors, and LTP modulation has been described at striato-nigral MSNs in slices (Shen *et al.*, 2015). Supporting a postsynaptic action at facilitatory M4 receptors on striato-nigral MSNs (Hernandez-Flores *et al.*, 2015), PD-102807 and tropicamide, in addition to LID and nigral GABA, also prevented the LID-associated elevation of nigral and striatal Glu, replicating the neurochemical profile of intrastriatal SCH23390, which blocks D1 receptors on striato-nigral MSNs (Mela *et al.*, 2012). Moreover, AFDX-116 counteracted LID and nigral GABA release inhibition induced by PD-102807, as if the elevation of striatal ACh levels produced by M2 autoreceptor blockade surmounted the postsynaptic M4 blockade and/or functionally counteracted it by overstimulating postsynaptic M1 receptors (Galarraga *et al.*, 1999). Finally, an fMRI study revealed that systemic M4 PAMs did not affect the D1 agonist-induced changes in cerebral blood volume (CBV), a hemodynamic response reflecting changes in neuronal activity, in striatum while inhibiting the amphetamine-induced CBV changes and DA release in the same area, along with the accompanying hyperlocomotion (Byun *et al.*, 2014). Thus, the main action of M4 PAMs in striatum has been proposed to be presynaptic, i.e. the modulation of DA release, and not postsynaptic (Moehle *et al.*, 2019; Moehle *et al.*, 2017). We can therefore speculate that VU0152100 reduced LID and striato-nigral MSNs activation by inhibiting ectopic DA release from serotonergic terminals, the predominant site of L-DOPA conversion to DA in the DA-depleted striatum (Bastide *et al.*, 2015), or even by rescuing M4 autoreceptor function in ChIs, which is inhibited due to upregulation of RGS4 proteins (Shen *et al.*, 2015). The failure of intrastriatal M4 PAM VU0152100 in modulating striatal Glu release would rule out the possibility that the inhibitory control operated by M4 receptors on Glu terminals (Pancani *et al.*, 2014) contributes to the antidyskinetic effect of VU0152100.

VU0152100 and PD-102807 in SNr

Intranigral reverse dialysis of VU0152100 prevented LID and the accompanying rise of nigral GABA release. This is consistent with the finding that M4 PAM VU0467154 inhibited the D1 agonist-induced GABA release from striato-nigral MSNs terminals *in vitro*, and CBV in SNr neurons *in vivo* (Moehle *et al.*, 2017). The profile of intranigral VU0152111 confirms the role of the cholinergic afferents arising from PPN in the modulation of D1 signaling in SNr (Moehle *et al.*, 2017) and shows that this modulation shapes LID. Indeed, the neurochemical responses to intranigral VU0152100, also considering the inhibition of striatal Glu, are consistent with the view that M4 receptor stimulation inhibits GABA release from striato-nigral MSN terminals, thus limiting the overinhibition of nigro-thalamic GABAergic neurons (i.e. the nigral output) associated with LID. *In vitro* electrophysiological experiments in nigral slices showed that the M4 inhibition of nigral D1 signalling and GABA release is tonically active (Moehle *et al.*, 2017). However, in the present *in vivo* model, blockade of nigral M4 receptors with tropicamide did not alter nigral LID and nigral GABA and Glu release, suggesting that endogenous ACh acting at nigral M4 receptors does not contribute to LID. Nonetheless, we should consider that LID is already maximal in these animals, thus making it difficult to

capture further increase of AIMS.

Conclusive remarks

Previous studies showed that striatal ChIs contribute to LID (Bordia *et al.* , 2016; Ding *et al.* , 2011; Won *et al.* , 2014) and that unspecific muscarinic antagonists dicyclomine and atropine prevented LID expression (Bordia *et al.* , 2016; Ding *et al.* , 2011). Consistently, intrastriatal telenzepine, PD-102807 and tropicamide attenuated LID and striato-nigral MSNs activation, further indicating that that M1 and M4 receptors are involved. Moreover, in keeping with the view that muscarinic receptors also mediate an inhibitory action of endogenous ACh on LID (Bordia *et al.* , 2016), potentiation of cholinergic transmission at striatal M4 receptors resulted in significant LID and striato-nigral MSNs inhibition. This suggest that M4 receptors, possibly located on different neuronal elements, can both facilitate and inhibit LID. During LID, the action of endogenous ACh at postsynaptic M4 receptors seems to prevail. Nonetheless, pharmacological potentiation of striatal M4 transmission during LID favors/unmasks other sets of M4 receptors, located on different neuronal elements, shifting the cholinergic control towards LID inhibition. This adds to a similar control in SNr. In fact, in line with previous evidence that PPN cholinergic inputs to SNr affect basal ganglia functions (Moehle *et al.* , 2017; Xiao *et al.* , 2016), the present study shows for the first time that nigral M4 receptor stimulation inhibited LID and nigral output. Altogether, these data reinforce the view that the M4 receptor is a promising target in LID therapy (Moehle *et al.* , 2019), providing *in vivo* evidence for powerful M4 receptor modulation of striato-nigral MSNs at both the striatal and nigral levels.

Author contributions

AB and CAP performed surgery, microdialysis experiments and HPLC analysis; MM and AB conceived the study and wrote the manuscript.

Conflict of interests : AB is postdoc at the University of Ferrara, CAP is PhD student at the University of Ferrara, MM is employed by the University of Ferrara.

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Figure legends

Figure 1. Behavioral and neurochemical effects of reverse dialysis of telenzepine in the dopamine-depleted striatum of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral substantia nigra reticulata (SNr). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with telenzepine (100 nM) through the probe. Telenzepine perfusion started 40 min prior to L-DOPA administration and continued until the end of experiment. A group treated with telenzepine alone was also included. ALO AIMs were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMs calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B) and glutamate (Glu; C-D) levels in dialysates were monitored in SNr and striatum. Data are means ± SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMs score using the Mann-Whitney test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-D). *p<0.05 different from Ringer + L-DOPA 6 mg Kg⁻¹; °p<0.05, °°p<0.01, different from Telenzepine 100 nM + saline.

Figure 2. Behavioral and neurochemical effects of reverse dialysis of PD-102807 in the dopamine-depleted striatum of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral substantia nigra reticulata (SNr). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with PD-102807 (3 µM) through the probe. PD-102807 perfusion started 40 min prior to L-DOPA administration and continued until the end of experiment. A group treated with PD-102807 alone was also included. ALO AIMs were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMs calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B) and glutamate (Glu; C-D) levels in dialysates were monitored in SNr and striatum. Data are means ± SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMs score using the Mann-Whitney test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-D). **p<0.01 different from Ringer + L-DOPA 6 mg Kg⁻¹; °°p<0.01, different from PD-102807 3 µM + saline.

Figure 3. Behavioral and neurochemical effects of reverse dialysis of tropicamide in the dopamine-depleted striatum of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral substantia nigra reticulata (SNr). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with tropicamide (100 nM) through the probe. Tropicamide perfusion started 40 min prior to L-DOPA administration and continued until the end of experiment. A group treated with tropicamide alone was also included. ALO AIMs were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMs calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B) and glutamate (Glu; C-D) levels in dialysates were monitored in SNr and striatum. Data are means ± SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMs score using the Mann-Whitney test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-D). *p<0.05, **p<0.01, different from Ringer + L-DOPA 6 mg Kg⁻¹; °°p<0.01, different from Tropicamide 100 nM + saline.

Figure 4. Behavioral and neurochemical effects of reverse dialysis of VU0152100 in the dopamine-depleted striatum of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral substantia nigra reticulata (SNr). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with VU0152100 (100 µM) through the probe. VU0152100 perfusion started 40 min prior to L-DOPA administration and continued until the end of experiment. A group treated with VU0152100

alone was also included. ALO AIMS were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMS calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B) and glutamate (Glu; C-D) levels in dialysates were monitored in SNr and striatum. Data are means \pm SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMS score using the Mann-Whitney test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-D). * $p < 0.05$; ** $p < 0.01$ different from Ringer + L-DOPA 6 mg Kg⁻¹; ° $p < 0.05$, °° $p < 0.01$, different from VU0152100 100 μ M + saline.

Figure 5. Behavioral and neurochemical effects of reverse dialysis of AFDX-116 in the dopamine-depleted striatum of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral substantia nigra reticulata (SNr). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with AFDX-116 (200 nM) or AFDX-116 (200 nM) + PD-102807 (3 μ M) through the probe. PD-102807 perfusion started 40 min prior to L-DOPA administration whereas perfusion with AFDX-116 started 60 min prior to L-DOPA or 20 min prior to PD-102807 and continued until the end of experiment. A group treated with AFDX-116 alone was also included. ALO AIMS were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMS calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B) and glutamate (C-D) levels in dialysates were monitored in striatum and SNr. Data are means \pm SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMS score using the Mann-Whitney test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-F). * $p < 0.05$, ** $p < 0.01$ different from Ringer + L-DOPA; # $p < 0.05$ different from PD-102807 3 μ M + L-DOPA; ° $p < 0.05$; °° $p < 0.01$ different from VU0152100 100 μ M + saline (B-D) or PD-102807 3 μ M + saline (E-F).

Figure 6. Behavioral and neurochemical effects of reverse dialysis of VU0152100 or PD-102807 in the substantia nigra pars reticulata (SNr) of dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned dorsolateral striatum and another in ipsilateral SNr. Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg kg⁻¹ plus 12 mg kg⁻¹ benserazide, i.p.) or in combination with VU0152100 (100 μ M) or PD-102807 (3 μ M) through the nigral probe. VU0152100 or PD-102807 perfusion started 40 min prior to L-DOPA administration and continued until the end of experiment. A group treated with VU0152100 or PD-102807 alone were also included. ALO AIMS were scored for 1 min every 20 min over 120 min after L-DOPA administration, then cumulative ALO AIMS calculated (A). Dialysate samples were collected every 20 min for 120 min after L-DOPA administration, and GABA (B, E) and glutamate (Glu; B-F) levels in dialysates were monitored in SNr or striatum. Data are means \pm SEM of 8 rats per group. Data were statistically analyzed as cumulative ALO AIMS score using the Kruskal-Wallis test for non parametric ANOVA followed by the Dunn test (A) or as AUC values using ANOVA followed by the Newman-Keuls test (B-F) * $p < 0.05$, ** $p < 0.01$ different from Ringer + L-DOPA; # $p < 0.05$ different from PD-102807 3 μ M + L-DOPA; ° $p < 0.05$; °° $p < 0.01$ different from VU0152100 100 μ M + saline (B-D) or PD-102807 3 μ M + saline (E-F).







