Università degli Studi di Cagliari

PHD DEGREE

IN NEUROSCIENCE

Cycle XXXII

Evaluation of pre- and post-synaptic events involved in the development of L-DOPA-induced dyskinesia in Parkinson's Disease.

Scientific Disciplinary Sector(s)

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List of abbreviations

5-HT: 5-hydroxytryptamine

6-OHDA: 6-hydroxydopamine

AADC: Aromatic L-amino acid decarboxylase

AC: Adenylyl cyclase

Ach: Acetylcholine

AIMs: Abnormal involuntary movements

ALO: Axial-limb-orolingual

AP: Antero-posterior

BG: Basal ganglia

cAMP: Cyclic adenosine monophosphate

CB_1R: Cannabinoid receptors type 1

ChaT: Choline acetyltransferase

ChI-D_2RKO: Cholinergic interneurons- D_2 receptor knock out

ChIs: Cholinergic interneurons

COMT: Catecol-O-metiltrasferasi

CPu: Caudate-Putamen nucleus

D_1R: Type 1 dopamine receptors

D_2R: Type 2 dopamine receptors

DA: Dopamine

DAR: Dopaminergic receptors

DARPP-32: Dopamine and cAMP-regulated neuronal phosphoprotein-32

dMSNs: Direct GABAergic medium spiny neurons

DV: Dorso-ventral

eCB-LTD: endocannabinoid-mediated-LTD

ERK: Extracellular signal-regulated kinase

fEPSP: Excitatory post-synaptic field potential

FSI: GABAergic fast spiking interneurons

GPe: External portion of the globus pallidus

GPi: Internal portion of the globus pallidus

HFS: High frequency stimulations

IEGs: Immediate early genes

iMSN-D_2RKO: Indirect medium spiny neurons- D_2 receptor knock out

iMSNs: Indirect GABAergic medium spiny neurons

KO: Knock out

LB: Lewy bodies

L-DOPA: L-3,4-dihydroxyphenylalanine

LFS: Low frequency stimulations

LFS-LTD: Low-frequency stimulation-induced long-term depression

LID: L-DOPA-induced dyskinesia

LTD: Long-term depression

LTP: Long-term potentiation

MAO-B: Monoamine oxidase type B

MFB: Medial forebrain bundle

mGluR: metabotropic glutamate receptors

ML: Medio-lateral

MRI: Magnetic resonance imaging

MSK: Mitogen- and stress-activated kinase 1

MSNs: GABAergic medium spiny neurons

NHP: Non-human primate

NMDA: N-methyl-D-aspartate

PB: phosphate buffer

PD: Parkinson's disease

pERK: Phospho- Extracellular signal-regulated kinase

PET: Positron emission tomography

PKA: Protein kinase type A

rpS6: Ribosomal protein S6

SNc: Substantia nigra *pars compacta*

SNr: Substantia nigra *pars reticulata*

SPECT: Single photon emission computed tomography

SSRIs: Selective serotonin reuptake inhibitors

STN: Subthalamic nucleus

TBS: Tris buffered saline

TH: Tyrosine hydroxylase

VMAT-2: Vesicular monoamine transporter type 2

WT: Wild type

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Introduction

1. Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder (De Lau et al., 2006) originally described by James Parkinson in his work *An Essay on the Shaking Palsy* published in the 1817.

Nowadays, PD affects approximately 1% of the population over 60 years old (De Lau et al., 2006) and the 0.1% of the entire population (von Campenhausen et al., 2005). Early onset of the pathology (< 50 years old of age) is rare and is reported in about the 4-10% of PD patients (Ferguson et al., 2015; Vela et al., 2016). Although several pathological changes in cortical and subcortical brain areas have been reported (Jenner, 2008), the most consistent neuropathological signs found in all PD patients are: (i) the extensive loss of dopaminergic neurons forming the nigrostriatal pathway, (ii) the presence of intraneuronal misfolded and insoluble proteins in the forms of Lewy bodies (LB; **Fig. 1**) (Dickson, 2018).

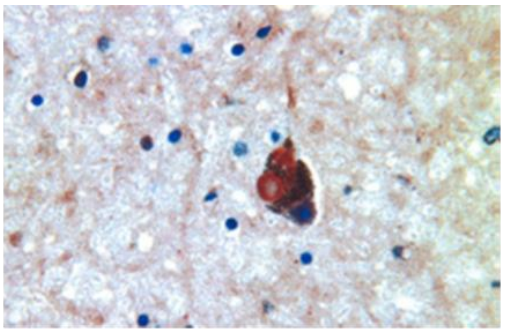

Fig. 1: Image showing Lewy bodies in a degenerating neuron. Blue dots represent alpha-Synuclein staining.
From: Sveinbjornsdottir (2016)

Diagnosis of PD is typically carried out when patients start suffering of disabling motor deficits. Importantly, when motor symptoms appear, approximately the 50-70% of striatal dopaminergic terminals are already lost (Burke and O'Malley, 2013). Resting tremors, bradykinesia and rigidity represent the cardinal PD symptoms and affect almost all PD patients. Beside the well-characterized motor symptoms, PD patients also suffer of a wide range of non-motor complications which are usually characterized by an early onset and critically affect the quality of life of affected patients (Schrag et al, 2015). **Table 1** summarises and briefly describes the most common motor and non-motor symptoms observed in PD patients.

Table 1: Principal motor and non-motor PD symptoms

Symptoms	Description	References
Bradykinesia	Slowness and difficulties in movement initiation. Hypomimia and micrographia are common.	[1] [2] [3]
Tremors	Asymmetrical onset which affects primarily one arm and/or hand (pill-rolling type are common). With the disease progression, involvement of the homolateral limb (≈80%) and the contralateral body side has been reported.	[1] [2] [3]
Rigidity	Diffuse muscles stiffness	[1] [2] [3]
Gait impairments	Shuffling gait accompanied by blocking (>60%), hesitancy and gait festination. Higher risk of falling among PD patients have been reported (>62-69%).	[1] [2] [3]
Oral motor disorders	Impaired speech fluency characterized by quiet and hurried speech (>50%), swallowing problems (40-80%), dribbling of saliva (25%).	[7]
Dystonia	Unilateral equinovarus foot position, writer's cramp, oro-mandibular dystonia. Usually, dystonia is reported 10 years before PD diagnosis.	[1] [2]
Sleep disturbances	Excessive daytime sleepiness, dream-enacting behaviour (≈27-35 %), increase of nightmare, fractionated sleep with frequent awakening in the night.	[3] [4] [6]
Gastrointestinal disfunction	Decrease of bowl motility (≈60-70%), post prandial fullness, gastric retention, constipation (>80%).	[3] [5]
Mood depression	Anxiety and depression (≈25%) accompanied by anhedonia, mood fluctuation, sadness, loss of energy.	[3] [6]
Senso-motor impairment	Loss of smell (Anosmia, ≈80%) and taste.	[3] [4] [6]
Autonomic disfunction	Excessive sweating; Orthostatic hypotension (≈30-40%); urinary control disturbances (higher frequency, urgency and incontinence); reduced saliva secretion.	[3] [4] [6]
Psycosis	Visual hallucinations (worsen by DA replacement therapy) and paranoid illusions have been reported.	[3]
Cognitive impariment	Mild cognitive impairment and dementia (worsen with PD progression) are commonly observed in PD.	[3] [4] [6]

References: [1] Postuma et al., 2015; [2] Tysnes and Storstein, 2017; [3] Obeso et al., 2017; [4] Lang, 2011 [5] Abbott et al., 2001 [6] Sveinbjornsdottir, 2016; [7] Canter, 1963.

1.1 Aetiology and diagnosis

Over the last decades, multiple genetic and environmental factors have been directly correlated with an increased risk of PD. In this respect, a large body of evidences suggest as most of PD cases may have a multifactorial aetiology (Ramsden et al., 2001). Several genes (Warner et al., 2003) as well as environmental factors (i.e. pesticides, coffee, cigarette, gender, beta-blockers, head injuries) have been found to be either positively or negatively involved in PD development (Warner et al., 2003; Bellou et al., 2016). These evidences partially explain why PD has always been considered a complex and heterogenous pathology in terms of symptoms, onset, clinical progression, treatment efficacy and related side effects. In this respect, a big effort has been made to classify PD in several subcategories characterized by specific clinical traits (e.g. genetic vs sporadic; tremor-dominant vs no-tremor), pathology progression and symptoms (Obeso et al., 2017).

Today, PD is diagnosed through a subjective symptom-based analysis performed by expert clinicians (see **Table 2** for details) which can misguidedly diagnose as PD other pathologies which share similar symptoms (e.g. tremor disorders, atypical parkinsonian conditions, secondary parkinsonism) (Hughes et al., 1992; Tolosa et al., 2006).

As more reliable biomarkers become established, problems related to the diagnosis and clinical heterogeneity of PD will be attenuated as has occurred with many other neurological disorders. Fortunately, objective results based on modern and reproducible imaging techniques (i.e. magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT)) are now frequently used to support clinical diagnosis (Brooks, 2010).

Table 2: Clinical diagnostic criteria for Parkinson's Disease

Step 1. Diagnosis of Parkinson's Disease

Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude)

And at least one of the following:

- Muscular rigidity
- 4-6 Hz rest tremor

Step 2. Supported criteria (at least two)

- No motor-symptoms (constipation, sleep-disturbances, anxiety ecc.)
- Responsiveness to dopaminergic therapy
- Presence of L-DOPA induced dyskinesia
- Asymmetric rest tremor
- Olfactory loss

Step 3. Absolute exclusion criteria (none of them must be present)

- Cerebellar abnormalities
- Supra nuclear gaze palsy
- Frontotemporal cognitive changes
- Slow progression or remission
- MPTP exposure
- Absence of L-DOPA response
- Normal DAT scan
- History of repeated: (a) strokes; (b) head injury; (c) encephalitis; (d) Neuroleptic treatment at onset of the symptoms

Step 4. Absence of red flags

- Early gait impairment
- Absence of progression
- Early bulbar dysfunction
- Respiratory dysfunction
- Severe automatic failure during the first year of PD
- Pyramidal tract signs
- Bilateral

References: Postuma et al. 2015; Tysnes and Storstein, 2017; Hughes et al., 1992

1.2 Basal Ganglia

The basal ganglia (BG) are a group of subcortical nuclei involved in goal-directed behaviours, habit formation, initiation and selection of appropriate movements in response to external stimuli (Florio et al., 2018). Alteration in BG functions have been found in several diseases, including PD, Huntington's disease, schizophrenia, dystonia, attention-deficit-hyperactivity-disorders, and depression (Surmeier et al., 2014). Anatomically, the BG may be divided in three groups: i) nuclei which received input form cortex and thalamus

(the Caudate-Putamen nucleus (CPu) and the nucleus accumbens); ii) nuclei which project back to the thalamus (the internal portion of the globus pallidus (GPi) and the substantia nigra *pars reticulata* (SNr)); and iii) intrinsic nuclei (the external portion of the globus pallidus (GPe), the subthalamic nucleus (STN), the substantia nigra *pars compacta* (SNc)) (Alexander, 1986).

The CPu is the major component of BG structures forming the motor circuit and is structurally formed by GABAergic medium spiny neurons (MSNs; ≈95%), cholinergic interneurons (ChIs; ≈1-2%), and GABAergic fast-spiking interneurons (FSI; ≈3-4%) (Pisani et al., 2007). The CPu receives complex sensory-motor inputs coming from widespread regions of the cerebral cortex (primary motor cortex, supplementary motor area, cingulate motor cortex and premotor cortex) and elaborates output signals convey by MSNs.

Striatal functions are principally regulated by the neuromodulator dopamine (DA) which is released from the massive dopaminergic innervations coming from the SNc. Together with acetylcholine (Ach) and GABA, released respectively from ChIs and FSI, DA affects MSNs responsiveness as well as the strength of cortico-striatal synapses (Calabresi et al., 2007; DeLong and Wichmann, 2009; Surmeier et al., 2014). Eventually, striatal MSNs send this re-elaborate information back to the ventral thalamus and the supplementary motor cortex by projecting to the canonical BG output nuclei, the GPi and the SNr (Alexander, 1985). In conventional models of BG (Albin et al., 1989; DeLong, 1990), MSNs are divided in two discrete sub-populations characterized by their differential expression of the two principal dopaminergic receptors (DAR) and by their output targets. MSNs forming the so-called direct pathway (dMSNs) express the type 1 of DAR (D_1R) and project monosynaptically to the SNr and the GPi. On the other hand, MSNs forming the so-called indirect pathway (iMSNs) express the type 2 of DAR (D_2R) and project to the GPe. Then, GABAergic neurons located in the GPe project to the GPi and SNr either monosinaptically or via the intercalated STN. Moreover, the STN receives motor inputs directly from the motor cortex (''hyperdirect'' pathway) likely to quickly delete the execution of inappropriate movements (Nambu, 2004). **Figure 2** shows a schematic representation of the motor cortex circuit. Accordingly to these models, release of DA from the SNc would facilitate movements by the concomitant D_1-mediated stimulation of dMSNs and the D_2-mediated inhibition of iMSNs. Following degeneration of the SNc, DA is no more able to balance MSNs activity causing the overactivation of the indirect pathway. This in turn inhibits glutamatergic neurons located in the thalamus and cortex and promotes the appearance of PD motor deficits (Albin et al., 1989; DeLong, 1990; Obeso et al., 2000). Although, conventional BG models have been useful to understand the relation between movement disorders and BG, they do not entirely consider several key aspects such as the dynamicity of the system, the complexity of the neuronal networks (within and between the BG) or alterations involving the autonomous generation of neuronal spikes (e.g dopaminergic neurons fire regularly at 5Hz of rate providing a physiological tone of dopamine which cannot be replicate by L-DOPA). Differently from what we should expect from classical models, ablation of either the ventrolateral thalamus or the GPe does not cause any motor

complication. This result demonstrates as the specific functions exert by each BG nucleus is still partially unclear and deserve further investigations.

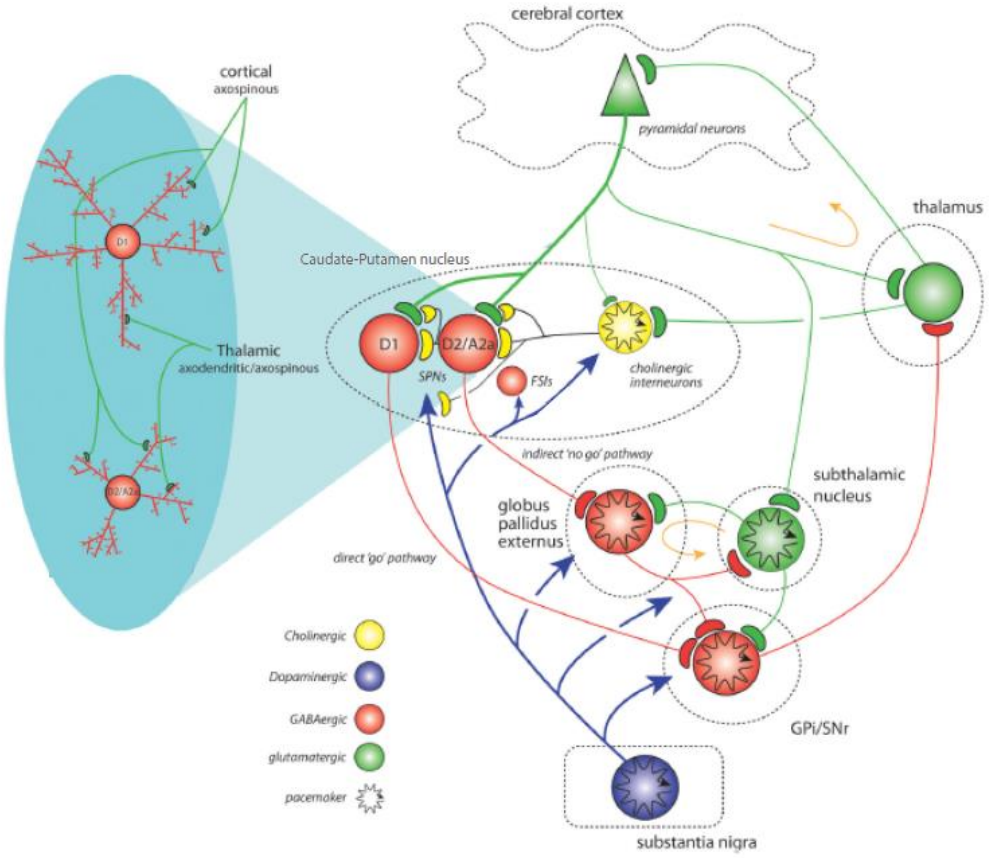

Figure 2. Schematic of the motor circuit. Figure depicts a schematic representation of the BG motor circuit and highlights the neuronal components of the Caudate-Putamen nucleus (CPu).

Excitatory glutamatergic projections arising from the primary motor cortex, supplementary motor area, cingulate motor cortex, premotor cortex and the thalamus form synaptic contacts with GABAergic medium spiny neurons (MSNs) and cholinergic interneurons (ChIs) located in the CPu. Moreover, cortex-related areas directly project onto the subthalamic nucleus (STN) forming the so called hyperdirect pathway.

The globus pallidus internus (GPi) and the substantia nigra *pars reticulata* (SNr), the output nuclei of the CPu, received either inhibitory inputs directly from D_1-expressing MSNs (dMSNs; direct pathway) or a mix of inhibitory/excitatory inputs from the D_2/ A_{2A} -expressing MSNs, which together with the GP externus (GPe) and the STN form the so called indirect pathway. Dopamine, acetylcholine and GABA released respectively from striatonigral afferents, ChIs and fast-spiking interneurons (FSI) regulate the GABAergic output activity of the CPu. Eventually, the SNr and the GPi send inhibitory afferents to the ventrolateral thalamus which, in turn complete the circuit, providing glutamatergic excitatory inputs to the motor cortex.

Figure has been modified from Handbook of Basal Ganglia Structure and Function (II edition; 2016)

2. DA replacement therapies and L-3,4-dihydroxyphenylalanine

Considering the complexity of PD, it is not surprising that medications currently available are limited in their therapeutic actions and do not guarantee a predictable response among PD patients.

Despite important efforts, today there are no disease-modifying or neuroprotective therapies clinically accepted for PD (AlDakheel et al., 2014).

Since its introduction in the late '60, the DA precursor L-3,4-dihydroxyphenylalanine (L-DOPA) it has been considered the first-choice treatment for relieving PD motor symptoms (Goetz, 2011). L-DOPA acts by restoring striatal DA content and is always co-administrated with aromatic L-amino acid decarboxylase (AADC) inhibitors to reduce its peripherical degradation and to extend its plasma half-life (Contin and Martinelli, 2010; LeWitt, 2015). In this respect, it has been reported as AADC inhibitors almost triple L-DOPA bioavailability (Contin and Martinelli, 2010; LeWitt, 2015).

Unfortunately, PD patients receiving chronic administrations of L-DOPA suffer of severely disabling and irreversible side effects which include motor fluctuations and L-DOPA-induced dyskinesia (LID) (Bastide et al., 2015). Motor fluctuations refer both to the unpredictable changes in the duration of the anti-parkinsonian effects of L-DOPA, which switch from ON (full effect) to OFF (no effect) periods, and to wearing-off phenomena (Schrag and Quinn, 2000). On the other hand, LID refers to a wide variety of irregular and purposeless abnormal involuntary movements (AIMs) which include chorea, dystonia, ballism and myoclonus (Tran et al., 2018).

Dopaminergic agonists (i.e. bromocriptine, pergolide, pramipexole, ropinirole) are generally used either during the early or late phases of PD to reduce L-DOPA consumption and to alleviate its side-effects. Differently from L-DOPA, dopaminergic agonists stimulate preferentially D_3 and D_2R, act for longer periods and do not require any metabolic conversion to be active (Rao et al., 2006). Unfortunately, they are less efficacious compared to L-DOPA in relieving PD motor symptoms (Rao et al., 2006) and serious adverse side effects have been reported following chronic treatment (Antonini et al., 2009)

Inhibitors of the monoamine oxidase-B (MAO-B; e.g. rasagiline, selergiline), as well as of the catechol-O-methyltransferase (COMT, e.g. entacapone, tolcapone, opicapone), two enzymes involved in DA metabolism, are also used in association with L-DOPA to elevate its concentration at central level. MAO-B inhibitors do not show substantial adverse effects and are principally used in patients with early PD (Rao et al., 2006). On the other hand, COMT inhibitors alleviate L-DOPA-induced motor fluctuations (reduction of OFF periods) but, in rare case, their use it has been associated with hepatotoxicity and fatal hepatic failure (Olanow et al., 2007).

2.1. L-DOPA mechanisms of action

Compelling evidences suggest that L-DOPA effects critically depend on the relative presence of nigrostriatal neurons. In this respect, while in healthy animals, L-DOPA produces modest elevation of striatal DA, in DA-denervated animals, L-DOPA significantly elevates striatal DA, especially when nigrostriatal neurons are almost completely degenerated (Kostrzewa et al., 2005).

It has been proposed that, once dopaminergic terminals are gone, exogenous L-DOPA is converted and released by non-dopaminergic terminals equipped with both the AADC and the vesicular monoamine transporter-type 2 (VMAT-2), two proteins involved respectively in the metabolism and storage of L-DOPA-derived DA (Deurwaerdère et al., 2017). Over the last decades, the involvement of either the adrenergic (Issidorides et al., 2004, Navailles et al., 2014), serotoninergic (Hollister et al., 1979; Lopez et al., 2001; Nevalainen et al., 2014) or the histaminergic (Deurwaerdère et al., 2017) system it has been associated with the conversion and release of L-DOPA-derived DA. Among these systems, studies performed in DA depleted animals, in which striatal 5-hydroxytryptamine (5-HT) terminals were either partially or completely removed, revealed the existence of a strong correlation between L-DOPA effects and the presence of 5-HT terminals (Hollister et al., 1979; Lopez et al., 2001; Nevalainen et al., 2014). Interestingly, increased level of DA were reported in several brain areas receiving 5-HT projections following L-DOPA, together with reduced levels of 5-HT (Carta et al., 2007; Navailles et al., 2011), supporting the notion that, when dopaminergic neurons are degenerate, exogenous L-DOPA is converted and released principally from serotonin terminals, and competes with endogenous 5-HT for the loading into synaptic vesicles.

Ultimately, L-DOPA should not simply be considered as an inactive precursor of DA rather as a real neuromodulator which is produced, stored and released from presynaptic catecholaminergic terminals and exerts its actions by binding pre and postsynaptic β-adrenergic, alpha-2 and 5-HT-2 receptors (Yoshimi and Yoshio, 1993; Hornykiewicz, 2002; Porras et al., 2014).

3. L-DOPA-Induced dyskinesia

LID have been observed and reported in scientific journal soon after the introduction of L-DOPA into the market (Barbeau, 1969; Klawans and Gamin, 1969; Duvoisin, 1974). It has been reported as LID affects approximately the 20-37% of PD patients after 5 years of L-DOPA treatment, and up to the 80% of the patients by 10 years of treatments (Rascol et al., 2000; Schrag and Quinn, 2000; Scott et al., 2016). Importantly, although L-DOPA is the compound producing the highest pro-dyskinetic effects, all dopaminergic agonists may generate dyskinetic behaviours in chronic treated PD patients.

LID is clinically classified in three categories which are based on their movement patterns and the different temporal correlation between AIMs onset and L-DOPA intake: (a) peak-dose dyskinesia, observes shortly after L-DOPA administration, (b) biphasic dyskinesia, that emerges when L-DOPA levels are rising or falling, and (c) OFF dyskinesia which is visible when L-DOPA positive effects are completely gone (Fabbrini et al., 2007; Bastide et al., 2015; Tran et al., 2018). Once established, AIMs occur upon every administration of L-DOPA or dopaminergic agonists (Jenner, 2008).

3.1. Animal models of LID

Despite efforts made to develop better animal models of LID, today peak-dose dyskinesia is the only form of LID reliably reproduced by animal models. Indeed, animal models of either diphasic dyskinesia or OFF dyskinesia are currently not available, and the mechanisms underlying their appearance remain to be elucidated (Cenci and Crossman, 2018). Following paragraphs offer a brief overview of the animal models which are included in the present thesis.

3.1.1 Rat model

Since its first description in the early 70' (Ungerstedt, 1971), the unilateral 6-hydroxydopamine (6-OHDA) rat model was considered the gold standard model for the study of PD (Bastide et al., 2015). 6-OHDA is a structural analogue of the catecholamine DA that, when administrated locally either in the CPu, SNc or in the medial forebrain bundle (MFB), triggers a series of powerful neurotoxic events leading to the death of dopaminergic neurons. 6-OHDA-lesioned rats suffer of motor deficits (bradykinesia, gait impairments, rigidity) which resemble those of PD patients (Simola et al., 2007).

In the late 1990s, Cenci and co-workers reported that unilateral 6-OHDA-lesioned rats treated with L-DOPA expressed AIMs affecting the side of the body contralateral to the lesion (Cenci et al., 1998). AIMs were observed soon after systemic L-DOPA administration (peak-dose dyskinesia) and increased in severity after 2-3 weeks of daily L-DOPA treatment. Qualitatively, 6-OHDA-lesioned rats express

dyskinetic behaviours affecting the forelimb (limb AIMs), the trunk (axial AIMs) and the masticatory muscles (orolingual AIMs) (Cenci and Lundblad, 2007).

Importantly several analogies can be found between the rat and human peak-dose dyskinesia, such as: (1) the time course in which AIMs are present; (2) the capacity to disrupt the execution of daily motor tasks (Lundblad et al., 2002); (3) the positive correlation between AIMs magnitude and L-DOPA dosage (Francardo et al., 2011); (4) the capacity of anti-dyskinetic agents to partially dampened LID (e.g. amantadine, clozapine) (Bido et al., 2011; Lundblad et al., 2002). It is important to note that 6-OHDA-lesioned rats develop LID only when approximately more than the 80% of striatal DA terminals are lost (Winkler et al., 2002).

3.1.2 Mouse models

Similarly to rats, mice unilaterally lesioned with 6-OHDA develop AIMs when repeatedly treated with L-DOPA. AIMs in mice are comparable in terms of topographic distribution, frequency and intensity to that observed in rats (Cenci and Crossman, 2018). Importantly, also the mouse model of LID is characterized by a robust predictive validity since dyskinetic mice well respond to compounds having anti-dyskinetic effects in humans (i.e. amantadine, buspirone, riluzole) (Lundblad et al., 2005). Nevertheless, important differences between the mouse and rat models exist, since: i) mice show an higher postoperative mortality which can be attenuated by the careful nursing of animals (Tronci and Francardo, 2018); ii) striatal 6-OHDA-lesioned mice requires a dosage 3-4-fold higher of L-DOPA in order to express AIMs comparable in intensity to that observed in MFB 6-OHDA-lesioned mice; (3) 6-OHDA-lesioned mice reach a constant level of dyskinetic movements soon after 2-3 daily injection of L-DOPA (Lundblad et al., 2005). Nowadays, the usage of the unilateral 6-OHDA-lesioned mouse model of LID has increased notably thanks to the possibility to generate genetically modified mouse models. In this respect, the Pitx-3-deficent *aphakia* mouse is a perfect example. Indeed, the lack of the transcriptional factor Pitx3 (required for the differentiation of DA neurons) results in the selective loss of nigrostriatal projections and in the appearance of the cardinal parkinsonian motor deficit (van den Munckhof et al., 2003; Ding et al., 2010).

3.3 Pathological hallmarks of LID

A large body of evidences suggest that LID is correlated with: (1) the pulsatile stimulation of post-synaptic striatal DAR; (2) alterations involving gene expression and protein production; (3) modifications in the release of non-dopaminergic neurotransmitters (e.g. Ach, glutamate, 5-HT) and (4) alterations in the morphology and reciprocal interaction of cortico-striatal synapses (Bastide et al., 2015).

When considering the pathological events which may be associated with LID, it is important to bear in mind their temporal location. Indeed, some are visible only after prolonged period of DA depletion, while others

after L-DOPA administration. As an example, it is thought that the first exposition to L-DOPA may produce a series of neuronal events (priming phase) which may significantly differ from those arise following subsequent L-DOPA administrations (expression phase) (Morelli and Di Chiara, 1987; Morelli et al., 1989), even though this distinction has been recently questioned (Nadjar et al., 2009).

In the following sections it will be described more in-depth pre- and post-synaptic modifications reported in preclinical animal models of LID.

3.3.1 Presynaptic changes in LID:

The degeneration of nigrostriatal neurons represents the first and most important risk factor correlated with LID. An increasing number of evidences suggest as in advance PD, L-DOPA-derived DA is released from serotoninergic terminals which lack the protein machinery required to tailor striatal DA concentration to external/internal DA-evoking inputs (Carta and Bezard, 2011).

A seminal clinical study performed by de la Fuente-Fernandez and collaborators demonstrated that advanced PD patients affected by severe motor complications showed a more marked pulsatile pattern of DA release when compared to parkinsonian controls well-responding to L-DOPA (de la Fuente-Fernandez et al., 2004). Pulsatile release of DA is thought to be a critical event in LID induction which promotes dramatic changes at post-synaptic level by altering receptor trafficking and by facilitating the appearance of long-term postsynaptic changes (Cenci and Konradi, 2010; Spigolon and Fisone, 2018).

It was reported that either the neurotoxin-induced degeneration or the pharmacological silencing of the serotoninergic system (by stimulation of 5-$HT_{1A/1B}$ receptors) almost completely supressed LID in both L-DOPA naïve and already dyskinetic 6-OHDA-lesioned rats, suggesting as L-DOPA-derived DA is improperly released as 'false neurotransmitter' from serotoninergic terminals (Tanaka et al., 1999; Carta el al., 2007; Muñoz et al., 2008). In line with this idea, pharmacological agents that lower serotoninergic transmission either directly (e.g. 5-$HT_{1A/B}$ agonist) or indirectly (e.g. SSRI) were efficacious in reducing AIMs when tested in rodents and non-human primate (NHP) models of LID as well as in PD patients (Eskow et al., 2007; Bezard et al., 2013; Svenningsson et al., 2015; Pinna et al., 2016).

It has been hypothesized that $5HT_{1A/1B}$ receptor agonists act by lowering the amount of L-DOPA-derived DA released from serotoninergic terminals and, consequently, by reducing the aberrant stimulation of post-synaptic DAR (Navailles et al., 2010; Carta and Bezard, 2011; Svenningsson et al., 2015). Besides, stimulation of post-synaptic $5HT_{1A}$ receptors may further contribute to LID reduction by dampening the release of glutamate from striatal thalamocortical afferents (Dupre et al., 2008).

These evidences demonstrating the importance of the presynaptic compartment in LID and may explain why new diagnosed PD patients, as well as partial DA-depleted animals, treated with clinical doses of L-DOPA do not develop LID (Perry et al., 1984; Marras et al., 2004), while completed DA-depleted animals, similarly to

advanced PD patients, manifest LID soon after the initiation of L-DOPA treatment (Carta et al., 2007; Cilia et al., 2014).

3.3.2 Dopamine-related postsynaptic mechanisms in LID:

Despite its origin, once resealed into the synaptic cleft, L-DOPA-derived DA exerts its functions by stimulating either D_1 $G_{\alpha s/olf}$-coupled receptors, located in dMSNs, or D_2 $G_{\alpha i/o}$-coupled receptors located in iMSNs, ChIs and both corticostriatal and dopaminergic terminals.

Preclinical findings have showed that prolong DA deprivation eventually leads to sensitization of DAR, likely in the attempt to counteract the failing signalling (Nadjar et al., 2009; Spigolon and Fisone, 2018). Importantly, DAR sensitization, particularly of D_1R, is critically involved in LID development (Gerfen, 2000; Picconi et al., 2003; Jenner, 2008). In this respect, D_1R sensitization is associated with an enhanced recruitment of D_1R, increased coupling with the $G_{\alpha s/olf}$ proteins and higher concentration of the adenylyl cyclase (AC) (Rangel-Barajas et al., 2011; Marcotte et al., 1994). It has been hypothesized that elevated levels of cyclic adenosine monophosphate (cAMP), generated from AC, over-activate the protein kinase type A (PKA)/ dopamine and cAMP-regulated neuronal phosphoprotein-32 (DARPP-32) signalling pathway which in turn promotes LID. Interestingly, pharmacological inhibition of the PKA (Lebel et al., 2010) as well as silencing of either the AC-type 5 (Park et al., 2014) or the DARPP-32-expressing genes (Santini et al., 2007) were found to reduce AIMs in dyskinetic animals.

The extracellular signal-regulated kinase (ERK) is the protein whose activation has been most frequently correlated with LID. The concomitant stimulation of D_1R and N-methyl-D-aspartate (NMDA) receptors is required for the phosphorylation (and activation) of ERK (pERK) (Valjent et al., 2005; Westin et al., 2007; Fasano and Brambilla, 2011). Importantly, dampening of ERK phosphorylation, as well as of its principal downstream mediator, the mitogen- and stress-activated kinase 1 (MSK1), counteracts the pro-dyskinetic effects of L-DOPA (Santini et al., 2007; Fasano et al., 2010; Feyder et al., 2016). Unfortunately, a global inhibition of ERK pathway is unfeasible in LID treatment since ERK is critically involved in cell survival (Fasano et al., 2010).

In the last decades, several immediate early genes (IEGs) have been causally related to LID. Elevated levels of *c-fos*, Arc, *zif-268* and other IEGs have been observed shortly after L-DOPA administration (Robertson et al., 1991; Andersson et al., 1999; Bastide et al., 2014; Pinna et al., 2016). IEGs play a vital role in cells since they encode transcriptional factors which modulate the expression of long-term responding genes (Robertson et al., 1991).

Particular attention was dedicated to the IEG encoding for the transcriptional factor FosB and its alternatively spliced isoform ΔFosB which was found to be highly expressed in the dorsolateral CPu of dyskinetic monkeys and rodents (Andersson et al., 1999; McClung et al., 2004; Cenci and Konradi, 2010; Bastide et al., 2014; Feyder et al., 2016). Interestingly, intrastriatal infusion of the FosB anti-sense oligonucleotide attenuated the

development of AIMs in rats treated daily for 3 weeks with a therapeutic dose of L-DOPA (Andersson et al., 1999).

For the sake of completeness, it is important to note that events previously mentioned take place in dMSNs and are strictly related to the stimulation of sensitized D_1R. Nevertheless, also D_2R play a critical role in LID as demonstrated by anatomical, pharmacological and electrophysiological evidences. In this respect, administration of selective $D_{2/3}$R agonists promote the development of chorea-like dyskinetic movements in drug-naïve MPTP-intoxicated NHPs (Luquin et al., 1992; Calon et al., 1995) and 6-OHDA-lesioned rats (Delfino et al., 2004), independently from the activation of D_1R on dMSNs (Luquin et al., 1992). On the other hand, administration of selective $D_{2/3}$R antagonist significantly improve LID in 6-OHDA-lesioned rats (Taylor et al., 2005; Shin et al., 2012; Sebastianutto et al., 2016).

Moreover, while D_1R-full knockout (KO) mice showed intact coordination and exhibited a mild hyperactivity (Xu et al., 1994), D_2R-full KO mice suffered of the core PD motor deficits (Baik et al., 1995).

Interestingly, stimulation of Gi-coupled D_2R not only decreases the activity of the canonical cAMP/PKA pathway but also promotes the formation of the Akt/β-arrestin/protein phosphate 2A complex which in turn activates (by phosphorylation) the glycogen synthase kinase 3 (GSK3) (Cenci and Konradi, 2010). The GSK3 is an important kinase that regulates cellular architecture, motility and survival (Beurel et al., 2004) adapting cellular responses to incoming stimuli. It has been reported as DA finely regulates the activation of the GSK3 and as its abnormal phosphorylation is positively correlated with the expression of LID in MPTP-intoxicated NHPs receiving L-DOPA (Morissette et al., 2010)

One should admit that LID studies focused on D_2R have been limited by the wide expression of D_2R on several striatal cell types and by the lack of appropriate experimental tools (Pisani et al., 2007; Bastide et al., 2015).

3.4 Electrophysiology

Electrophysiological studies performed in rodents and NHPs have contributed substantially in improving our understanding of the neuropathological events involved in PD and LID. Long-term forms of synaptic plasticity have been object of numerous investigations since they are critically involved in BG functions (Kreitzer and Malenka, 2008).

In the CPu, DA produces two opposite forms of long-term synaptic plasticity known as long-term depression (LTD) and long-term potentiation (LTP). LTD is promoted by D_2R stimulation and makes glutamatergic synapses less excitable to future stimulations (Calabresi et al., 1997; Kreitzer and Malenka, 2005). On the other hand, LTP is promoted by D_1R stimulation and strengthens cortico-striatal synaptic connections (Surmeier et al., 2014). Reversal of LTP is termed depotentiation and brings synaptic transmission to the naïve

state, making cortico-striatal synapses ready to respond to incoming stimuli (Picconi et al., 2003; Picconi et al., 2018).

Loss of bi-directional plasticity has been frequently observed in movement-related disorders such as PD, Huntington's disease and dystonia (Kreitzer and Malenka, 2007; Shen et al., 2008; Peterson et al., 2010).

In the dorsolateral CPu, the best characterized form of long-term plasticity is the endocannabinoid-mediated-LTD (eCB-LTD). eCB-LTD is generated post-synaptically, at level of MSNs, and expressed pre-synaptically at excitatory thalamo- and cortico-striatal afferents (Choi and Lovinger, 1997) and requires the activation of D_2R, mGluR-5 receptors and L-type calcium channels (Calabresi et al. 1997; Choi and Lovinger, 1997; Gerdeman et al., 2002).

Loss of DA signalling at cortico-striatal synapses results in the elevation of iMSNs firing and the loss of excitatory axospinous glutamatergic contacts (Suarez et al., 2016), events that contribute to the genesis of PD symptoms (Calabresi et al., 2007; Kreitzer and Malenka, 2007; Shen et al., 2008).

In the 2003, Calabresi's group and collaborators described for the first time the critical loss of bi-directional plasticity which occurred both in L-DOPA naïve and in dyskinetic rats unilaterally lesioned with 6-OHDA (Picconi et al., 2003). It was reported as drug naïve 6-OHDA-lesioned rats were unable to express both striatal LTP and LTD, while LTP was observed after chronic L-DOPA treatment. However, differently from what observed in non-dyskinetic rats, dyskinetic rats did not express either depotentiation of previously induced LTP or LTD (Picconi et al., 2003).

One of the limits of this study was the inability to distinguish between synaptic alterations taking place either in the direct or the indirect pathway. In this respect, it was reported as in 6-OHDA-lesioned rats, the delivery of high-frequency stimulations (HFS) in corticostriatal afferents, approach used to promote LTD, revealed that iMNSs, but not dMSNs, where unable to express LTD (Kreitzer and Malenka, 2007). Interestingly, under similar conditions, iMSNs expressed LTP instead of LTD after chronic treatment with L-DOPA (Belujon et al., 2010). Nevertheless others found that DA depletion was able to induce LTP in iMSNs and LTD in dMSNs, respectively (Thiele et al., 2014). Moreover, while in non-dyskinetic 6-OHDA-lesioned rats L-DOPA restores bidirectional plasticity, in dyskinetic rats L-DOPA restored the expression of LTD in the indirect pathway and of LTP in the direct one (Thiele et al., 2014). Thus, despite some differences, switching from bidirectional to unidirectional plasticity is thought to be a critical factor involved in LID (Thiele et al., 2014; Picconi et al., 2003, 2018).

3.5 Cholinergic interneurons

CPu homeostasis strictly depends on the achievement of the right balance between DA and Ach (Lehmann and Langer, 1983). Despite ChIs represent approximately the 2% of the entire striatal population, they are the main source of striatal Ach and are characterized by richly arborized axons with large terminal fields (Pisani et al., 2007).

Interestingly, only a small percentage of ChIs form synapses with surrounding neurons, indicating that Ach acts principally as neuromodulator (Pisani et al., 2007). Differently from other striatal neurons, ChIs have an intrinsic pacemaker activity since they can spike (3-9 Hz) in the absence external stimulations (Bennett et al., 2000).

Phasic and tonic release of Ach is regulated both by DA (through D_5 and D_2R) and Ach (through M_2 and M_4 auto-receptors) (Tanimura et al., 2018).

In PD, DA depletion is initially accompanied by a higher release of Ach principally due to the loss of the muscarinic M_4-mediated inhibitory feedback (Fox, 2013). Elevation of Ach critically affects striatal functions by either reducing or elevating GABA, glutamate or DA release (through M_2, M_3 and nicotinic receptors), and by affecting MSNs activity through the stimulation of both M_4 (selectively located on dMNSs) and M_1 receptors (located in dMNSs and iMSNs) (Shen et al.,2007, 2016). Not surprisingly, lack of D_2R modulation on ChIs was recently associated with deficits related to the expression of striatal LTD (Augustin et al., 2018), while the selective photo-inhibition of striatal ChIs resulted in a significant improvement of parkinsonian symptoms in 6-OHDA-lesioned mice (Maurice et al., 2015; Ztaou et al., 2016)

It has been hypothesised that aberrant activation of striatal ChIs may be casually correlated with LID. Indeed, while either acute or sub-chronic L-DOPA treatment induced ERK phosphorylation predominately on MSNs, chronic L-DOPA induced a shift in pERK activation from MNSs (about 72% reduction) to ChIs (Ding et al., 2010). This phenomenon was observed in both unilaterally 6-OHDA-lesioned mice and in the genetic PD model of aphakia mice (Ding et al., 2010). Moreover, selective ablation of striatal ChIs critically dampened LID severity without affecting the anti-parkinsonian effects of L-DOPA in 6-OHDA-lesioned mice (Won et al., 2014).

4. Anti-dyskinetic treatment strategies

4.1 Prevention and management of dyskinesia in PD

As discussed in Chapter 3, LID is the result of a wide variety of functional and pathological neuronal adaptations which initiate following the degeneration of dopaminergic neurons located in the SNc and are furtherly exacerbated by the pharmacological replacement of DA. To compound issues further, not all DA-depleted animals, as well as PD patients, treated chronically with L-DOPA develop dyskinetic behaviours. This suggests that other, not yet defined, factors must be involved in LID appearance (Manson et al., 2012; Cenci, 2014).

It is important to underline that these maladaptive adaptations develop gradually over the time in a manner which could be significantly influenced by several individual-related factors (e.g. age, environment, genetic) (Linazasoro, 2005).

Ideally, the best anti-dyskinetic treatment should counteract the development of LID by protecting dopaminergic neurons from internal/external threats able to trigger the neurodegenerative process (Thanvi et al., 2007).

Unfortunately, today not only we do not have neuroprotective or disease-modifying agents able to halt, or at least to slow down, the degeneration of dopaminergic neurons, but we also lack valid biomarkers which can be clinically screened to verify early signs of PD and the development of the pathology (Thanvi et al., 2007).

Nevertheless, considerable progresses have been made in understanding the molecular events leading to dyskinesia and their associated physio-pathological hallmarks. While LID are managed with difficulty once established (Cenci, 2014), increasing clinical evidences have pointed out that LID prevention is crucial in LID therapies (Cenci et al., 2012) and that it can be better pursued in the temporal window spacing the diagnosis of PD to the first dyskinetic episodes.

Given the inability of counteracting the progression of PD, current therapeutic strategies aimed to prevent LID are principally based on patient symptoms and are mainly focused at: (a) reducing L-DOPA intake, (b) providing a continuous, rather than pulsatile, dopaminergic stimulation, or (c) contrasting the generation of postsynaptic changes (Cenci et al., 2012; Pilleri and Antonini, 2015). **Figure 3** depicts a schematic representation of suggested LID treatments.

Delaying the initiation of L-DOPA was considered for years the best therapeutic option to prevent the development of dyskinetic behaviours in PD patients (Grandas et al., 1999; Schrag and Quinn, 2000; Poewe and Mahlknecht, 2009). This idea was supported by experimental and clinical evidences showing that, compared to dopaminergic agonists (e.g. pramipexole), initial treatment with L-DOPA was associated with earlier appearance of dyskinesias and wearing-off fluctuations (Rascol et al., 2000; Holloway, 2000).

However, recent investigations have depicted a more complex scenario indicating that: (1) the delivery of L-DOPA, rather than the duration of L-DOPA treatment, should be improved to ensure a continuous and physiological stimulation of striatal DAR and to slow down pathological adaptations which take place in the parkinsonian brain (Thanvi et al., 2007; Cilia et al., 2014); (2) higher L-DOPA doses are associated with higher incidence of LID (Fahn et al., 2004, 2005; Olanow et al., 2013). In light of these considerations, the efficacy of DA agonists in delaying LID development is thought to be principally related to the possibility of reducing L-DOPA dosage over the course of the day rather than due to L-DOPA removal *per se*.

As discussed more in depth in the following paragraphs, one of the biggest problem associated with the development of valid anti-dyskinetic therapies depends on the fact that brain networks and cellular mechanisms involved in the therapeutic and dyskinetic effects of L-DOPA are highly interconnected, especially those involving the dopaminergic transmission. Thus, once LID appears, treatment options are limited by the worsening of the anti-parkinsonian effects of L-DOPA (Cenci and Crossman, 2018). In this scenario, the development of non-dopaminergic remedies that can be added to L-DOPA plays a pivotal role in LID treatment in order to offer new therapeutic strategies for reducing dyskinetic behaviours without interfering with the beneficial effects of L-DOPA.

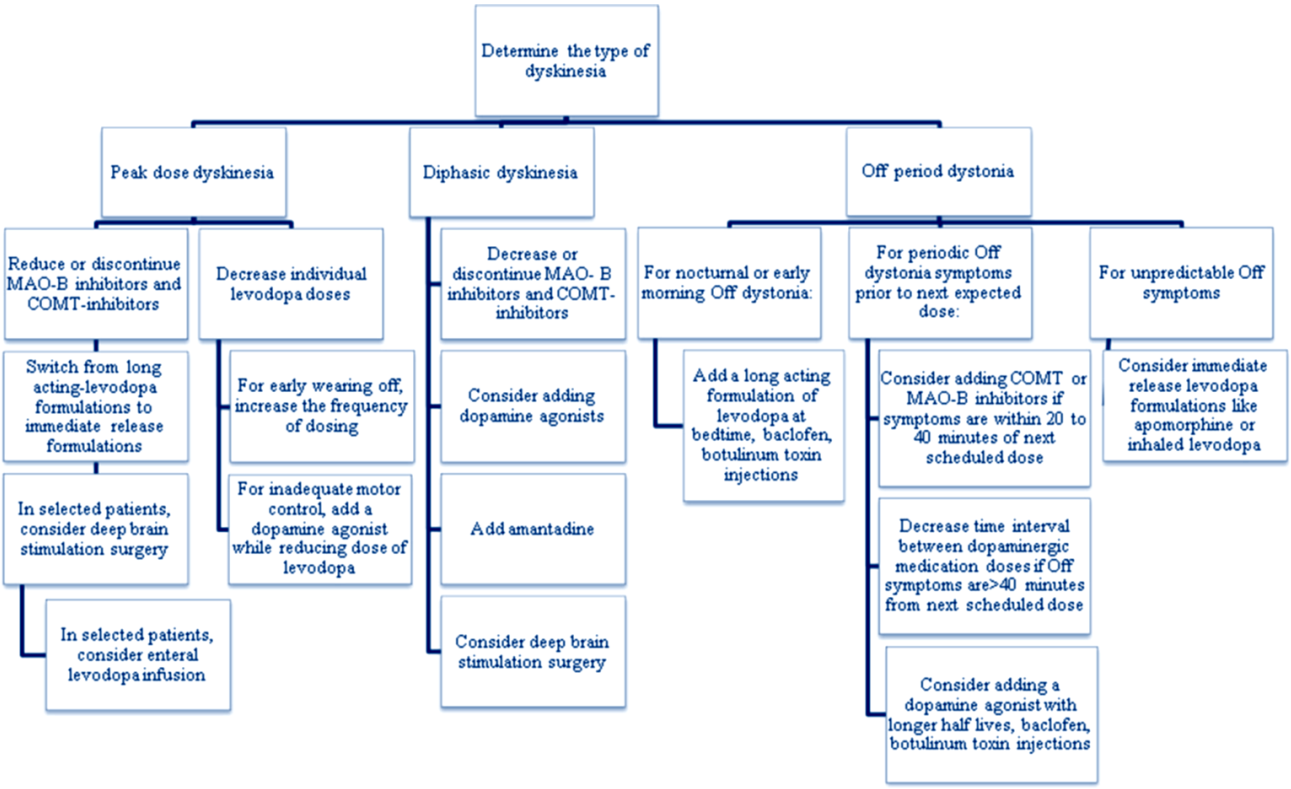

Figure 3. Block diagram showing the suggested therapeutic options based on the different type of LID. Abbreviations: COMT catechol-O-methyltransferase, MAO-B monoamine oxidase B. Figure from *Vijayakumar and Jankovic, 2016*

4.2 Treatment targeting the dopaminergic transmission

Correlation between dyskinesia, L-DOPA dosage and route of administration have been frequently reported (Durif, 1999; Cenci et al., 2012; Pilleri and Antonini, 2015). As suggested by the Elldopa study (Fahn et al., 2004, 2005), to postpone the development of LID, L-DOPA dosage should not exceed the minimum providing symptomatic relief. In this respect, in early PD either the sparing of L-DOPA, by combining L-DOPA with MAO or COMT inhibitors, or the use of dopaminergic agonists as monotherapy, it has been proposed to be a good therapeutic strategy to delay the appearance of motor fluctuations and dyskinesia (Grandas et al., 1999; Schrag and Quinn, 2000), even though its efficacy on LID is still matter of debate (Cilia et al., 2014; Xie et al., 2015).

Differently from L-DOPA, DA agonists do not require any metabolic activation and possess a better pharmacokinetic profile (better absorption, lower or absent interaction with food, longer stimulation of postsynaptic striatal DAR) (Pilleri and Antonini, 2015).

Among dopaminergic agonists used, ropinirole and its extended-release form, was found to be clinically efficacious both in the prevention and delay of LID (Seppi et al., 2011; Batla et al., 2013). Nevertheless, monotherapy with several dopaminergic agonists has been reported to induce a high rate of dropouts due to their relative side effects (especially for ergolinic derivates) and the minor symptomatic relief when compared to L-DOPA (Clarke and Guttman, 2002).

To overcome these problems, preparation such as Sinemet CR, an oral pharmaceutical form providing a controlled-release of L-DOPA/carbidopa, have been developed and marketed to improve L-DOPA pharmacokinetic by stabilizing plasmatic L-DOPA-derived DA concentration (LeWitt et al., 1989).

Although significant improvement in the execution of daily living activities as been observed (Block et al., 1997), two independent 5-year follow-up clinical studies showed that in PD patients receiving either Sinemet CR or Madopar (L-DOPA plus Benserazide) CR the incidence of dyskinesia was unchanged when compared to PD patients receiving the chemically equivalent immediate-release preparation (Block et al., 1997; Duriff et al., 1999). In this regard it is important to note that CR oral preparations, similarly to their immediate-release analogues, have a variable absorption due to the phasic gastric emptying and need to be administered in a twice-daily regimen. This could explain, at least partially, the failure of oral CR form in the prevention of LID (Duriff et al., 1999; Thanvi et al., 2007).

Nevertheless, a recent new pharmaceutical forms of L-DOPA, having an improved modified pattern of release, has been developed to reduce motor fluctuations and dyskinesia. In this respect, the L-DOPA extended-release capsule (IPX-066) has been found to reduce L-DOPA-related motor complications and increase on time periods without troublesome dyskinesia (Hauser et al., 2013). Moreover, intrajejunal administration of L-DOPA by the mean of intestinal gels was found to be a valuable approach able to extend L-DOPA efficacy and lower LID (Antonini et al., 2015; Nyholm et al., 2005). The continuous delivery of L-DOPA

from the jejunum permits to bypass the stomach, avoiding the irregular systemic absorption derived by phasic gastric emptying. Nevertheless, the high frequency of complications (e.g. age of implantation, risk of infections, tube dislocation or occlusion), as well as the low patient compliance, has reduced its clinical usage (Vijayakumar and Jankovic, 2016).

Ultimately, despite initial investigations have raised the possibility that the administration of the MAO-B inhibitor rasagiline may exert a disease-modifying action in PD patients (Maruyama et al., 2004; Olanow et al., 2009), subsequent follow-up studies have not confirmed this hypothesis (Rascol et al., 2016). However, initiation of antiparkinsonian treatment using MAO-B inhibitors in addition to L-DOPA treatment do not decrease the occurrence of motor fluctuations and dyskinesias (Brannan et al., 1995).

4.3 Treatment targeting non dopaminergic pathways

4.3.1 Anti-glutamatergic drugs

To date, the only drug available for the treatment of LID is amantadine (Bastide et al., 2015). Amantadine is a multi-target drug initially developed to treat influentia A, which acts principally as non-competitive NMDA receptor antagonist (Fox et al., 2011). Interestingly, a randomized double-blind placebo-controlled study performed in 19 dyskinetic patients found as amantadine did not affect the severity of dyskinesia, rather, its duration and impact on the execution of daily tasks (Pereira Da Silva-Júnior et al., 2005). Beyond amantadine, memantine, milacemide, dextromethorphan and other NMDA antagonists were tested as anti-dyskinetic medications even though none of them was found to be clinically superior to amantadine (Bastide et al., 2015).

Inhibition of glutamatergic AMPA receptors was also reported to reduce the expression of LID in a rat model of dyskinersia (Kobylecki et al., 2010). Nevertheless, the severe side effects related to AMPA inhibition have critically limited their clinical use in PD patients (Cenci et al., 2012).

Apart from ionotropic receptors, several preclinical evidences demonstrated as LID is associated with the abnormal expression and activation of mGlu-1 and mGlu-5 receptors in striatal neurons (Breysse et al., 2003; Coccurello et al., 2004; Levandis et al., 2008). In line with previous preclinical investigation (Mela et al., 2007), selective antagonists (mavoglurant) or negative allosteric modulator (dipraglurat) of mGluR-5 have been clinically tested as anti-dyskinetic remedies (Tison et al., 2016; Trenkwalder et al., 2016), showing potential to reduce LID in PD patients without affecting the anti-parkinsonian effects of L-DOPA (Amalric, 2015). However, further characterizations of these compounds are needed especially in relation to their long-term side effects.

4.3.2 Anti-serotoninergic drugs

An increasing number of evidences support the notion that, in advance PD, exogenous L-DOPA is mostly released from presynaptic striatal serotoninergic terminals and that dyskinesia is associated with several pathological plastic changes (e.g. terminal sprouting, altered expression of 5-HT receptors) which affect serotonin projections (Politis et al., 2010; Cenci, 2014). In this respect, several agents which lower the serotoninergic transmission showed excellent anti-dyskinetic properties in rodents and NHPs, and their effects have been tested in clinical trials, despite the concern related to their negative action on the therapeutic effects of L-DOPA (Bastide et al., 2015).

Sarizotan, a selective $5HT_{1A}$ receptor agonist which partially blocks D_3, D_4 and D_2R (Bartoszyk et al., 2004), was initially reported to decrease the AIMs scores of dyskinetic PD patients approximately by the 40% without modifying the UPDRS motor scores (Bara-Jimenez et al., 2005). Unfortunately, these encouraging results were not confirmed in a large randomized, placebo-controlled phase II dose finding trial (Goetz et al., 2007).

Interestingly, a recent Phase II clinical trial which evaluated the effects of eltoprazine, a mixed $5\text{-}HT_{1A/1B}$ partial agonist, supported the use of eltoprazine against dyskinesia (Svenningsson et al., 2015). However, the observed reduction of LID was not superior to that observed with amantadine (Bastide et al., 2015).

Since their ability to indirectly elevate serotoninergic stimulation of presynaptic $5HT_{1A/B}$ receptors, several selective serotonin reuptake inhibitors (SSRIs) have been tested. Among them, clozapine, a DAR antagonist having antiserotoninergic, antimuscarinic, antiadrenergic and antihistaminergic properties, was found to be clinically effective in reducing LID (Fox et al., 2011; Durif et al., 2004). Importantly, clozapine had no effects against the anti-parkinsonian actions of L-dopa (Durif et al., 2004). Adverse events were not more frequent with clozapine except for drowsiness and hypereosinophilia, the latter rapidly resolved after treatment discontinuation (Vijayakumar and Jankovic, 2016).

4.3.3 Agents affecting the adenosinergic system

In the last decades, great interest has been raised for the potential role in PD of agents acting on the adenosinergic system (Morelli et al., 2007; Pinna et al., 2018). In particular, A_{2A} receptors have been object of intensive investigations since they are located on iMSNs and corticostriatal afferents and their blockade was found to decrease iMSNs activity and to reduce glutamate release in preclinical models of PD (Quiroz et al., 2009; Armentero et al., 2011).

In this respect, preclinical studies demonstrated as several A_{2A} antagonists (i.e. istradefylline, preladenant, ST-1535) possess anti-akinetic properties (Pinna, 2014) and potentiate the anti-parkinsonian effects of L-DOPA without worsening LID, when administrated in combination with low doses of L-DOPA (Pinna et al. 2001; Tronci et al. 2007).

Although these compounds cannot reduce already established dyskinesia, they allow to spare L-DOPA, thus delaying the development of motor fluctuations (Cenci et al., 2012). Interestingly, genetic deletion of the gene expressing the A_{2A} receptor was found to prevent the sensitization of rotational behaviours and LID in 6-OHDA-lesioned mice (Fredduzzi et al. 2002; Xiao et al. 2006). Among A_{2A} receptor antagonists tested in clinical trials, Istradefylline (KW-6002) was the only compound approved first in Japan, and recently in the US, as add-on therapy to L-DOPA/carbidopa for the reduction of OFF periods (Pinna, 2014; Takahashi et al., 2018).

Interestingly, recent preclinical results clearly demonstrated as selective A_{2A} receptor antagonists may be used in association with L-DOPA and 5-$HT_{1A/B}$ receptor agonists to reduce LID without affecting the therapeutic effects of L-DOPA (Pinna et al., 2016; Ko et al., 2017).

4.3.4 Agents affecting the endocannabinoids system

Nabilone is a synthetic derivate of the tetrahydrocannabinol and acts as partial CB_2 and CB_1 receptor (CB_1R) agonist. It was reported as Nabilone administration improved LID in NHP models of PD (Fox et al., 2002). In a small randomized, placebo-controlled study involving 7 dyskinetic PD patients, it was showed as Nabilone decreased dyskinesia by the 22% compared to placebo (Sieradzan et al., 2001). However, similar antidyskinetic effects were not seen with other CB_1R agonists (Carroll et al., 2004) so further studies are needed to clarify whether non-cannabinoids-related mechanisms are involved in the anti-dyskinetic effects of Nabilone.

AIM of the study

Pathological events associated with PD first, and LID later, are far from being completely understood, despite significant improvements have been made. In this respect, amantadine, a multi-target drug introduced more than 20 years ago (Verhagen Metman et al., 1998) is still the only clinically accepted pharmacological treatment prescribed against LID.

Experiments performed during my PhD have been designed starting from the unquestionable evidences that today the DA precursor L-DOPA remains the most effective symptomatic medication for the treatment of PD motor symptoms, increases the life expectancy of PD patients (Joseph et al., 1978) and it is globally used thanks to its competitive price.

To extend our knowledge about the pro-dyskientic effects of L-DOPA, two studies, both directed to the comprehension of LID-like mechanisms, have been performed which are here described separately for the sake of clarity.

As it will be discussed in detail later, the two studies differ in terms of species tested (rat vs mouse), genetic background, and pharmacological treatments.

Nevertheless, in both studies, animals were treated in order to develop severe peak-dose dyskinesia following the instauration of pathological maladaptive modifications induced, in first place, by the net deprivation of DA, and later by the pulsatile stimulation of striatal DAR. Thus, despite the objective differences across the two studies, the specific events leading to LID, as well as its reduction, are here carefully examined.

In the study (1), we asked whether the combined administration of eltoprazine, a partial 5-$HT_{1A/B}$ receptor agonist, with preladenant, a selective A_{2A} receptor antagonist, may prevent the onset of LID in a rodent model of advance PD. This study was based on previous evidences demonstrating that, while inhibitors of the serotoninergic system possess excellent anti-dyskinetic properties at the expenses of the anti-parkinsonian effects of L-DOPA, selective A_{2A} receptor antagonists boost L-DOPA efficacy without affecting the severity of dyskinesia (Pinna et al., 2016; Ko et al., 2017). Although promising preclinical results have been reported in rats chronically treated with eltoprazine, preladenant and L-DOPA (Pinna et al., 2016), none have ever evaluated the behavioral and molecular events derived from an early combined administration of eltoprazine, preladenant and L-DOPA in drug naïve 6-OHDA-lesioned rats in the prevent of LID induced by a subsequent challenge with L-DOPA.

To pursue this aim, we treated unilateral 6-OHDA-lesioned drug naïve rats for fourteen days with eltoprazine (0.6mg/kg) and/or preladenant (0.3mg/kg), alone or in association with L-DOPA (4mg/kg). Over the treatment, AIMs as index of dyskinesia were evaluated. In order to unveil whether the sole or combined pharmacological manipulation of the adenosinergic and serotoninergic system may prevent the development

of LID, all rats were challenged with L-DOPA after 4 days of wash-out and their dyskinetic behaviours evaluated. Moreover, immunoreactivity for the IEG *zif-268,* as index of long-term changes correlated with dyskinesia, was tested.

In the study (2), in collaboration with prof. Emiliana Borrelli at University of Irvine, California, we focused our research on the involvement of striatal D_2R located either on iMSNs or ChIs in the modulation of LID. Indeed, despite a large body of evidence suggest a major role of striatal D_1R in LID, inhibition of the D_2 transmission has been associated with the appearance of parkinsonism as well as with the inability to express either low or high frequency stimulations-evoked striatal LTD, which is essential to properly paired synaptic responses to external stimuli (Baik et al., 1995; Kreitzer and Malenka, 2008).

Moreover, pharmacological stimulation or inhibition of $D_{2/3}R$ has been reported to either increase (Luquin et al., 1992; Calon et al., 1995) or decrease (Delfino et al., 2004; Taylor et al., 2005) the expression of dyskinetic behaviours, respectively. Nevertheless, available dopaminergic agonist/antagonists cannot discriminate among D_2 and D_3R and, to the best of our knowledge, no one has never tested how D_2R located on different striatal cellular population are involved in the development of LID.

In addition, we investigated whether the pharmacological manipulation of cholinergic M_1R located on iMNS-D2RKO may attenuate LID.

To this aim, experiments were performed in unilateral 6-OHDA-lesioned drug naïve mice carrying a cell-specific deletion of the D_2R either in the iMSNs (iMSN-D_2RKO) or in the ChIs (ChIs-D_2RKO), as well as in wild-type (WT) controls. Mice were treated either with: (i) saline; (ii) 15 mg/kg of L-DOPA (once a day for 11 consecutive days) or (iii) an ascending-dose regimen of L-DOPA (1.5, 3, 6 mg/kg; dosage was changed every 3 days, 9 days of treatment in total). Moreover, after 10 days of wash-out, dyskinetic mice from treatment (iii) received an acute administration of L-DOPA (6 mg/kg) either alone, or in combination, with the selective M1 antagonist VU-0255035 (60 mg/kg). AIMs were evaluated immediately after L-DOPA administration. To investigate molecular events taking place both before and after pharmacological treatments, 6-OHDA-lesioned mice and sham-lesion controls were sacrificed to perform electrophysiological analyses, to evaluate the expression of low-frequency stimulation (LFS)-induced LTD (LFS-LTD), and immunohistochemical analyses, to evaluate the activation of *c-fos,* pERK and the phosphorylated form of the ribosomal protein S6 (rpS6).

Study 1

New therapeutic strategy to prevent the onset of dyskinesia in the Parkinson's disease

Materials and Methods

Animals

Male Sprague–Dawley rats (Charles River, Calco, Italy) were housed in groups of 4-6 with free access to food and water and maintained under standard conditions (lights: 08.00–20:00, temperature 23°C). Behavioral tests were performed during the light cycle. Experiments were performed in accordance with European-Communities-Council Directive (2010/63/EEC; D.L.27.01.1992-number116) and guidelines for animal experimentation were approved by Cagliari's University.

Drugs

Preladenant (0.3 mg/kg) was purchased from Sequoia-Research-Products (UK), suspended in sterile saline containing 0.4% methylcellulose and administered orally (p.o). The 0.4% methylcellulose suspension served as vehicle control for preladenant. Eltoprazine (0.6mg/kg) (Sequoia-Research-Products) was dissolved in sterile saline and injected subcutaneously (s.c.). 6-OHDA, L-DOPA-methyl-ester-hydrochloride, desipramine-hydrochloride, and benserazide were purchased from Sigma-Aldrich. L-DOPA-methyl-ester (4 mg/kg) and benserazide (4 mg/kg) were injected s.c., while desipramide-hydrocloride (10 mg/kg) was injected intraperitoneally (i.p.) ten minutes before stereotaxic surgery. Preladenant and eltoprazine were administered 60 min before L-DOPA administration and simultaneously with L-DOPA, respectively. Dosagse of drugs used were based on a previous dose-finding study performed in our laboratory (Pinna et al., 2016).

6-OHDA lesion

Rats (275–300g) were anesthetized with chloral-hydrate and placed in a stereotaxic frame (David Kopf Instruments). Thereafter, rats were infused, through stainless steel cannula, into left MFB (coordinates (mm): anteroposterior (AP): −2.2; mediolateral (ML): +1.5; dorsoventral (DV): −7.9) with 6-OHDA (8µg/4µl in saline containing 0.05% ascorbic acid) (Pellegrino et al., 1979). 6-OHDA solution was delivered by an external pump in which flow rate was set to 1 µL/min. All rats were pretreated with desipramine (10 mg/kg i.p.) 30 min before 6-OHDA to prevent damage of noradrenergic neurons.

AIMs evaluation

AIMs were analyzed between 11 a.m. and 4 p.m. by visual observation. AIMs evaluation was based on a well-established rating scale for rodents (Lundblad et al., 2002). Briefly, rats were place individually in transparent cages, without bedding material, and observed for 1 min every 20 min over a total period of 120 min, immediately after the administration of L-DOPA or its vehicle. Dyskinetic behaviors, clearly different from natural stereotyped movements, were classified into three subtypes according to their topographic distribution: axial (dystonic posturing of the upper part of the body toward the side contralateral to the lesion), limb (abnormal movement of the forelimb contralateral to the lesion), and orolingual AIMs (vacuous jaw movements and tongue protrusion toward the side contralateral to the lesion). Each of these AIMs subtypes were assessed using scores ranging from 0 to 4 (0: no dyskinesia 1: AIMs present during less than half of the observation time; 2: AIMs present during more than half of the observation time; 3: AIMs present all the time but stoppable by the presentation of external threatening stimuli; 4: AIMs present all the time and not stoppable). The theoretical maximum score that could be accumulated by one animal in one testing session was 96 (maximum score per observation point, 16; number of observation points per session, 6). Data are presented as total AIMs (axial+limb+orolingual (ALO) AIM scores) ±SEM in 120-min after L-DOPA.

Experimental plan

Experimental timeline is shown in **figure 4**. Three weeks after surgeries, L-DOPA-naïve 6-OHDA-lesioned rats were randomly divided into 5 groups, and chronically treated (14 days) with: (1) L-DOPA (4 mg/kg); (2) L-DOPA (4 mg/kg) plus preladenant (0.3 mg/kg); (3) L-DOPA (4 mg/kg) plus eltoprazine (0.6 mg/kg); (4) L-DOPA (4 mg/kg) plus preladenant (0.3 mg/kg) plus eltoprazine (0.6 mg/kg) or (5) Saline. Moreover, sham-lesioned rats were treated either with saline or L-DOPA as controls. AIMs evaluation was performed on day 1, 4, 10 and 14. After 4 days of wash-out, L-DOPA (4 mg/kg) was administrated to all rats for the pharmacological challenges and AIMs was scored again. 90 minutes after L-DOPA, rats were anesthetized and transcardially perfused with sterile saline followed by 4% PFA in 0.1M phosphate buffer (PB; pH= 7.4). Afterwards, brains were removed, postfixed overnight in the same solution at 4 °C, and then processed for immunohistochemical evaluations.

Immunohistochemistry

PFA-fixed 40 µm thick coronal sections containing the SNc and the CPu were cut on a vibratome (Leica). For each rat, three coronal sections were collected from +1.60 mm to +0.48 mm to the bregma for the CPu and from -5.20 mm to -5.80 mm away from the bregma for the SNc, according to the rat brain atlas of Paxinos and Watson (1998). Free-floating sections were rinsed in 0.1 M PB, blocked in a solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.1 M PB at room temperature for 1h, and incubated at 4°C in the same solution with the primary antibody directed against either *zif-268* (1:1000, Santa Cruz

Biotechnology) or tyrosine hydroxylase (TH) (1:1000,Millipore) for 2 or 1 night, respectively. After being rinsed, sections were incubated with the appropriate AlexaFluor 594-labeled secondary antibody (1:400, Jackson ImmunoResearch) at room temperature for 1h. After secondary antibody incubation, sections were rinsed and mounted onto gelatine-coated glasses using the Mowiol mounting medium. Omission of either the primary or secondary antibodies served as negative controls and yielded no cellular labelling.

Image analyses

Images of single wavelength were obtained with an epifluorescence microscope (Axio Scope A1, Zeiss, Germany) connected to a digital camera (1.4 MPixels, Infinity 3–1, Lumenera, Canada). Brain sections immunostained either for *zif-268* or TH was captured using a 20X objective in order to acquire the whole dorsal part of the CPu as well as the whole SNc. The Image J software (National Institutes of Health, USA) was used to quantify the density of immunoreactive fibers positive for TH or to manually count the number or the mean grey intensity of immunoreactive neurons positive either for the TH or *zif-268,* respectively.
All animals included in the present study showed a reduction ≥ 90 % in TH-immunoreactivity at the level of both the SNc and the CPu.

Statistical analysis

Statistical analysis was performed with Statistica (StatSoft) and with Prism (GraphPad v.6) for Windows. Data were statistically analysed using parametric one-way or two-way ANOVA followed by post hoc Tukey's test or Newman-Keuls multiple comparisons test. All data are presented as mean ± SEM. Results were considered significant when the p value was < 0.05.

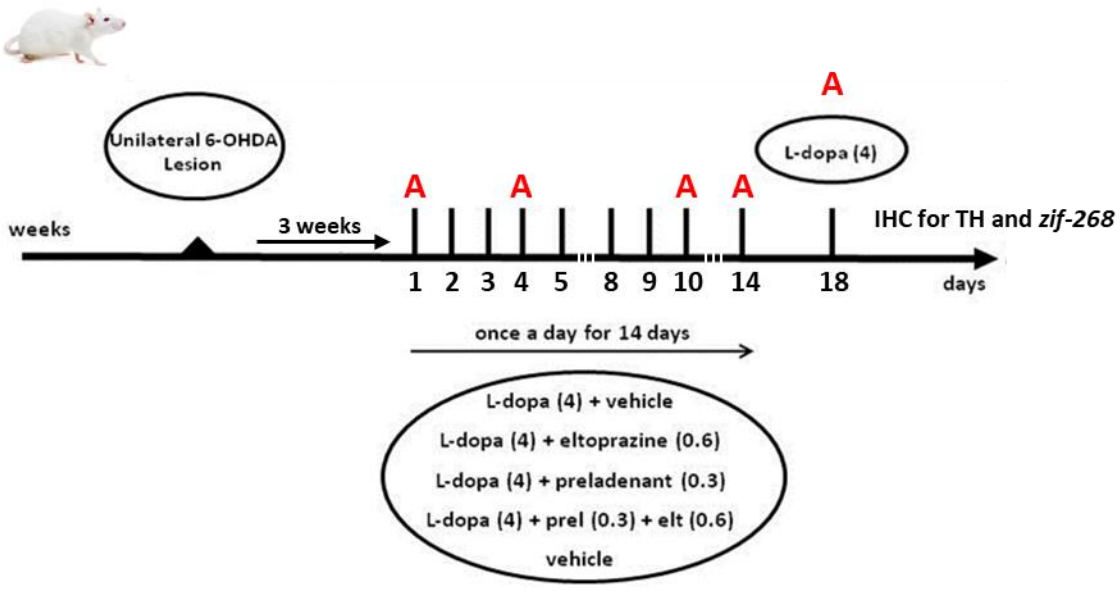

Figure 4. Image showing a schematic representation of the experimental plan. Abbreviations: A: abnormal involuntary movements; elt: eltoprazine; IHC: immunohistochemistry; prel: preladenant; TH: tyrosine hydroxylase.

Results

Effect of chronic pharmacological treatments in L-DOPA-naïve hemi-parkinsonian rats

To determine whether the early combined administration of eltoprazine (0.6 mg/kg) and preladenant (0.3 mg/kg) prevents the development of dyskinetic behaviours in rats treated with a low dose of L-DOPA (4 mg/kg), we examined the effects produced by chronic (14 days) L-DOPA administrations, alone or in combination with eltoprazione and/or preladenant, in drug naïve 6-OHDA-lesioned rats.

Statistical analyses performed on behavioural results obtained at the 14th day of chronic treatment clearly demonstrated as the different pharmacological treatments significantly affected the total number of AIMs (**Figure 5**, A-B; treatment effect: F3,46=88.88, p<0.0001; one-way ANOVA). Our results showed that chronic L-DOPA administration promoted a consistent expression of AIMs in 6-OHDA-lesioned rats.

Interestingly, statistical analysis revealed as 6-OHDA-lesioned rats treated with L-DOPA plus preladenant expressed the highest score of AIMs during the 14th day of the chronic treatment (Figure 5. LD + preladenant vs: LD, p<0.05; LD + eltoprazine, p<0.0001; LD + eltoprazine + preladenant, p<0.0001). The higher score of AIMs induced by L-DOPA plus preladenant relative to that induced by L-DOPA alone, is substantially due to the longer duration of the pharmacological effects of L-DOPA (**Figure 5**). As expected, L-DOPA plus eltoprazine treatment significantly reduced dyskinetic behaviours when compared to L-DOPA (**Figure 5;** p<0.0001) suggesting the critical involvement of serotoninergic terminals in L-DOPA pro-dyskinetic effects. Importantly, we observed a significant reduction of the total AIMs expressed by L-DOPA plus eltoprazine plus preladenant-treated rats when compared to L-DOPA-treated rats (**Figure 5;** p<0.0001). This reduction was similar to that observed in rats treated with L-DOPA plus eltoprazine (**Figure 5;** p> 0.05).

Moreover, the rotational behaviour observed during the chronic treatment, showed that rats receiving the triple association performed fluent contralateral rotations which differed qualitatively from the contralateral rotation associated to stereotyped behaviours observed following L-DOPA.

Sham-lesioned and vehicle-treated 6-OHDA-lesioned rats were not included in **figure 5** since no AIMs were observed during the whole treatment.

To further test whether the combined administration of eltoprazine, preladenant and L-DOPA may delay the development of LID, we challenged all rats with L-DOPA (4 mg/kg), after 4 days of pharmacological wash-out. This approach allowed us to estimate how previous treatments affected the pro-dyskinetic effects produced by L-DOPA. Results clearly demonstrated as dyskinetic behaviours promoted by L-DOPA were critically affected by previous treatments (**Figure 5, C-D;** $F_{3,46}$= 10.80, p<0.0001; one-way ANOVA). Subsequent Turkey's test indicated that, while no differences were observed in the total AIMs scorses between L-DOPA and L-DOPA plus preladenant pre-treated rats, both L-DOPA plus eltoprazine (**Figure 5;** p<0.001) and L-DOPA plus eltoprazine plus preladenant pre-treatment (**Figure 5;** p<0.001) significantly reduced the total AIMs scored after the challenge with L-DOPA

when compared to L-DOPA pre-treated rats. The extent of AIMs reduction was similar between rats pre-treated with L-DOPA plus eltoprazine, given alone or in combination, with preladenant (**Figure 5;** p> 0.05).

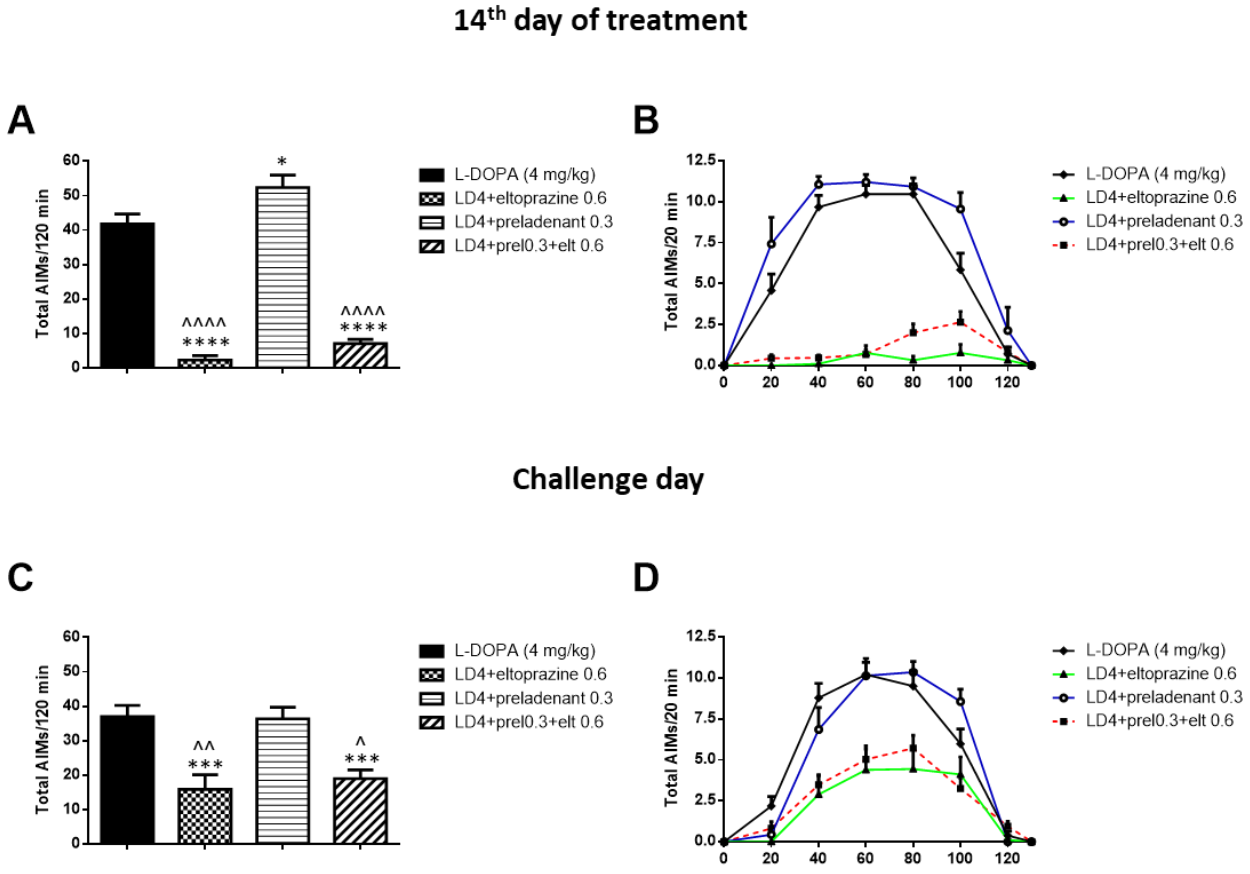

Figure 5. chronic administration of eltoprazine and preladenant reduces the expression of AIMs induced by L-DOPA. (A) Bar graph showing the total ALO AIMs scores measured in 120 minutes at day 14 of the chronic treatment; * p<0.05, **** p<0.0001 vs L-DOPA, ^^^^ p<0.0001 vs L-DOPA plus preladenant; **(B)** Time course showing the total AIMS expression at day 14 of the chronic treatment; **(C)** Bar graph showing the total AIMs scores over 120 minutes measured after L-DOPA challenge (18th day); *** p<0.001 vs L-DOPA, ^ p<0.05 vs L-DOPA, ^^ p<0.01 vs L-DOPA plus preladenant; **(D)** Time course showing the total AIMS expression after L-DOPA challenge (18th day).

Immunoreactivity for *zif-268*

To estimate how the manipulation of the adenosinergic and/or serotoninergic system/s differently affects the L-DOPA-mediated activation of post-synaptic striatal MSNs, we measured the activation of the IEG *zif-268* in the dorsal CPu by immunofluorescence.

One-way ANOVA analysis of the mean intensity of *zif-268* immunoreactivity in the dorsal CPu showed as the different treatments significantly affected the striatal activation of *zif-268* (**Figure 6**; $F_{5,48}$=8, p<0.001).

As expected, L-DOPA pre-treatment induced a strong elevation of the mean intensity of the IEG *zif-268* in the dorsal CPu of L-DOPA-challenged 6-OHDA-lesioned rats.

In particular, high levels of *zif-268* were found in 6-OHDA-lesioned rats which received chronic administrations of either L-DOPA (4 mg/kg) or L-DOPA plus preladenant (0.3 mg/kg) before the pharmacological challenge with L-DOPA (4 mg/kg), when compared with both drug-naïve sham operated or vehicle pretreated 6-OHDA-lesioned rats (**Figure 6**; p< 0.001, Tukey's post-hoc test). Importantly, 6-OHDA-lesioned rats pre-treated either with L-DOPA plus eltoprazine or L-DOPA plus eltoprazione plus preladenant showed significant lower levels of the mean intensity values of *zif-268* when compared to L-DOPA pre-treated 6-OHDA-lesioned rats (**Figure 6;** p< 0.001 L-DOPA + eltoprazine vs L-DOPA; p< 0.01 L-DOPA + eltoprazine + preladenant vs L-DOPA).

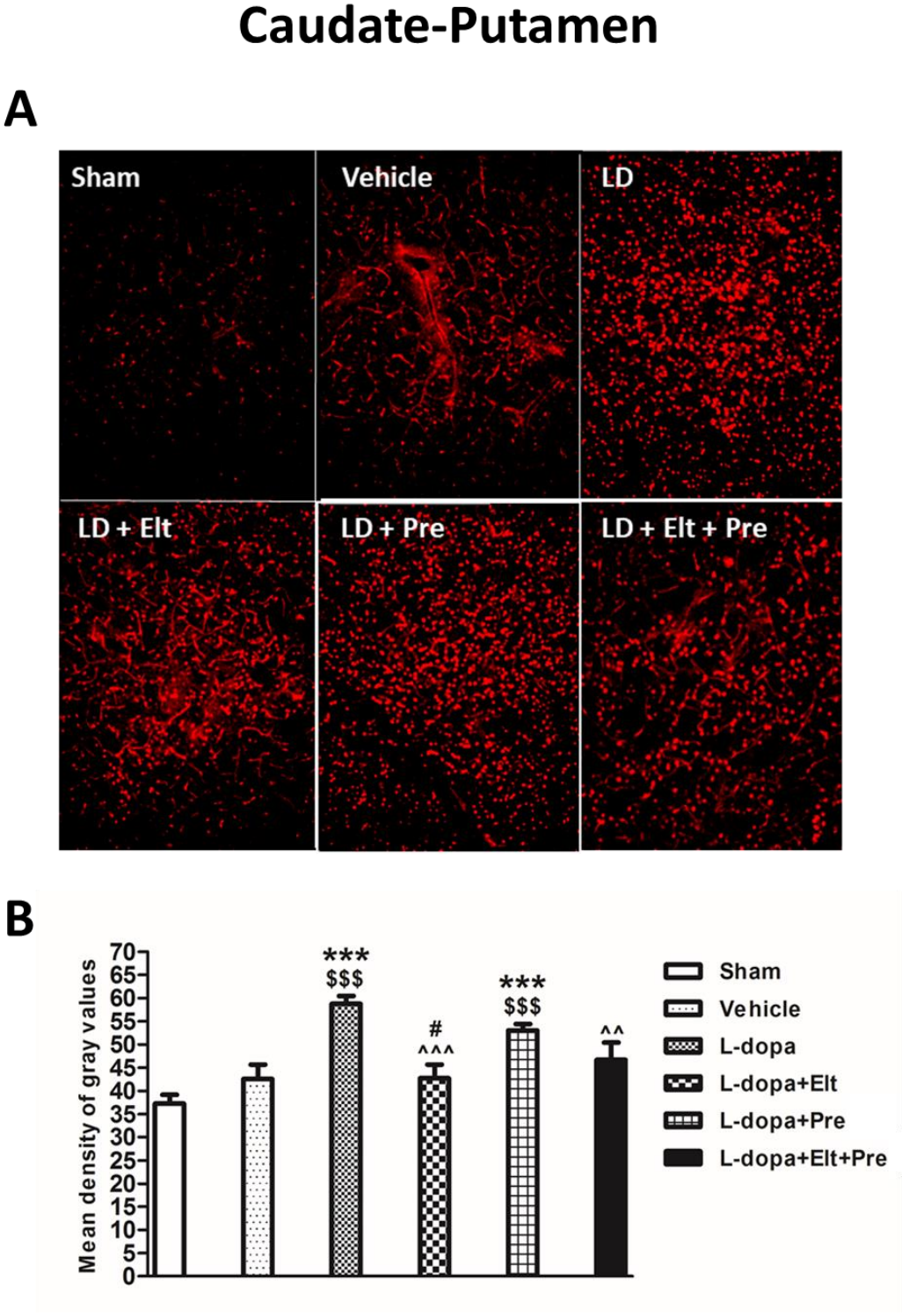

Figure 6. Pre-treatment dependency on *zif-268* activation in the CPu after L-DOPA challenge. (A) Representative fluorescent images from the dorsal CPu showing the immunoreactivity for *zif-268* in DA-depleted pretreated rats after L-DOPA challenge. White title inside each box described the pre-treatment received by rats. **(B)** Bar graph showing the mean density of the grey value for *zif-268*; ***p<0.001 vs Sham, $$$ p<0.001 vs vehicle, ^^ p<0.01 vs L-DOPA, ^^^ p<0.001 vs L-DOPA, # p<0.05 vs L-DOPA plus preladenant. Abbreviations: LD, L-DOPA; Elt, Eltoprazine; Pre, Preladenant.

Discussion

Dyskinesia remains a major therapeutic problem in PD patients receiving L-DOPA. Despite several preclinical studies have reported that compounds manipulating different neurotransmitters may effectively reduce LID, their effects in humans were found to be unexpectedly variable and limited by their negative impact on the therapeutic action of L-DOPA (Rascol et al., 2015).

Nevertheless, recent preclinical studies performed in unilaterally 6-OHDA lesioned rats (Pinna et al., 2016) and MPTP-intoxicated NHPs (Ko et al., 2017) have reported a promising non-dopaminergic pharmacological strategy which could alleviate LID, without worsening PD-like symptoms. These studies demonstrated that the combined administration of eltoprazine, a selective 5-$HT_{1A/1B}$R agonist exerting potent antidyskinetic effects at the expense of the therapeutic efficacy of L-DOPA (Bezard et al., 2013; Pinna et al., 2016; Ko et al., 2017), with preladenant, a selective A_{2A}R antagonist which potentiates the antiparkinsonian effects of L-DOPA (Hauser et al., 2011; Pinna, 2014), significantly reduced LID in already dyskinetic and drug-naïve DA-depleted animals, without interfering with the therapeutic action of L-DOPA (Pinna et al., 2016; Ko et al., 2017).

Starting from the important translational implications of these findings, with the present study we sought to extend our knowledges about this novel anti-dyskinetic treatment by assessing whether the combined and early administration of eltoprazine and preladenant may prevent LID as well as the instauration of long-term changes associated with the production of the transcriptional factor *zif-268,* an IEG whose expression had been positively correlated with LID (Carta et al., 2011; Bastide et al., 2014; Pinna et al., 2016). To this end, unilaterally 6-OHDA-lesioned drug naïve rats were first chronically treated (14 days) with L-DOPA (4 mg/kg) alone or in association with eltoprazine (0.6 mg/kg) and/or preladenant (0.3 mg/kg), and then challenged with L-DOPA (4 mg/kg) after 4 days of drug wash-out. This protocol was specifically designed to unveil whether the different pre-treatments may affect the development of abnormal and long-lasting neuronal adaptations leading to dyskinesia.

Dosage of tested drugs was based on a previous dose-finding study (Pinna et al., 2016), who utilized AIMs as an index of dyskinesia and rotational behaviour as an index of motor activation, to determine the best combination of doses that could produce maximal anti-dyskinetic effects without compromising motor disabilities. Importantly, thanks to the anti-akinetic effects of preladenant (Pinna, 2014), the dose of L-DOPA used in the present study was lower compared to that typically employed (4 mg/kg vs 6-20 mg/kg), thus reducing the dyskinesiogenic potential of the proposed combined treatment (Fahn et al., 2004, 2006; Olanow et al., 2013).

The main finding of the present study was that the early and combined pre-administration of eltoprazine (0.6 mg/kg), either alone or in combination with preladenant (0.3 mg/kg), efficaciously prevented the

development of LID in unilateral 6-OHDA-lesioned rats subsequently challenged with L-DOPA (4 mg/kg) as well as the production of the IEG *zif-268* in the dorsolateral CPu. On the other hand, pre-administration of preladenant did produce neither prophylactic effects against LID development nor influenced the striatal production of the IEG *zif-268*.

While these results suggested a predominant influence of the serotoninergic system over the adenosinergic one in the generation of pro-dyskinetic events, they also corroborated the concept that the symptomatic effects of selective $A_{2A}R$ antagonist against PD motor symptoms involved mechanisms which are not directly interconnected with dyskinesia.

Altogether, these results strengthened the concept that a combinative drug treatment approaching different non-dopaminergic targets may be optimal to alleviate LID and improve PD disability at the same time.

Based on previous studies focusing the involvement of 5-$HT_{1A/1B}R$ and $A_{2A}R$ in LID, two different, but complementary, mechanisms could be envisioned to explain the results obtained:

The first is strictly implicated in LID reduction and involves 5- $HT_{1A/1B}$ auto-receptors located in serotonin cell bodies and terminals. Specifically, we hypothesized that chronic pre-administration of eltoprazine prevented dyskinesia enhancing the pharmacokinetic properties of L-DOPA by rendering DA release more stable and, consequently, stabilizing the stimulation of post-synaptic striatal DAR. In this respect, previous studies revealed as an increase stimulation of 5- $HT_{1A/1B}$ auto-receptors not only reduced the firing rate (Blier et al., 1998) but also dampened the excessive and pulsatile released of L-DOPA-derived DA from serotoninergic terminals (Carta et al., 2007; Lindgren et al., 2010), thus providing a more prolonged and physiological stimulation of sensitized DAR.

Indeed, the oscillatory release of DA combined with the pulsatile stimulation of sensitized DAR are considered to be the driving force for the induction of pathological alterations involving both the pre-synaptic (e.g. sprouting of serotoninergic terminals) and the post-synaptic striatal compartment (e.g. aberrant DAR trafficking, expression of IEG) (Cenci and Konradi, 2010; Cenci, 2014).

Not surprisingly, interventions aimed to improve L-DOPA delivery by providing a lower and continuous rather than higher and pulsatile release of L-DOPA (e.g. intrajejunal infusion), were less susceptible of inducing dyskinesia (Nutt, 2007; Antonini et al., 2013, 2015).

Nevertheless, since 5-HT_{1A} and 5-HT_{1B} are also expressed post-synaptically in the CPu and their respective stimulation inhibits the striatal release of glutamate and GABA (Dupre et al., 2008, 2011), further studies are needed to define the precise mechanisms related to the anti-dyskinetic action of eltoprazine.

The second mechanism related to $A_{2A}R$ is critical for the improvement of the therapeutic efficacy of L-DOPA and involves the blockade of post-synaptic $A_{2A}R$ together with the potentiation of the dopaminergic transmission on iMSNs. Normally, activation of post-synaptic $A_{2A}R$ counteracts the motor stimulation induced by D_2R (Armentero et al., 2011). However, under condition of dopamine depletion, the interaction between

$A_{2A}R$ and D_2R is critically disrupted, contributing to the strong motor inhibition which characterized PD symptoms. Pharmacological and toxicological preclinical studies have demonstrated as the administration of several $A_{2A}R$ antagonists in DA-depleted animals not only produces anti-akinetic effects (Hodgson et al., 2010; Pinna and Morelli, 2014) but, in addition, potentiates the beneficial effects of L-DOPA by extending its duration (Fenu et al., 1997; Hudgson et al., 2010; Morelli et al., 2007; Pinna and Morelli, 2014).

In this respect, the slight but significant worsening of the AIM score that we observed in unilateral 6-OHDA-lesioned rats chronically treated with L-DOPA plus preladenant, compared to L-DOPA alone, was not due to the exacerbation of observed dyskinetic behaviours, but likely to the extended duration of its effects.

Moreover, in agreement with a recent meta-analysis (Wang et al., 2017), and in contrast with preclinical study evaluating the pro-dyskinetic effects of L-DOPA in unilateral 6-OHDA-lesioned $A_{2A}R$ null mice (Xiao et al., 2006; Yin et al., 2017), we showed that the blockade of $A_{2A}R$ by preladenant did not prevent the development of dyskinesia.

Discrepancies between results may derive from important variation in the experimental design involving the animal species, the specific properties of the $A_{2A}R$ antagonist as well as the duration and the dosage of L-DOPA used; this may suggest that in specific circumstances, the adenosinergic system may affect LID development. Therefore, further studies, having comparable variables, are warranted to obtain a comprehensive estimation of the role of $A_{2A}R$ in LID prevention.

Overall these findings improve our understanding of how the adenosinergic and serotoninergic systems are involved in LID prevention, adding further information about the potential application of the combined treatment in a clinical setting.

Study 2

Involvement of the dopamine D2 receptor in the modulation of L-DOPA-induced dyskinesia

Materials and Methods

Animals

Mutant mice were generated by mating $D_2R_{flox/flox}$ males either with D_1-Cre females, to generate the $D_2R_{flox/flox}$/D_1-Cre+ line (iMSN-D_2RKO mice), or with ChaT-Cre females, to generate the $D_2R_{flox/flox}$/ChAT-Cre+ line (ChI-D_2RKO mice). Eight- to fifteen-weeks-old male iMSN-D_2RKO, ChI-D_2RKO and WT mice, weighting between 25-30g at the beginning of the study, were used. Mice were group housed and maintained in standard conditions (12-h light/dark cycle) with food and water ad libitum. For genotyping iMSN-D_2RKO, ChI-D_2RKO and WT littermates, genomic DNA was extracted from tail biopsies and analysed by Southern Blot, as previously described (Anzalone et al., 2012; Kharkwal et al., 2016). All protocols were approved by the Institutional Animal Care and Use Committee in accordance with the NIH guidelines.

Drugs and Reagents

L-DOPA (Sigma) was injected s.c. at a dose of 1.5, 3, 6 or 15 mg/kg always in combination with benserazide hydrochloride (Sigma) (12 mg/kg). VU 0255035-HCl were synthetized (Weaver et al., 2009) and injected i.p. at a dose of 60 mg/kg, 15 minutes before L-DOPA administration. All drugs were dissolved in physiological sterile saline (0.9% NaCl) and injected either s.c or i.p. using a volume of 10 ml/kg of body weight. When mice were not treated with L-DOPA or VU 0255035-HCl, they received an equivalent volume of vehicle. 6-OHDA HCl (Sigma) was dissolved in sterile saline with 0.05% of ascorbic acid.

Unilateral 6-OHDA mouse model of LID

WT, ChI-D_2RKO and iMSN-D_2RKO mice (25-28g) were anesthetized with isoflurane (Primal) and placed in a stereotaxic frame equipped with a mouse adaptor (model 963, David Kopf Instruments). Afterward, each mouse received either two unilateral injections of 2µL of 6-OHDA (Sigma) into the right dorsolateral CPu (coordinates (mm): [1]. AP: +1.0, ML: -2.1, DV: -2.9 and [2]. AP: +0.3, ML: -2.3, DV: -2.9) or one unilateral injection of 1µL of 6-OHDA into the right MFB (AP: -1.2, ML: -1.2, DV: -4.75) (Cenci and Lundblad, 2007; Francardo et al., 2011). 6-OHDA was dissolved in sterile saline with 0.05% of ascorbic acid to obtain a final concentration of 3mg/ml (calculated as free base). The neurotoxin was delivered using a 10 µL WPI syringe (equipped with a 35G needle) connected with an external pump (model UMC4, WPI) in which flow rate was

set to 500 µL/min. After the end of each 6-OHDA injection, needle was left in place for 10 min to optimize tissue retention of the solution. 3 weeks after surgeries, the extent of dopaminergic degeneration was evaluated by placing each mouse in an open field arena within a white box (30X30cm; 70 lux) and scoring the number of 6-OHDA-induced asymmetric turnings together with the total horizontal locomotor activity over a period of 10 minutes. All animals included in the present study showed a reduction ≥ 85 % in TH immunoreactivity of nigral dopaminergic neurons as confirmed by immunofluorescence analyses.

Experimental plan

A series of experiments were performed to test whether, in L-DOPA-treated 6-OHDA-lesioned mice, D_2R expressed either by ChIs or iMSNs may: (1) modulate the development and expression of LID; (2) be involved in alteration affecting the generation of striatal LTD. Moreover the interplay between D_2R and M_1R in the modulation of iMSNs activity and LID severity was also assessed (3).

Experiments (1) and (2) were carried out in striatal 6-OHDA lesion mice and sham-controls. For experiments (1), mice were treated with a daily injection of 15 mg/kg of L-DOPA + 12 mg/kg of benserazide (s.c.) for 11 consecutive days. AIMs were assessed at day 1, 4, 7 and 10 of L-DOPA treatment. The eleventh day, mice were sacrificed 30 min after L-DOPA and their brains removed for immunohistochemical analyses. For experiments (2), drug naïve 6-OHDA- and sham-lesioned WT and iMSN-D2RKO mice were sacrificed, three weeks after the surgeries, and their CPu extracted for electrophysiological analyses.

Experiments (3) were performed in MFB 6-OHDA-lesioned mice and sham-lesioned controls. Three weeks after the lesion, mice were treated daily with escalating doses of L-DOPA (1.5, 3, and 6 mg/kg) + 12 mg/kg of benserazide over 9 consecutive days (dosage was changed every 3 days). AIMs evaluation was performed following each L-DOPA injection.

After 10 days of wash-out, 6-OHDA-lesioned dyskinetic mice received an injection of L-DOPA (6 mg/kg), to evaluate the basal expression of AIMs. The following day, mice were randomly treated with saline or the selective M1 antagonist VU 0255035 (60 mg/kg, i.p.) followed 15 min later by L-DOPA (6mg/kg), to evaluate how pharmacological treatments affect AIMs expression. AIMs were assessed after each L-DOPA injection. Finally, after 10 days of wash-out, mice were randomly treated with saline or VU 02255035 followed by L-DOPA and sacrificed after 30 min for the brain extraction. All experiments were designed in order to minimize the number of animals used. **Figure 7** offers a schematic representation of the experimental plan.

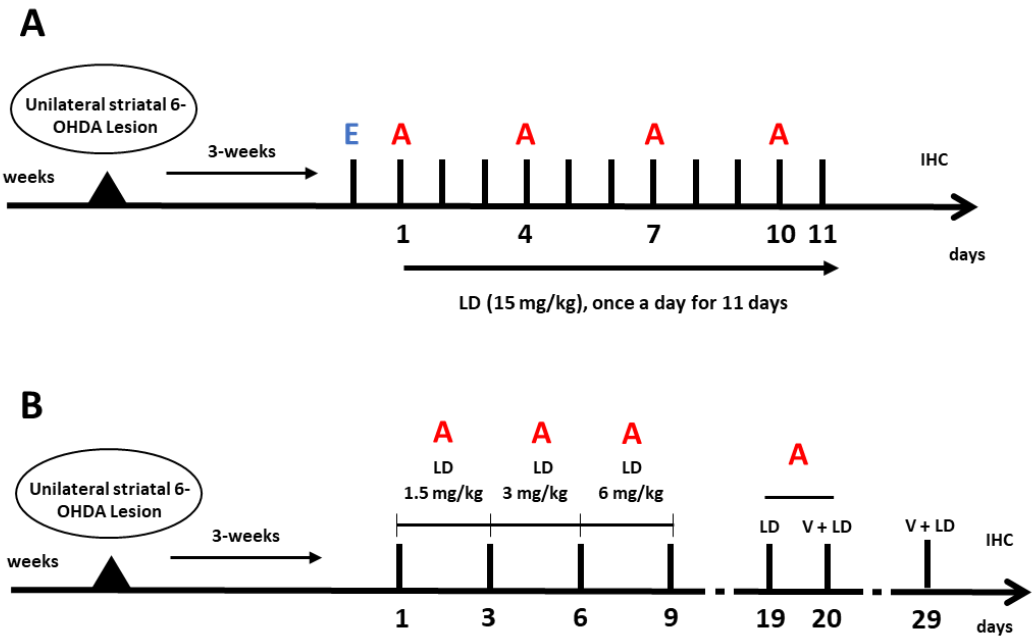

Figure 7. Image showing a schematic representation of the experimental plans. **(A)** Scheme showing experiments performed in striatal 6-OHDA-lesioned iMNS-, ChI-D_2RKO and WT mice, and sham-lesioned controls; **(B)** Scheme showing experiments performed in MFB 6-OHDA-lesioned iMNS-, ChI-D_2RKO and WT mice, and sham-lesioned controls. Abbreviations: A: abnormal involuntary movements; E: electrophysiology; IHC: immunohistochemistry; LD: L-DOPA; V: VU-0255035.

Abnormal involuntary movements evaluation

AIMs were scored using a pharmacologically validated rating scale of mouse model of LID (Cenci and Lundblad, 2007; Francardo et al., 2011) by an observer blind to the mouse genotype, using the same parameters previously described in the materials and methods section of study 1.

Immunofluorescence

Thirty minutes after the last L-DOPA administration, mice were anaesthetized with euthasol (Virbac AH, Inc., Fort Worth, TX), transcardially perfused with 4% paraformaldehyde in PBS 1X and their brains were extracted and post-fixed overnight at 4°C. Afterward, striatal and mesencephalic coronal brain sections were prepared (30 µm) using a vibratome (Leica) and kept in cryoprotective solution at -20°C. During the immunohistochemical analyses, free floating sections were first washed 3 times for 8 minutes in tris-buffered saline (TBS) and then incubated and permeabilized in 5% NGS + 0.05% BSA + 0.3% Triton in TBS for 1 hour. Finally, sections were incubated overnight at 4°C in 1% NGS-TBS solution containing primary antibodies directed against: 1) TH (1:1000, Santa Cruz Biotechnology), 2) pERK (1:200, Cell Signaling), 3) *c-fos* (1:1000, Santa Cruz Biotechnology), 4) choline Acetyltransferase (ChAT) (1:500, Millipore), 5) phospho-rpS6 (Ser235/236) (p-rpS6-$S^{235/236}$; 1:600, Cell Signaling) and 6) phospho-rpS6 (Ser240/244) (p-rpS6-$S^{240/244}$, 1:600,

Cell Signaling). After incubation, sections were rinsed three times for 10 minutes in TBS and incubated for 1h at RT with the appropriate secondary antibody. Afterwards, slices were rinsed three times in TBS and finally incubated in the fluorescent DNA dye Draq7 (Biostatus, 1:800, 15 min in TBS), and glass-mounted using the Prolong Gold (Thermofisher). Pictures were taken either from the dorsolateral CPu (from 1.10 to 0.62 mm anterior to Bregma, Paxinos and Franklin, 2001) or from the SNc (from -2.92 to -3.40 mm to Bregma, Paxinos and Franklin, 2001) using a sequential laser scanning confocal microscopy (DMRE; SP5 microscope, Leica) or a fluorescent microscope (MD IL LED, Leica), respectively. Neuronal quantification and measurement of the mean intensity/cell for pERK, *c-fos*, p-rpS6-$S^{235/236}$, p-rpS6-$S^{240/244}$ immunostainings were performed in images (387,5 x 387,5 µm) covering the whole dorsolateral CPu (3 images x hemisphere x sections) from 3 consecutive sections. For the analyses assessing the mean intensity/cell, ROIs were manually drawn around each individual cell using the freehand selection tool of ImageJ software (National Institutes of Health, Bethesda, MD, USA). Mean grey value per cell were then obtained and background subtracted. pERK/ p-rpS6-$S^{235/236}$/p-rpS6-$S^{240/244}$ intensity specifically in cholinergic interneurons was obtained overlaying ROIs previously drawn on ChAT (+) neurons.

To quantify the dopaminergic degeneration following 6-OHDA administration, TH-immunoreactive neurons were manually counted in images (1,243.8 x 932.73 µm) covering the whole SNc from 3 consecutive sections (2 images x hemisphere x section).

Field Potential Recordings

Field potential recordings were conducted in coronal slices of both control (no surgery), sham- and 6-OHDA-lesioned iMSN-D_2RKO and WT mice containing the dorsolateral CPu. Extracellular recordings were conducted in the presence of the GABA-A receptor antagonist picrotoxin (50 mM), with micropipettes (2.0–3.5 MU) filled with 1 M NaCl. A twisted bipolar electrode was placed in the dorsolateral CPu near the border of the external capsule. All experiments were done at room temperature. The magnitude of LTD was calculated by comparing the baseline responses with the average responses recorded 30–40 min after delivery of a train of LFS (10 Hz for 10 min).

Statistics

Statistical analysis was performed using the analysis software Prism (GraphPad 6). Data were statistically analysed using parametric one-way, repeated measures (rm) or two-way ANOVA, in which genotype and time or genotype and treatment were the independent variables, followed by either post hoc Tukey's test or Newman-Keuls multiple comparisons test. All data are presented as mean ± SEM. Results were considered significant when the p value was < 0.05.

Results

Effect of cell specific D_2R deletion on AIMs expression

To determine the involvement of D_2R in the modulation of LID, we examined the effect of chronic (11 days) L-DOPA administration in striatal 6-OHDA- and sham-lesioned iMSN-, ChI-D_2RKO and WT mice (**Figure 8**). Chronic administrations of L-DOPA (15 mg/kg) promoted a sustained expression of AIMs among all 6-OHDA-lesioned mice. One-way ANOVA analysis related to the cumulative score of the ALO AIMs was significantly affected by the genotypes (**Figure 8A**; $F_{2,30}$= 4.509, p=0.0194). Subsequent Tukey's test indicated that 6-OHDA-lesioned iMSN-D_2RKO mice showed a significant worsening of LID when compared to both WT and ChI-D2RKO mice (**Figure 8A**; $p < 0.05$).

To unveil whether L-DOPA was differently affecting the development of discrete dyskinetic movements, ALO AIMs scores were evaluated individually. Two-way ANOVA revealed that the total score for axial, limb and orolingual AIMs was affected both by the genotypes and by the type of dyskinetic movements considered (**Figure 8B;** ALO effect: $F_{2, 90}$= 11.85; $p < 0.0001$**;** Genotype effect**:** $F_{2,90}$= 9.873, p = 0.0001).

Although iMNS-D2RKO mice showed a marked trend toward worsening of all dyskinetic movements evaluated, Tukey's test evidenced as only the total axial AIMs score was statistically higher in iMSN-D_2RKO mice as compared to either WT or Chi-D_2RKO mice (**Figure 8B**; $p<0.05$); while no statistical differences were observed either in limb (**Figure 8B**; iMSN-D2RKO: vs WT, p= 0.07; vs ChI-D2RKO, p=0.13) or orolingual (**Figure 8B**; iMSN-D2RKO: vs WT, p= 0.09; vs ChI-D2RKO, p=0.09) AIMs among genotypes.

Based on these results, we asked whether the worsening of the dyskinetic behaviours observed in iMSN-D_2RKO mice was present or not since the first L-DOPA administration. Thus, AIMs scores obtained at day 1, 4, 7 and 10 of L-DOPA treatment were analysed.

Two-way repeated measures ANOVA showed that the cumulative number of AIMs observed during the chronic L-DOPA treatment was affected both by the genotypes ($F_{2, 90}$= 14.64, $p < 0.0001$) and by the day in which L-DOPA was administrated ($F_{3, 90}$= 3.137; p = 0.0293). Tukey's test revealed as iMSN-D_2RKO mice started to suffer of a significant worsening of LID from day 4 of L-DOPA treatment (**Figure 8C;** $p<0.05$ vs WT and ChI-D2RKO mice), reaching the peak on day 10 (**Figure 8C**; $p<0.01$ vs WT and ChI-D2RKO mice).

Interestingly, time course analyses of the ALO AIMs scores obtained on day 10 of L-DOPA treatment revealed that the widest difference between iMSN-D_2RKO, ChI-D_2RKO and WT mice took place during the first 40 minutes following L-DOPA injection, when DA concentration is expected to be higher (**Figure 8D;** two-way repeated measures ANOVA followed by Tukey's test: $p<0.05$ vs WT and ChI-D2RKO).

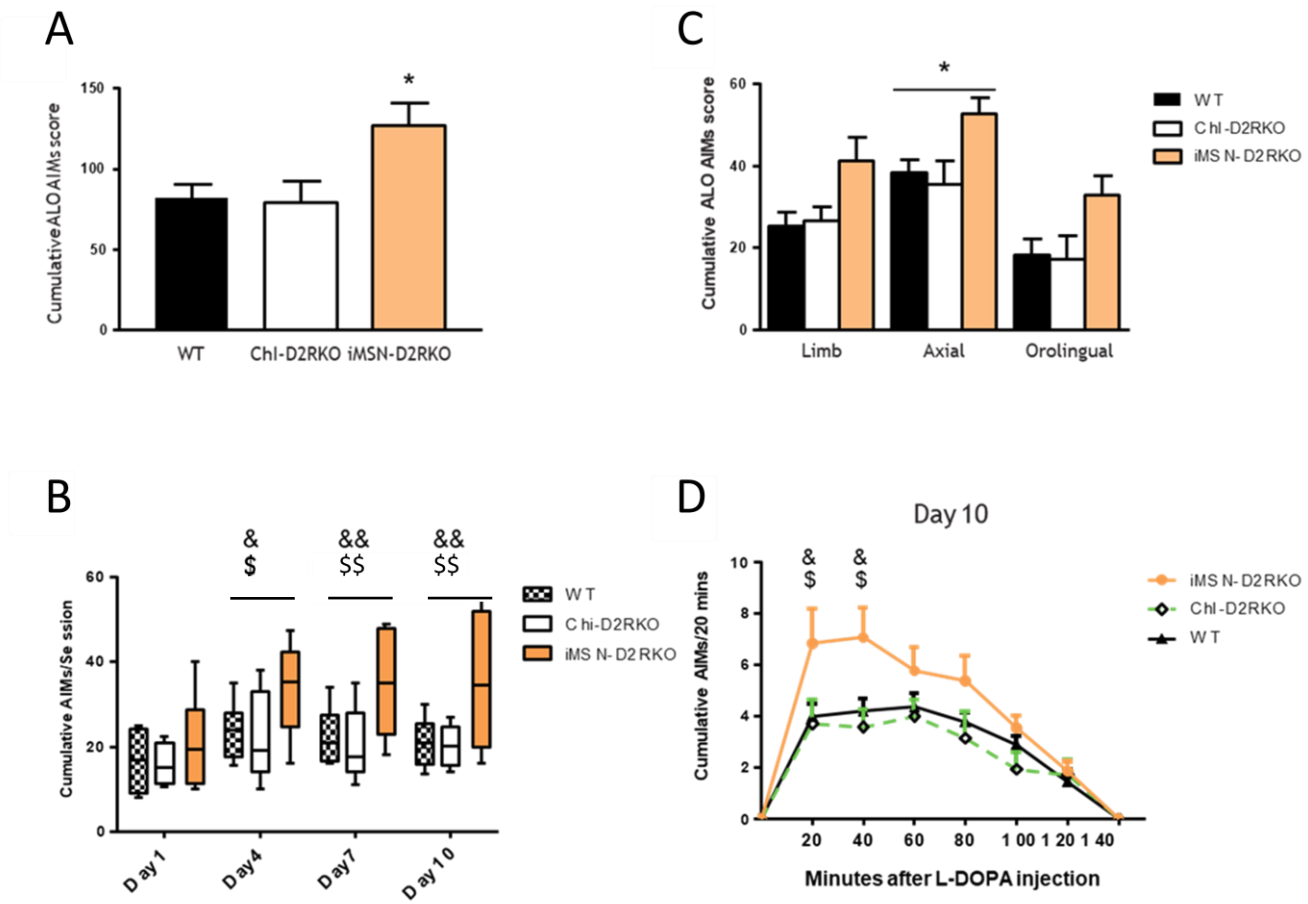

Figure 8. Selective D_2R deletion on iMSNs worsen dyskinetic behaviour after chronic L-DOPA administration. (A) Bar graph showing the cumulative ALO AIMs scores measured during day 1, 4, 7 and 10 of L-DOPA treatment; *p<0.05 vs WT and ChI-D_2RKO; **(B)** Bar graph showing the cumulative limb (left), axial (middle) and orolingual (right) AIMs scores measured at day 1, 4, 7 and 10 of L-DOPA treatment; *p< 0,05 vs WT and ChI-D_2RKO; **(C)** Box plot showing the ALO AIMs scores assed at day 1, 4, 7 and 10 of L-DOPA treatment. Box plot indicate the top and bottom quartiles; whiskers refers to top and bottom 90%. $ p<0.05, $$ p<0.01 ChI-D_2RKO vs iMSN-D_2RKO; & p<0.05, && p < 0.01 WT vs iMSN-D_2RKO; **(D)** Time course showing the ALO AIMs expression after L-DOPA treatment (day 10). $$ p<0.01 ChI-D_2RKO vs iMSN-D_2RKO; & p<0.05 WT vs iMSN-D_2RKO. Data are expressed as mean value, error bars represent SEM.

Effects of acute and chronic L-DOPA on *c-fos* and pERK activation in the dorsolateral CPu.

Since LID appearance is directly correlated with the overactivation of dMSNs (Picconi et al., 2003; Santini et al., 2010), we evaluated, in the dorsolateral CPu, the acute and chronic effects of L-DOPA on the activation of pERK and its downstream target *c-fos*, two biomarkers previously correlated with LID (Valjent et al., 2000; Santini et al., 2007; Westin et al., 2007) (**Figure 9**). Results showed that both acute and chronic L-DOPA administration induced a significant increase of the total number of *c-fos* positive neurons in the dorsolateral CPu of 6-OHDA-lesioned mice when compared to sham-lesion controls (**Figure 9 A-B, D-E**). Two-way ANOVA analysis revealed that the total number of *c-fos* positive neurons was significantly affected by the lesion (6-OHDA vs sham lesion) (**Figure 9 A-B, D-E;** Acute: $F_{1,16}$ = 67,3; $p < 0,0001$; Chronic: $F_{1,16}$ = 249.7, $p < 0.0001$). Importantly, Newman-Keuls post-hoc test showed that acute L-DOPA significantly enhanced the number of *c-fos* positive neurons in the dorsolateral CPu of iMSN-D_2RKO mice compared to ChI-D_2RKO mice (**Figure 9B;** $p<0.05$), while in chronic L-DOPA treated mice, *c-fos* levels from iMSN-D_2RKO mice was statistically higher compared to both ChI-D_2RKO and WT mice (**Figure 9E;** $p<0.05$).

Similarly, both acute and chronic L-DOPA produced a critical elevation of pERK intensity/cell in the dorsolateral CPu of 6-OHDA-lesioned mice. Indeed, two-way ANOVA analysis revealed as the mean intensity/cell of pERK positive neurons was significantly affected by the lesion (6-OHDA vs sham lesion) (**Figure 9 A,C,D,F;** Acute: $F_{1,16}$ = 84.22, $p < 0.0001$; Chronic: $F_{1,26}$ = 249.2, $p < 0.0001$). A significant difference in pERK intensity/cell was observed between iMSN-D_2RKO and ChI-D_2RKO mice after acute L-DOPA treatment (**Figure 9 A,C;** $p<0.05$; Newman-Keuls post-hoc test) and between iMSN-D_2RKO and WT and ChI-D_2RKO mice after chronic L-DOPA treatment (**Figure 9 D,F;** $p<0.05$; Newman-Keuls post-hoc test). Nevertheless, no differences were observed in the total number of pERK positive neurons in the 6-OHDA-lesioned dorsolateral CPu among genotypes after either acute or chronic L-DOPA.

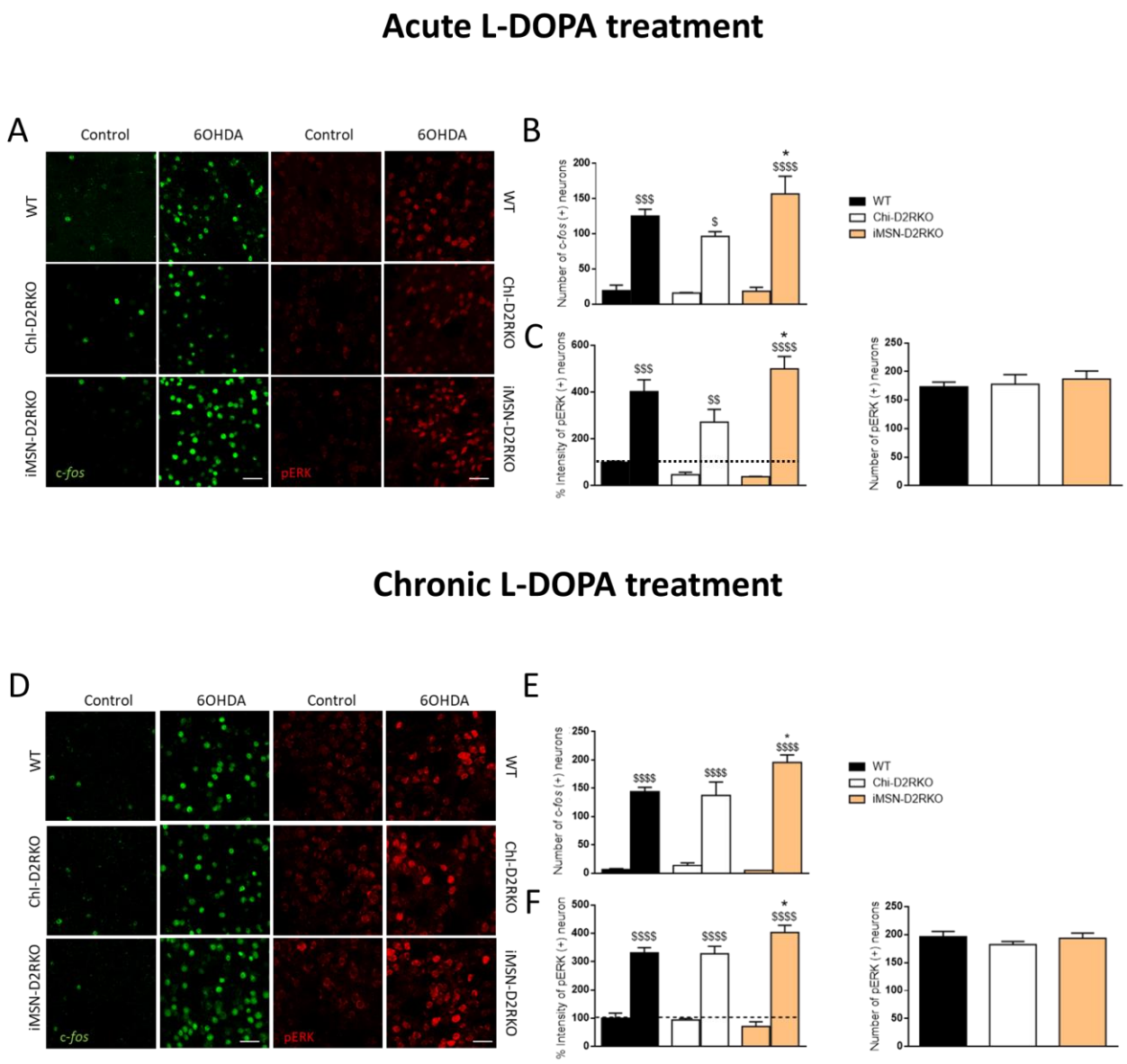

Figure 9. Activation of *c-fos* and pERK after either acute or chronic L-DOPA treatment. Representative confocal images from the dorsolateral CPu showing the immunoreactivity for *c-fos* (green) and pERK (red) after single (**A**) or multiple (11 injections, once a day) (**D**) injections of L-DOPA. Acute L-DOPA treatment: (**B**) Bar graph showing the number of *c-fos* positive neurons; * p<0.05 vs ChI-D$_2$RKO 6-OHDA-lesion CPu; $ p< 0.05 vs control CPu; $$$ p< 0.001 vs control CPu; $$$$ p< 0.0001 vs control CPu. (**C**) Bar graphs showing the percentage of the mean intensity/cell (left) and the total number (right) of pERK positive neurons; * p<0.05 vs ChI-D2RKO 6-OHDA-lesion CPu; $$ p< 0.01 vs control CPu; $$$ p< 0.001 vs control CPu; $$$$ p< 0.0001 vs control CPu. Chronic L-DOPA treatment: (**E**) Bar graph showing the number of *c-fos* positive neurons; * p<0.05 vs WT and ChI-D2RKO 6-OHDA-lesion CPu; $$$$ p< 0.0001 vs control CPu; (**F**) Bar graphs showing the percentage of the mean intensity/cell (left) and the total number (right) of pERK positive neurons; * p<0.05 vs WT and ChI-D2RKO 6-OHDA-lesion CPu; $$$$ p< 0.0001 vs control CPu. Data are expressed as mean value, error bars represent SEM.

Effects of chronic L-DOPA on rpS6 phosphorylation in the dorsolateral CPu

The rpS6 is a component of the ribosomal complex which, differently from ERK, is phosphorylated exclusively in response to dopaminergic stimulation (Biever et al., 2015). Since rpS6 phosphorylation has been associated with states of sustained neuronal activation as well as modification of the synaptic plasticity, we asked whether L-DOPA may produce an abnormal phosphorylation of rpS6 specifically at residues $Ser^{235/236}$ and $Ser^{240/244}$ in the dorsolateral CPu of 6-OHDA-lesioned iMSN-, Chi-D_2RKO and WT mice (**Figure 10**).

Regardless the genotype, results showed that chronic L-DOPA treatment induced a significant increase of the total number of both p-rpS6-$S^{235/236}$ and -$S^{240/244}$ positive neurons in dorsolateral CPu of the 6-OHDA-lesioned mice when compared to the sham-lesioned one (**Figure 10 A-C**; One-way ANOVA, p-rpS6-$S^{240/244}$: $F_{1,17} = 231.8$, $p < 0.0001$; p-rpS6-$S^{235/236}$: $F_{1,17} = 801.1$, $p < 0.0001$).

However, no statistical differences were observed among 6-OHDA-lesioned WT, ChI-D2RKO and iMSN-D2RKO mice when either the total number or the intensity/cell of p-rpS6 positive neurons were compared (**Figure 10 A-C;** Newman-Keuls post-hoc test, $p>0.05$).

Effect of chronic L-DOPA on striatal cholinergic interneurons activity

Acetylcholine released from ChIs plays a pivotal role in the regulation of striatal functions (Tanimura et al., 2018). Since removal of striatal ChIs it has been reported to reduced LID in 6-OHDA-lesioned mice (Won et al., 2014), we asked whether the cell-specific ablation of D_2R either from iMSNs or ChIs may affect ChIs activity and consequently LID expression. To investigate ChIs activation in the dorsalateral CPu, we used antibodies directed against either p-rpS6-$S^{240/244}$ or pERK in combination with choline acetyltransferase (ChAT), an exclusive marker of ChIs.

Results showed that chronic L-DOPA administration produced a strong activation of p-rpS6-$S^{240/244}$ and pERK in the 6-OHDA-lesioned dorsal CPu of WT, ChI- and iMSN-D_2RKO mice **(Figure 10 D-F)**. One-way ANOVA analysis revealed as the mean intensity/cell of either p-rpS6-$S^{240/244}$ (**Figure 10 D-E;** $F_{2,11} = 10.16$, $p < 0.0049$) or pERK (**Figure 10 D,F;** $F_{2,12} = 5.233$, $p < 0.0232$) on ChaT positive ChIs was significantly affected by the genotypes. Indeed, Newman-Keuls post-hoc test showed that ChIs from ChI-D_2RKO and iMSN-D_2RKO mice presented a significant elevation of the mean intensity/cell when compared to WT mice (**Figure 10 D-E;** *$p<0.05$; **$p<0.01$). Interestingly, our results indicated that after chronic L-DOPA, ERK phosphorylation was statistically higher in ChaT positive neurons coming from iMSN-D_2RKO mice compared to both WT and ChI-D2RKO mice (**Figure 10 D,F;** *$p<0.05$).

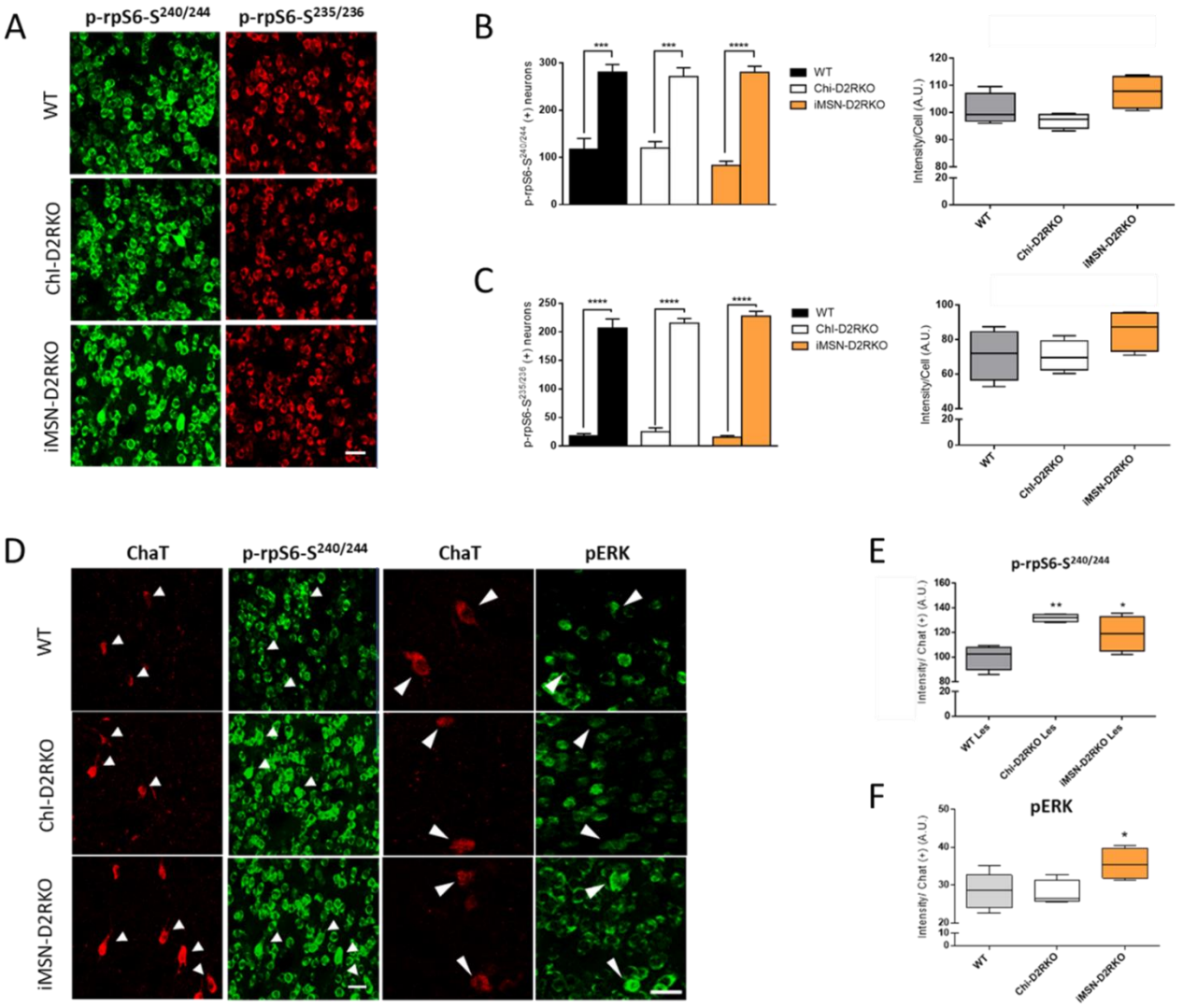

Figure 10. Lack of D_2R differentially affects striatal ChIs activity in 6-OHDA-treated mice treated with L-DOPA. Representative confocal images from the dorsolateral CPu showing the immunoreactivity either for p-rpS6-$S^{240/244}$ (green) and p-rpS6-$S^{235/236}$ (red) in striatal neurons (**A**), or for p-rpS6-$S^{240/244}$ (green) and pERK (green) in ChaT (+) striatal cholinergic interneurons (red) (**D**). **(B)** Left: Bar graph showing the total number of p-rpS6-$S^{240/244}$ positive neurons; *** p<0.001, **** p<0.0001 vs 6-OHDA lesioned CPu; Right: Box plot showing the % mean intensity/cell of p-rpS6-$S^{240/244}$ positive neurons. **(C)** Left: Bar graph showing the number of p-rpS6-$S^{235/236}$ positive neurons; **** p<0.0001 vs 6-OHDA-lesioned CPu; Right: Box plot showing the % mean intensity/cell of p-rpS6-$S^{235/236}$ positive neurons; **(E)** Box plot showing the % mean intensity/cell of double p-rpS6-$S^{240/244}$ and $ChaT^+$ positive ChIs; * p<0.05, ** p<0.01 vs WT; **(F)** Box plot showing the % mean intensity/cell of double pERK and $ChaT^+$ positive ChIs; * p<0.05 vs WT and ChI-D_2RKO. Data are expressed as mean value, error bars represent SEM.

L-DOPA worsening of LID expression in 6-OHDA-lesioned iMSN-D_2RKO mice are dose-dependent

Preclinical evidences suggest as dyskinetic behaviours are also strongly influenced by how L-DOPA is administrated (i.e. dosage, frequency and route of administration) (Carta and Bezard, 2011; Deurwaerdère et al., 2017). In order to uncover differences in AIMs expression related to L-DOPA dosage, we tested whether in unilaterally MFB 6-OHDA-lesioned WT, ChI-D_2RKO and iMSN-D_2RKO mice the application of an ascending dose regimen of L-DOPA (1.5, 3, 6 mg/kg, s.c.; once a day for 9 consecutive days) may differently affect the severity of LID (**Figure 11A**).

Results showed that, despite the genotype, increased dosages of L-DOPA were correlated with the worsening of the cumulative AIMs scores (**Figure 11A)**. Two-way ANOVA revealed that the cumulative AIMs score was significantly affected both by the ascending L-DOPA treatment ($F_{2,79}$ = 78.99, $p < 0.0001$) as well as by the interaction between the ascending L-DOPA treatment and the genotypes ($F_{4,79}$=4.114, p=0.004) (**Figure 11A**). Importantly, subsequent Tukey's test showed as the administration of 6 mg/kg of L-DOPA statistically worsen the expression of AIMs in 6-OHDA-lesioned iMSN-D_2RKO mice when compared to both WT and ChI-D_2RKO mice (**Figure 11A**; $p<0.01$).

The selective M1R antagonist VU 0255035 reduced LID severity

The D_2 protein is a G_i-coupled metabotropic receptor whose activation inhibits AC activity. Thus, D_2R stimulation reduces intracellular cAMP/PKA-related events and leads to an overall suppression of the neuronal activity. Starting from our previous results, we asked whether the worsening of LID observed in iMSN-D_2RKO mice was solely due to the lack of the D_2R-medated inhibition of iMSNs. Since the M_1 G_q-coupled receptor is expressed by iMSNs and acts in opposition to D_2R by elevating intracellular [Ca^{2+}] and increasing iMSNs activity (Pisani et al., 2007), we tested the acute anti-dyskinetic effect of the selective M_1-antagonist VU 0255035 (60 mg/kg; i.p.) in combination with L-DOPA (6 mg/kg) in already dyskinetic WT, iMSN- and ChI-D_2RKO mice (**Figure 11 B-C**). Results indicated that, although 6-OHDA-lesioned WT and Chi-D_2RKO mice showed a trend toward a decrease, 6-OHDA-lesioned iMSN-D2RKO mice treated with VU 0255035 plus L-DOPA were the only one showing a significant reduction of the cumulative AIMs scores when compared to L-DOPA treated iMSN-D_2RKO mice (**Figure 11B**). Two-way ANOVA showed that the cumulative AIMs scores were affected both by the treatments (L-DOPA vs VU 0255035 plus L-DOPA) ($F_{1,34}$=10.83; p=0.0023) and by the genotypes ($F_{2,34}$=8.359; p=0.0011) (**Figure 11B**). Tukey's test further evidenced a statistical difference in the cumulative AIMs scores between L-DOPA and L-DOPA + VU 0255035-treated iMSN-D_2RKO mice (**Figure 11B**; $p<0.05$). Interestingly, time course analyses indicated that, in iMSN-D_2RKO mice, VU 0255035 treatment significantly reduced L-DOPA pro-dyskinetic effects mostly during the first 40 minutes following L-DOPA administration (**Figure 11C;** rm two-way ANOVA followed by Bonferroni's test, $p<0.05$).

Behavioural observations were supported by immunohistochemical analyses aimed to investigate how the blockade of the M_1-related signalling was affecting the phosphorylation of ERK and its downstream target *c*-

fos in the dorsolateral CPu (**Figure 11 D-E**). Overall, results showed a reduction of both the number of pERK and *c-fos* as well as of the mean grey intensity/cell of pERK positive neurons in WT, ChI-D_2RKO and iMSN-D_2RKO mice receiving VU 0255035 in combination with L-DOPA when compared with L-DOPA treated mice sharing the same genotype. For *c-fos,* statistical analyses revealed that the total number of immunoreactive neurons was significantly reduced in the dorsolateral CPu of iMSN-D_2RKO mice treated with VU 0255035 plus L-DOPA when compared with iMSN-D_2RKO mice treated with L-DOPA (**Figure 11D**; two-way ANOVA followed by Newman-Keuls post-hoc test, $p<0.01$). For pERK, two-way ANOVA indicated that both the number ($F_{1,23}=29.07$; $p<0.0001$) and the mean grey intensity/cell ($F_{1,23}=43.67$) of pERK positive neurons was significantly affected by the treatments (**Figure 11E)**. Interestingly, while we observed a significant difference between treatments in the reduction of the number of pERK (+) neurons exclusively in iMNS-D_2RKO mice (**Figure 11E;** Newman-Keuls post-hoc test, $p<0.05$), the mean grey intensity/cell of pERK (+) was statistically reduced among all genotypes ($p<0.001$ vs either WT, ChI-D_2RKO or iMSN-D2RKO L-DOPA treated mice).

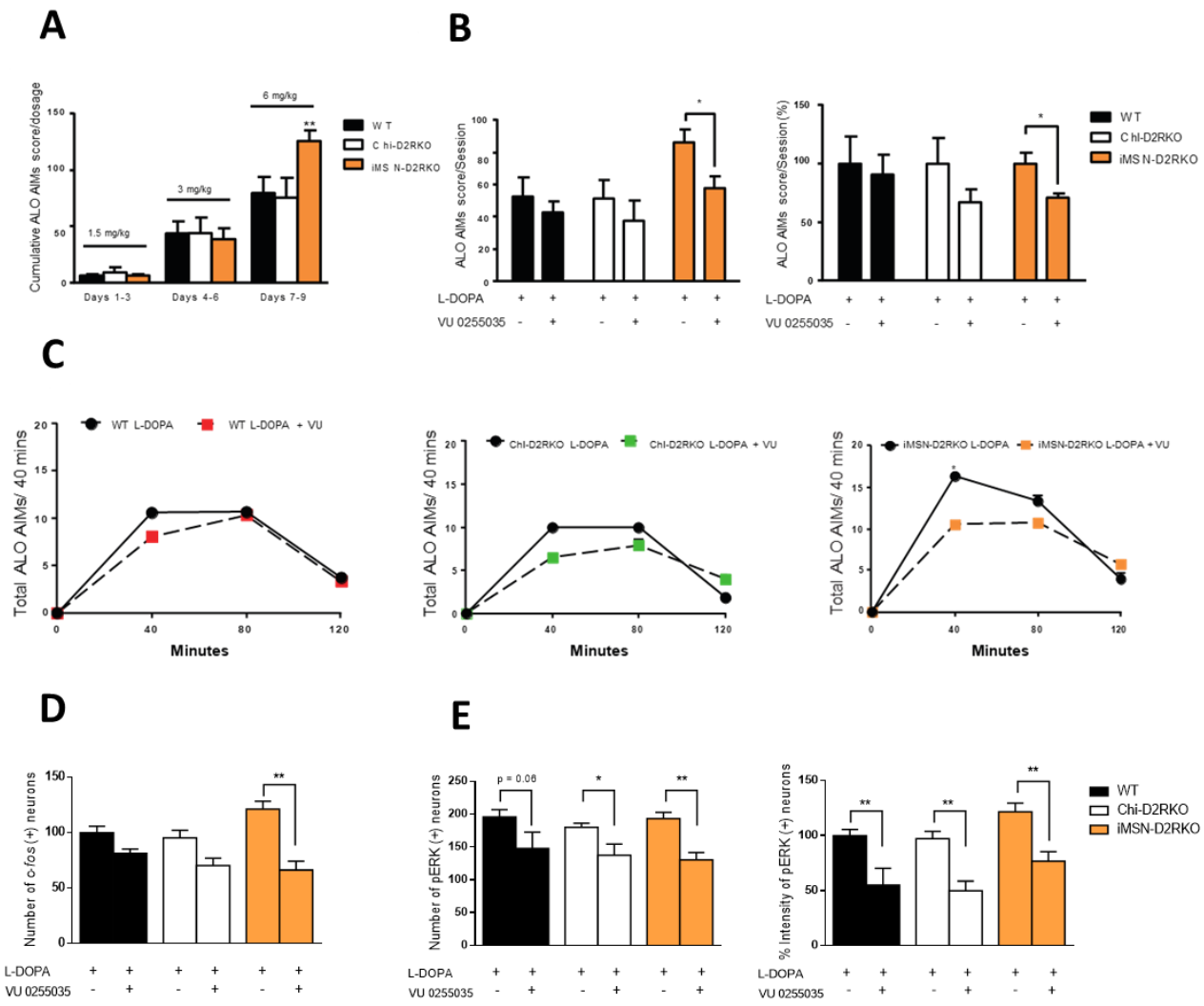

Figure 11. DA and Ach modulation of iMSNs activity is correlated with LID severity (A) Bar graph showing the cumulative AIMs scores measured after each L-DOPA injection; **p<0.05 vs WT and ChI-D_2RKO; **(B)** Bar graphs showing the ALO AIMs scores measured after the acute administration of 6 mg/kg of L-DOPA combined either with the M1 selective antagonist VU 0255035 or its vehicle (right: raw value; left: % value); *p< 0,05 vs L-DOPA treatment **(C)** Time course of the ALO AIMs scores measured after the co-administration of L-DOPA either with VU 0255035 (dash-line) or its vehicle (full-line) in WT (left), ChI-D_2RKO (middle) and iMSN-D_2RKO (right) mice. * p<0.05 vs L-DOPA + VU 0255035. **(D)** Bar graphs showing the total number of *c-fos* (+) neurons after either L-DOPA (control) or L-DOPA + VU 0255035. * p<0.05 vs L-DOPA; **(E)** Bar graphs showing the total number and the % mean grey intensity/cell of pERK (+) neurons after either L-DOPA (control) or L-DOPA + VU 0255035. * p<0.05 vs L-DOPA; ** p<0.01 vs L-DOPA. Data are expressed as mean value; error bars represent SEM.

Striatal eCB-LTD in iMSN-D_2RKO and 6-OHDA lesioned mice is restored following the selective inhibition of the cholinergic M_1 receptor

The eCB-LTD is the best-characterized form of synaptic plasticity in the CPu and makes glutamatergic synapses less excitable to subsequent stimulations (Bassi et al., 2017). Acting through the D_2R, DA promotes eCB-LTD expression by inhibiting the cAMP/PKA/RGS-4 signalling pathway and facilitating the mGluR5-dependent production of eCBs (Lerner and Kreitzer, 2012). Since 6-OHDA-lesioned iMSN-D_2RKO mice manifested more severe AIMs following L-DOPA compared to ChI-D_2RKO and WT mice, we asked whether the lack of the D_2R specifically on iMSNs was sufficient to inhibit the genesis of LFS-LTD, making corticostriatal synapses unable to respond adequately to external stimuli of different nature. To this end, we measured the excitatory post-synaptic field potential (fEPSP) in the dorsolateral CPu from control (no surgery), 6-OHDA- and sham-lesioned iMSN-D2RKO and WT mice. To test the expression of LTD, we delivered a train of LFS (10Hz) over 10 minutes in cortical afferents.

In both control and sham-lesioned WT mice, LFS-LTD induced a significant long-lasting depression of fEPSP **(Figure 12B, left;** control: 64% ± 4%, sham-lesioned: 65% ± 5%; rmANOVA; p<0.0001**)** which was not detected in recordings obtained from both control and sham-lesioned iMSN-D_2RKO mice (**Figure 12C, left;** control: 102% ± 4%, sham-lesioned: 103% ± 3%; rmANOVA; p>0.05). These results suggest that, in our experimental condition, activation of the D_2R on postsynaptic iMSNs is required for the generation of LFS-LTD. To investigate the relation between DA-depletion and LFS-LTD, we tested LFS-LTD expression in the dorsolateral CPu from both the 6-OHDA-lesioned and the non-lesioned hemisphere of iMSN-D_2RKO and WT mice (**Figure 12 B-C, right)**. In agreement with previous studies (Kreitzer and Malenka, 2007; Bagetta et al., 2011; Lerner and Kreitzer, 2012), the removal of nigrostriatal afferents prevents the generation of any LFS-LTD in WT mice (**Figure 12B, right;** 96% ± 3.5%, rmANOVA; p>0.05). Unsurprisingly, the 6-OHDA-lesioned dorsolateral CPu of iMSN-D_2RKO mice did not express any LFS-LTD similarly to what observed in the non-lesioned side (**Figure**

12C, right; 101% ± 5%, rmANOVA; p>0.05). These results remark that striatal LFS-LTD is finely regulated by circulating DA.

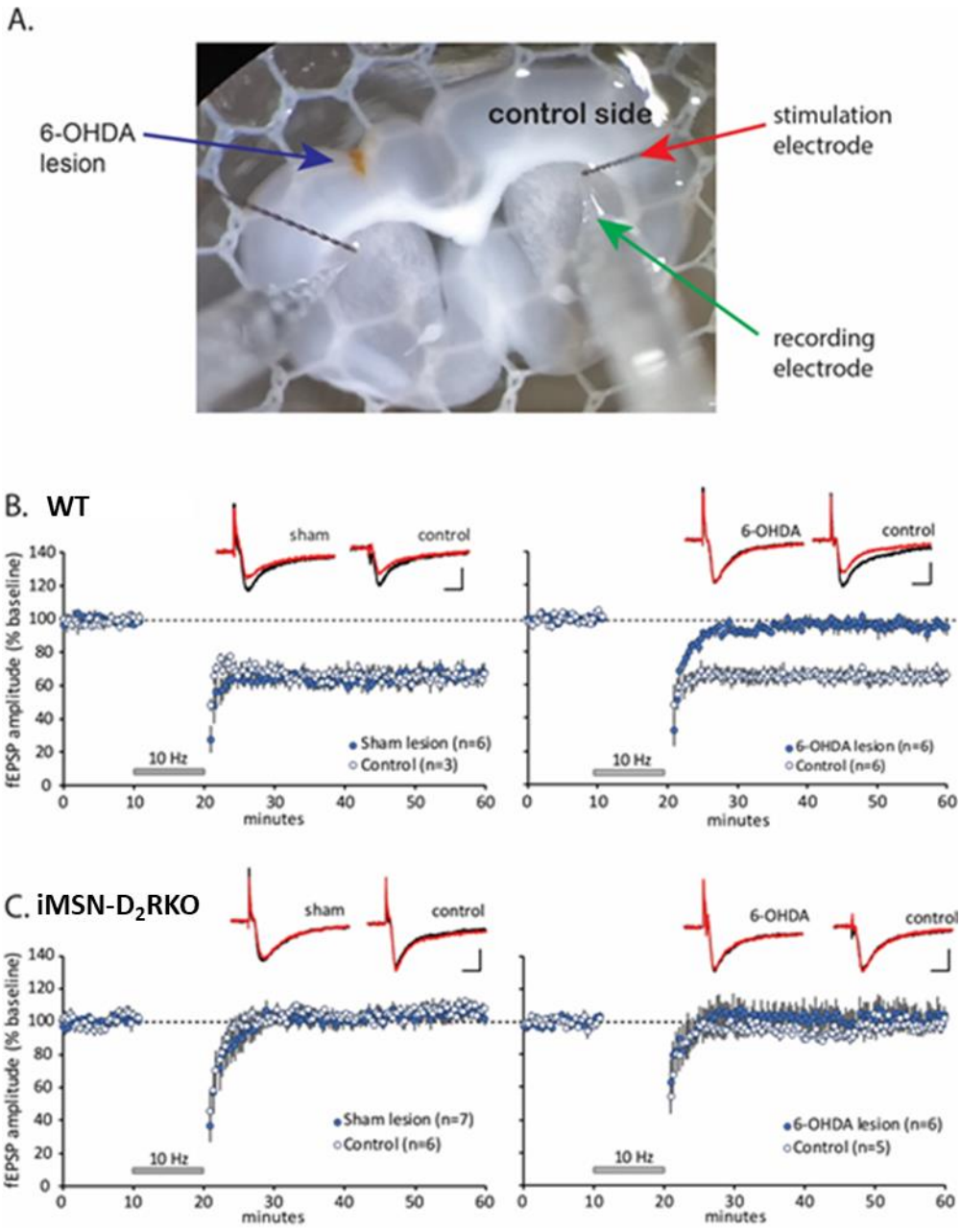

Figure 12. Delivery of LFS promotes LTD in the dorsolateral CPu of WT but not iMSN-D2RKO mice. Photo showing how LFS were delivered in the dorsal CPu (**A**). Results showed as LFS promoted the expression of a robust LTD in the dorsolateral CPu of control and sham-lesion WT mice (**B, left**) but not in 6-OHDA-lesioned WT mice (**B, right**). Results showed as LFS were not able to promote the generation of LTD in the dorsolateral CPu of neither control, sham-lesion (**C, left**) or 6-OHDA-lesioned iMSN-D_2RKO mice (**C, right**). PS amplitude were normalized to baseline, average (mean ± SEM), and plotted as a function of time.

Behavioural and immunohistochemical results demonstrated that already dyskinetic mice acutely treated with L-DOPA in combination with the selective M_1R antagonist VU 0255035 showed an attenuation of their

dyskinetic behaviours. Thus, we asked whether the pre-application of VU 0255035 (10 µM) in the bath solution may restore the LFS-LTD in the dorsolateral CPu of 6-OHDA-lesioned mice WT and iMSN-D_2RKO. Our recordings indicated that bath-application of VU 0255035 restored the expression of a robust LFS-LTD in the dorsolateral CPu of 6-OHDA lesioned WT mice (**Figure 13A, left**; 68% ± 9%, rmANOVA; p<0.001). Importantly, the selective inhibition of M_1R restored the LFS-LTD in both the control and 6-OHDA lesioned dorsolateral CPu of iMSN-D_2RKO mice (**Figure 13A, right;** control: 76% ± 3%, lesioned: 82% ± 2%, rmANOVA; p<0.0001). These results suggested that acetylcholine-mediated stimulation of postsynaptic M_1R strongly inhibit LTD generation in cortico-striatal synapsis, acting in opposition to D_2R.

Lastly, bath application of the selective CB_1R antagonist AM 251 (5 µM) blocked the generation of VU 0255035-induced LFS-LTD in both control and 6-OHDA-lesioned dorsolateral CPu of both WT and iMSN-D_2RKO mice (**Figure 13B;** rmANOVA, p>0.05). These results indicated that, by blocking the postsynaptic M_1R, VU 0255035 likely enhanced the endogenous production of eCB which in turn stimulate the presynaptic CB_1R located in corticostriatal afferents.

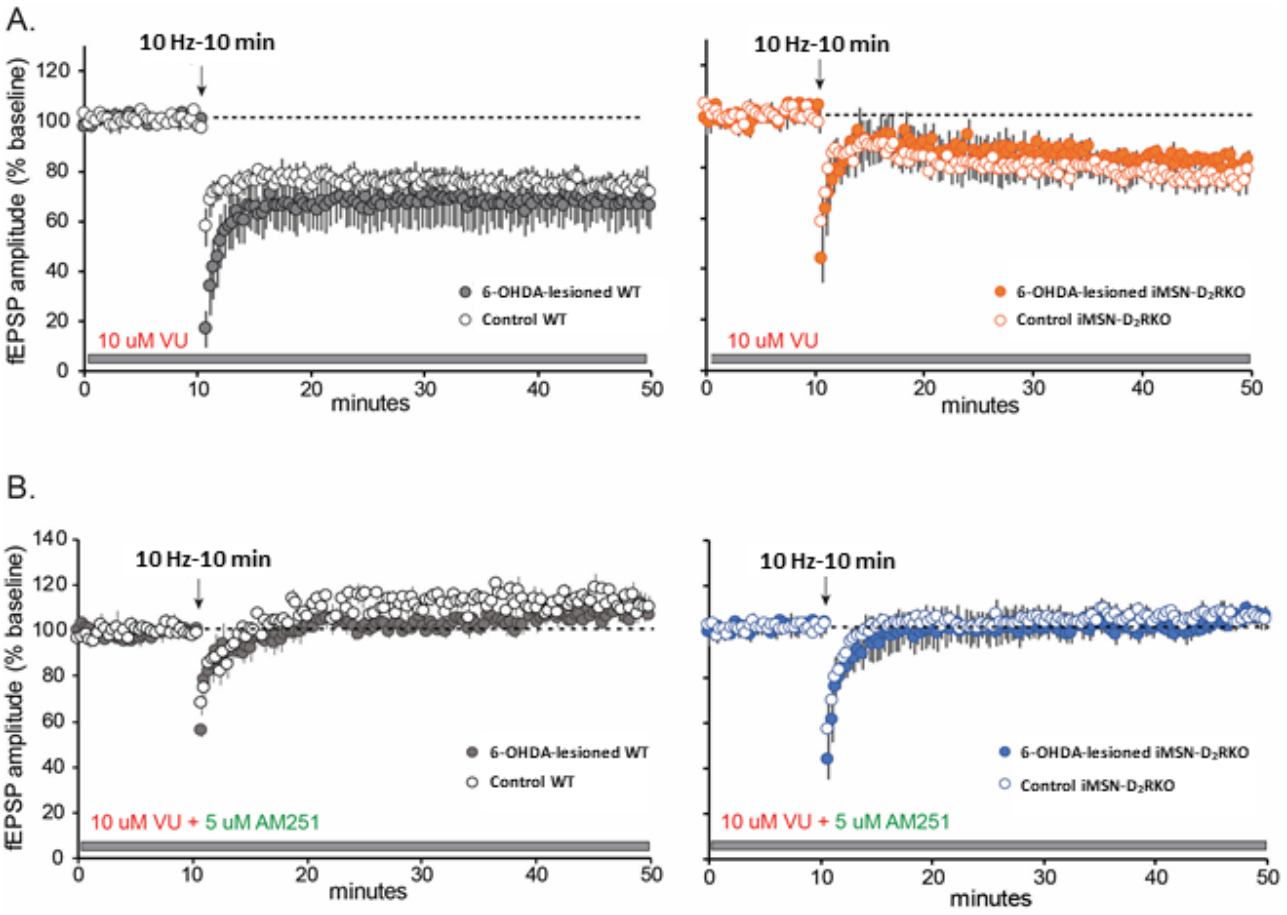

Figure 13. VU 0255035 restored the LFS-LTD elevating the stimulation of presynaptic CB_1R. Results demonstrated as the bath application of the selective M_1R antagonist VU 0255035 restored the expression of LFS-LTD in the dorsolateral CPu of both 6-OHDA-lesioned WT mice (**A, left**) and sham-,6-OHDA-lesioned iMNS-D_2RKO mice (**A, right**). Results

demonstrated as bath co-application of VU 0255035 and of the selective CB_1R antagonist AM251 completely blocked the generation of any LFS-LTD in both sham- and 6-OHDA-lesioned WT (**B, left**) and iMSN-D_2RKO (**B, right**) mice, suggesting the involvement of eCB in the M_1-dependent LFS-LTD. PS amplitude were normalized to baseline, average (mean ± SEM), and plotted as a function of time.

Discussion

The aim of the present study was to evaluate: i) the involvement of D_2R, located either in iMSNs or in ChIs, in the expression of LID; ii) whether the pharmacological manipulation of Ach may alleviate the severity of AIMs in already dyskinetic mice. Our results clearly demonstrated that the lack of D_2R in iMSNs, but not in ChIs, critically worsened the expression of LID in 6-OHDA-lesioned mice and was associated to the overactivation of ERK signalling as well as the disruption of the striatal synaptic plasticity. Interestingly, administration of the selective M_1R antagonist VU 0255035, significantly ameliorated dyskinetic behaviours, as well as LID-related pathological events, in L-DOPA-treated iMSN-D_2RKO mice, while a mild effect was observed both in WT and ChI-D_2RKO mice.

Stimulation of D_2R located on iMSNs mediates the pro-dyskinetic effects of L-DOPA

Despite a large body of evidences pointed out that $D_{2/3}R$ are critically involved in striatal motor functions as well as in the development of LID, little is known about the specific contribution of D_2R versus D_3R in LID as well as about the functions of D_2R located on different striatal neurons.

In this respect, the hemi-parkinsonian mouse model represents a unique resource in LID research since it models the cardinal motor symptoms observed in PD and it is suitable for genetic manipulation. Thus, unilateral 6-OHDA-lesioned cell-specific KO mice, lacking the D_2R either in iMSNs or in ChIs, were employed. Although healthy iMSN-D_2RKO mice have a normal life span and reproductive capacity, they suffer of severe motor disabilities (lack of coordination, stiffness, akinesia) which resemble that observed in PD patients and D_2R-null mice (Baik et al., 1995; Anzalone et al., 2012). On the other hand, healthy ChI-D_2RKO mice have normal weight, size, fertility and show normal motor behaviours when comparted to WT littermates (Kharkwal et al., 2016).

Previous studies performed in DA-depleted rodents (Drake et al., 2013; Delfino et al., 2004), NHPs (Luquin et al., 1992; Calon et al., 1995; Grodin et al., 1996; Goulet et al., 1997) and PD patients (Guttman, 1997; Rascol et al., 2006) demonstrated as the chronic administration of selective $D_{2/3}R$ agonists not only ameliorated parkinsonian symptoms, but also promoted the development of dyskinesia, especially when given in a pulsatile fashion. On the other hand, the administration of selective $D_{2/3}R$ antagonists (e.g. eticlopride, raclopride) reduced LID expression (Monville et al., 2005; Taylor et al., 2005; Sebastianutto et al., 2016).

The anti-dyskinetic effects of $D_{2/3}R$ antagonists have been related to their ability to prevent the stimulation of sensitized post-synaptic $D_{2/3}R$ located in the CPu (Taylor et al., 2005), hence rebalancing the physiological MSN neuronal activity.

In contrast, our results clearly showed that while the genetic removal of D_2R from ChIs produced no effects in LID expression, the same approach at the level of iMSNs significantly worsened the expression of dyskinetic behaviours in unilaterally 6-OHDA-lesioned, following chronic L-DOPA administration.

Indeed, despite the surgical approach, we observed a significant worsening of LID severity in unilateral 6-OHDA-lesioned iMSN-D_2RKO mice chronically treated with high doses of L-DOPA (15 mg/kg, for 6-OHDA-striatal injection; 6 mg/kg, for 6-OHDA MFB injection) when compared to both WT and ChI-D_2RKO mice. LID worsening involved principally, but not exclusively, muscles located in the trunk and was particularly evident immediately after the administration of L-DOPA (from minute 0 to minute 40), when striatal L-DOPA-derived DA reached its peak of concentration (Lee et al., 2008).

How these differences could be explained? Differently from previous pharmacological studies, which could not discriminate among D_2 and D_3R, our knock-out models have the great advantage of showing the specific role exerted by striatal D_2R in LID, in a cell-specific fashion, without affecting the DA-mediated stimulation of striatal D_3R.

The segregation of D_2 and D_3R involvement in LID represents an essential necessity in LID-related studies since, in the CPU, D_3R are located in dMSNs and their activation potentiates the D_1R-mediated signalling (Marcellino et al., 2008). In this respect, previous pharmacological studies showed as the repeated administration of selective D_3R agonists (e.g. PD-128.907), as well as partial agonists (e.g. BP897), may induce dyskinesia (Blanchet et al., 1997; Bezard et al., 2003; Lanza et al., 2018), while their selective blockade (e.g. S-33.084, PG01037) or genetic removal (Solis et al., 2017) significatively alleviated LID without worsening the therapeutic effects of L-DOPA (Visanji et al., 2009; Sebastianutto et al., 2016). Moreover, a significant elevation in D_1/D_3R co-localization was also observed in the dorsal CPu of dyskinetic rats following chronic treatment with L-DOPA (Bordet et al., 1997; Bezard et al., 2003; Azkona et al., 2014).

Considering these evidences, we inferred that the lack of specificity among D_2 and D_3R, combined with the blockade of D_2R on multiple striatal sites underpinned the important differences observed between the present and previous studies and that the blockade of D_3R, alone or in combination with D_2R, may be required to replicate the anti-dyskinetic effects observed following the administration of $D_{2/3}$R antagonist .

Alternatively, although no alterations were found either in the expression of D_1R located in dMSNs (Anzalone et al., 2012; Kharkwal et al., 2016), or in the amount of DA released from nigrostriatal terminals (Anzalone et al., 2012; Kharkwal et al., 2016), we cannot exclude that following a dramatic event, such as the complete removal of dopaminergic nigrostriatal afferents, the lack of D_2R specifically in iMSNs may trigger a series of adaptative events which may facilitate the L-DOPA-induced overactivation of the direct pathway. Unfortunately, to the best of our knowledge, no one has ever tested whether the chronic administration of selective $D_{2/3}$R antagonists, far before the removal of dopaminergic nigrostriatal afferents, may facilitate the activation of the dMSNs following chronic L-DOPA and further studies are needed to address this point.

Activation of ERK signalling is directly correlated with LID

In the CPu, the loss of dopaminergic innervation is associated with the sensitization of post-synaptic DAR (Cenci and Konradi, 2010; Spigolon and Fisone, 2018). Stimulation of sensitized DAR produces the abnormal activation of canonical and non-canonical intracellular pathways, event which is thought to be directly correlated with LID (Cenci and Konradi, 2010).

In accordance, our results indicated as 6-OHDA-lesioned mice acutely or chronically treated with 15 mg/kg of L-DOPA displayed a significant activation of *c-fos,* p-ERK, p-rpS6-$S^{235/236}$ and p-rpS6-$S^{240/244}$ in the dorsolateral CPu when compared to sham-lesioned controls. Interestingly, while no differences were observed among genotypes in the activation of either p-rpS6-$S^{235/236}$ or p-rpS6-$S^{240/244}$, chronic L-DOPA produced a significant elevation of *c-fos* and pERK immunoreactivity specifically in the dorsolateral CPu of 6-OHDA-lesioned iMSN-D_2RKO mice when compared to both ChI-D_2RKO and WT mice, suggesting a direct correlation between the pro-dyskinetic effects of L-DOPA and the activation of the ERK pathway. In this respect, previous investigations revealed as the pharmacological blockade of ERK was correlated with a significant reduction of AIMs (Santini et al., 2007; Lindgren et al., 2010).

On the other hand, considering that rpS6 phosphorylation depended principally on the stimulation of D_1R (Biever et al., 2015), we hypothesized that our results on p-rpS6 simply reflected the higher release of L-DOPA-derived DA from presynaptic terminals and suggested a comparable stimulation of sensitized post-synaptic D_1R among genotypes.

In addition, we also found a significant activation of both p-rpS6-$S^{240/244}$ and pERK specifically in ChIs located in the dorsolateral CPu of chronically L-DOPA-treated iMSN-D_2RKO mice, suggesting that ChIs over-activation may contribute to the worsening of LID. In this respect, previous studies demonstrated as chronic L-DOPA treatment was associated with a significant increase of pERK immunoreactivity specifically in striatal ChIs (Ding et al., 2010), and that selective ablation of striatal ChIs markedly ameliorated LID (Won et al., 2014).

Expression of striatal LFS-LTD is impaired in mice lacking D_2R on iMSNs and in 6-OHDA-lesioned mice

Recordings of the fEPSP performed in the dorsolateral CPu of either control (no surgery), sham- or 6-OHDA-lesioned iMSN-D_2RKO and WT mice revealed as the delivery of LFS on corticostriatal afferents promoted a robust and long-lasting LTD exclusively in control and sham-lesioned WT. Indeed, neither 6-OHDA-lesioned WT mice nor control, sham- or 6-OHDA-lesioned iMSN-D_2RKO mice expressed LTD following the application of LFS.

Similar results were obtained either in striatal sections from D2R-null mice (Calabresi et al., 1997) or after the bath-application of the selective D2R antagonist sulpiride (Kreitzer and Malenka, 2005, 2007) following the delivery of HFS. Moreover, in accordance to our findings, DA depletion was found to prevent the expression of the HFS-evoked LTD (Calabresi et al., 2007; Kreitzer and Malenka, 2007; Shen et al., 2008).

These results suggested that DA stimulation, especially at the level of post-synaptic D_2R located on iMSNs, was critical for the generation of striatal LTD.

On these bases, we hypothesized that basal motor deficits as well as the severe LID observed in iMSN-D_2RKO mice may derive from the lack of a physiological neuronal plasticity at the level of corticostriatal synapses.

The selective M_1 antagonist VU 0255035 ameliorates LID and restores striatal LFS-LTD

In the CPu, iMSNs activity is oppositely modulated by DA and Ach. Indeed, while D_2R stimulation inhibits iMSNs activity, stimulation of M_1R promotes the opposite effect (Tanimura et al., 2018). Since the pro-dyskinetic effects of L-DOPA increased significantly in mice lacking the D_2R in iMSNs, we asked whether the selective blockade of M_1R may be consider as a valid pharmacological strategy able to normalize iMSNs activity and reduce LID in already dyskinetic mice.

Our results clearly demonstrated that co-administration of L-DOPA with the selective M_1R antagonist VU 0255035 significantly reduced the expression of AIMs and the activation of pERK and *c-fos* in the dorsolateral CPu of iMSN-D_2RKO mice when compared to L-DOPA-treated iMSN-D_2RKO mice. Interestingly, a mild reduction of LID was also observed in WT and ChI-D_2RKO mice receiving VU 0255035 in combination with L-DOPA, even though a statistical reduction was found exclusively when the immunoreactivity for pERK was considered. The latest result suggests that, although D_2R-modulation of iMSNs activity affects LID, other events are involved in the expression and sustainment of dyskinetic behaviours following L-DOPA.

Moreover, we demonstrated as bath application of VU 0255035 was enough to recovery the expression of LFS-LTD that we found to be prevented either by the removal of D_2R from iMSNs or by DA depletion. The VU 0255035-mediated LFS-LTD was eCB dependent since the pharmacological blockade of CB_1R totally prevented the generation of LTD.

These results are in line with previous findings in which striatal HFS-LTD was either blocked by the bath application of the selective $D_{2/3}R$ antagonist sulpiride or rescued by the addition of the M_1 antagonist pirenzepine (Wang et al., 2006).

Overall, considering the higher neuronal activity of striatal ChIs observed following chronic L-DOPA treatment, the superior anti-dyskinetic effects produced by the selective M_1R antagonist VU 0255035 and the role played by DA and Ach in the generation of eCB-LTD, we inferred that in iMSN-D2RKO mice, the worsening of LID arose from the critical lower production of striatal eCB at the level of iMSNs, which derived from the lack of D_2R stimulation and the simultaneous overactivation of M_1R. Figure 14 depicts a schematic representation of the proposed mechanisms.

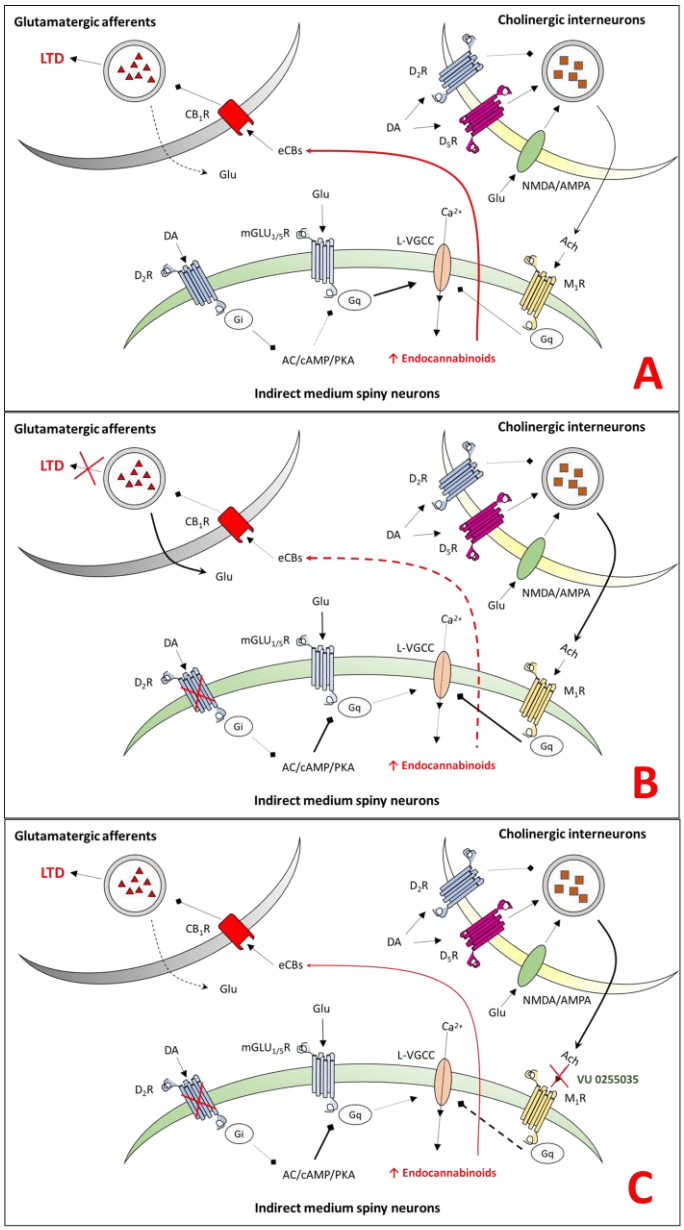

Figure 14. Stimulation of D_2R in iMSNs critically modulates LID by elevating eCB production. Schematic representation showing how DA and Ach oppositely regulate the endogenous production of eCBs from post-synaptic iMSNs in WT (**A**) and iMSN-D2RKO mice before (**B**) and after (**C**) the administration of selective M_1R antagonist VU 0255035. In WT mice (**A**), DA stimulates D_2R located on iMSNs and indirectly enhances the activation of the G_q-coupled metabotropic $mGLU_{1/5}R$ by lowering the intracellular production of cAMP (Kreitzer and Malenka, 2005). Activation of the $mGLU_{1/5}R$, in turn, increases the opening of the L-type voltage gate calcium channels (L-VGCC), elevating the intracellular $[Ca^{2+}]$ and promoting the production of eCBs (Tanimura et al., 2018). At the same time, Ach released form ChI, reduces eCB production by inhibiting Ca^{2+} entrance through the stimulation of excitatory M_1R located on iMSNs (Wang et al., 2006). Importantly, Ach release is regulated, in a time-dependent manner, not only by cholinergic $M_{2/4}$-autoreceptors, but also by DA and glutamate (Pisano et al., 2007). New synthetized eCBs, eventually, retrogradely activate inhibitory CB_1R located on presynaptic cortical terminals, reducing the strength of corticostriatal synapses, by lowering the release of glutamate. In iMSN-D2RKO mice (**B**), the lack of D_2R stimulation on iMSNs severely impairs the production of striatal eCBs, fostering an excessive release of glutamate from cortical afferents. High concentration of glutamate, in turn, may increase the activation of both MSNs and ChIs, exacerbating the expression of LID. Administration of the selective M1 antagonist VU 0255035 (**C**), partially ameliorates LID by increasing the endogenous production of eCBs, lowering glutamate release, and restoring the expression of LTD.

In this respect, our results not only corroborates previous studies showing the critical role exerted by D2R on iMSN in the generation of eCB-LTD (Calabresi et al., 1997; Gerdeman et al., 2002; Kreitzer and Malenka, 2005, 2007), but they also showed that, following chronic L-DOPA, the absence of D_2R on iMSN may significantly increase the neuronal activity of ChIs located in the dorsolateral CPu. Since DA release from pre-synaptic terminal should not differ among genotypes (Anzalone et al., 2012; Kharkwal et al., 2016), we believed that the higher ChIs activation observed in iMSN-D2RKO mice was strictly correlated with the circulating levels of glutamate which should be retrogradely lowered by the eCB-mediated stimulation of CB_1R located on presynaptic glutamatergic afferents. Indeed, glutamate not only increase MSNs excitability at post-synaptic level, but also depolarize the membrane potential of ChIs by inducing the opening of AMPA and NMDA ionotropic receptors (Pisano et al., 2017).

Importantly, we inferred that, high levels of Ach released from overactive ChIs, furtherly reduced the production of striatal eCBs, by stimulating M_1R located on iMSN and promoting the closing of L-type voltage gate calcium channels (Tanimura et al., 2018).

In accordance with this model, administration of the selective M_1R antagonist VU 0255035 not only rescued the generation of the striatal eCB-LTD, but also produced the higher anti-dyskinetic effects in iMSN-D2RKO mice as compared to both WT and ChI-D_2RKO mice. Nevertheless, further studies are required to clarify the involvement of other neurotransmitters (e.g. GABA, adenosine), how striatal levels of DA, glutamate and Ach changed following L-DOPA administration, and the role exerted by other striatal cells such as FSI and astrocytes.

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