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Novel therapeutic targets in levodopa-induced dyskinesia: in vivo studies in rodent models of Parkinson's disease

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ABSTRACT

Dyskinesia is probably the most debilitating side-effect of L-DOPA therapy of Parkinson's disease. Development of L-DOPA-induced dyskinesia (LID) reflects a process of sensitization to the drug, taking place in D1 receptor expressing striatonigral medium-sized spiny neurons (MSNs), i.e. the so-called "direct pathway", which leads to their abnormal overactivation in response to L-DOPA and dopamino-mimetics.

In the present thesis, I used in vivo microdialysis combined to behavioural assessment in 6-OHDA hemilesioned rats and mice, validated rodent models of Parkinson's disease, to investigate the involvement of striato-nigral MSNs in the mechanisms of action of different antidyskinetic treatments. Specifically, I adopted a dual probe approach to investigate the dynamics of nigral GABA and striatal glutamate following the appearance of abnormal involuntary movements (the rodent correlate of dyskinesia) in response to the administration of L-DOPA in combination with pharmacologically active compounds targeted on serotonergic 5-HT_{1A} and 5-HT_{1B} receptors, the mTORC1/Rhes complex and, lastly, cholinergic muscarinic receptors.

In the first part, I investigated the mechanism of action of eltoprazine, a 5-HT_{1A} and 5-HT_{1B} receptor mixed agonist, which has been proposed to inhibit LID appearance by reducing ectopic release of DA from striatal serotonergic terminals. I confirmed the acute and chronic antidyskinetic effects of this drug, proving in addition that eltoprazine prevented striato-nigral MSNs sensitization. Moreover, contrary to what expected, I discovered that eltoprazine attenuated LID expression at a dose that simultaneously inhibited striatal glutamate but not striatal dopamine release, suggesting the involvement of 5-HT_{1A} and/or 5- HT_{1B} heteroreceptors rather than autoreceptors.

In the second part, I investigated the role of mTORC1 and Rhes, two proteins that are along the non-canonical D1 signaling cascade. I showed that the antidyskinetic effect of rapamycin, a pharmacological inhibitor of mTORC1, could be replicated by genetic deletion of Rhes, a striatal upstream regulator of mTORC1. Moreover, I found that both approaches were successful in preventing the LID-associated rise of nigral GABA, although they differentially modulated striatal glutamate, suggesting differential effects on basal ganglia circuitry.

Finally, in the third part I attempted to investigate the role of striatal cholinergic interneurons in LID expression, specifically targeting M1 and M4 muscarinic receptors, which are highly expressed in striato-nigral MSNs. Here, I adopted a reverse dialysis approach to specifically target striatal muscarinic receptors, and minimize the issue of the

poor pharmacological selectivity of muscarinic antagonists. Striatal perfusion of the M1 receptor preferential antagonist telenzepine, and the M4 receptor preferential antagonists PD-102807 and tropicamide, inhibited LID expression along with the rise of nigral GABA and striatal glutamate, indicating both receptor subtypes contribute to striato-nigral MSNs overactivation underlying LID expression.

Overall, this study confirms that serotonergic 5-HT_{1A} and 5-HT_{1B} receptors, M1 and M4 muscarinic receptors, and the Rhes/mTORC1 complex represent promising targets in LID therapy. It shows that combined 5-HT_{1A} and 5-HT_{1B} receptor stimulation, or M1/M4 receptor and mTORC1 blockade share the ability of reducing both LID emergence and the rise in nigral GABA associated with it, providing an in vivo evidence for the crucial role of striato-nigral MSNs in their mechanisms of action. Methodologically, microdialysis proved a useful tool for identifying the neurochemical correlates of LID, and shedding light on the mechanisms of action of antidyskinetic drugs.

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INTRODUCTION

Parkinson's disease and L-DOPA-induced dyskinesia

Parkinson's disease (PD) is one of the most frequent neurodegenerative disorders, first described in 1817 by James Parkinson (Parkinson, 1817), and affecting approximately 1% of the population over 60 (Tarsy, 2012).

Neuropathological hallmarks of PD are the (progressive) loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) and the presence of Lewy bodies in the surviving neurons.

PD is clinically characterized by motor and non-motor symptoms (Beitz, 2014).

Typical motor symptoms are tremor at rest, which usually disappears when voluntary movement is performed, rigidity, expressed as a defect to obtain a complete muscular relaxation, postural instability, caused by the loss of postural reflex, and akinesia, often referred as slowness in the movement (bradykinesia). As the disease progresses, these symptoms use to get worse. Difficulty in swallowing is often a cause of death in parkinsonian patients (Jankovic, 2008).
Typical early non-motor manifestations include mood and sleep disorders, loss of the sense of smell, orthostatic hypotension. Meta-analysis studies highlighted the value of these symptoms as valuable diagnostic tools (Noyce et al., 2012). Others non-motor symptoms might be also related to PD medications.

In the late 60s, a novel strategy to counteract the motor symptoms of PD was developed, which is based on the replacement of dopamine (DA) with its precursor 3,4-dihydroxy-L-phenylalanine (L-DOPA) (Cotzias et al., 1967).

Even though L-DOPA represents the gold standard in PD therapy, L-DOPA pharmacotherapy of PD is burdened by major long-term side-effects such as motor fluctuations and dyskinesia. With the progression of the disease, the therapeutic effect of L-DOPA wears off, both in terms of extent and duration, and the patient starts to fluctuate between "on" and "off" periods in which symptoms re-appear. In addition, involuntary choreic and dystonic movements, i.e. dyskinesia, appear in the "on" and "off" phases (Fig. 1). After ten years of L-DOPA therapy, 70-80% of patients develop dyskinesia and almost 100% of patients with early onset of disease is affected by this severe side-effect (Fahn, 1982; Quinn et al., 1987).

The clinical phenomenology of dyskinesia is complex, and a wide range of presentations have been described, including neck, truncal, facial and limb chorea and dystonia (Nutt, 1990; Luquin et al., 1992).

The pattern of dyskinesia varies with respect to the time of onset in relation to L-DOPA intake. Peak-dose dyskinesia occurs when plasma levels of L-DOPA are high, and tends to be predominantly represented by chorea with some dystonia (Muenter et al., 1971).

Diphasic dyskinesia, or "onset and end-of-dose dyskinesia," occurs when plasma levels of L-DOPA are either rising or falling ("low-dose dyskinesia"), but not when they are stable, and tends to be predominantly dystonic (Muenter & Tyce, 1971).

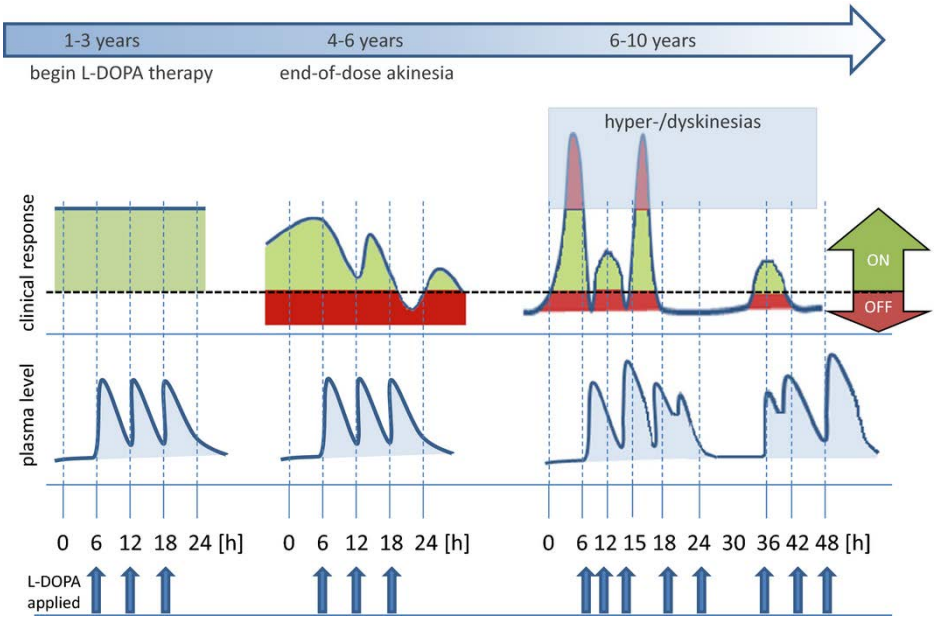

Figure 1 During the first 1–3 years of therapy with L-DOPA, the clinical response, measured as 'ON' phase, is constant ("honeymoon period"). In the following years, despite continuous plasma level responses, patients experience end-of-dose akinesia, in which clinical responses are frequently lower and transgress into 'OFF'-phases with the reappearance of the cardinal motor symptoms and signs as well as of non-motor symptoms. Some 6–10 years after therapy onset, increase in plasma levels still follow L-DOPA applications, yet the patients commonly suffer from dyskinesia during the 'ON' phase and/or the L-DOPA dose does not necessarily result in a measurable clinical response (Oertel et al., 2016).

Etiology of L-DOPA-induced dyskinesia (LID) is still unclear but we can distinguish some individual risk factors such as age, lesion extension, dosage and frequency of L-DOPA administration (Huot et al., 2013b; Bastide et al., 2015).

LID can be seen as an aberrant form of neuronal plasticity where DA newly formed from L-DOPA causes long-lasting biochemical changes in a DA-depleted striatum. Progressive

neurodegeneration causes maladaptive changes in basal ganglia circuits, altering the pathophysiological response to antiparkinsonian therapy.

Overactive glutamatergic transmission is believed to be an important contributor of both the development and expression of LID (Chase et al., 2003; Sgambato-Faure et al., 2012). In particular, the classical model suggests overactivity of glutamatergic cortico-striatal projections as being instrumental to overactivity of striatal GABAergic medium spiny neurons (MSNs) which project to the substantia nigra (SN) pars reticulata (SNpr) (i.e. the "direct" striato-nigral pathway), via both NMDA and AMPA receptors (Chase et al., 2003; Huot et al., 2013b; Bastide et al., 2015).

DA depletion can also influence other non-dopaminergic neurons, like serotonergic neurons, which are able to convert L-DOPA in DA, and release it in a non-physiological way (Carta et al., 2007). Consistently, it was observed that activation of serotonergic 5-$HT1_{A/B}$ autoreceptors results in alleviation of LID (Carta et al., 2007; Huot et al., 2011; Huot et al., 2013b; Bastide et al., 2015). I will examine this subject in the following chapter.

The loss of nigro-striatal input is accompanied by profound modifications of the response of GABAergic MSNs to drugs that modulate dopaminergic signaling, including L-DOPA (Cenci, 2007a). Chronic intermittent stimulation of DA receptors brings about alterations in canonical and non-canonical D1 receptor signaling in striato-nigral MSNs which results in an enhancement of neuron excitability (Santini et al., 2007; Santini et al., 2009; Santini et al., 2010).

LID-associated pathological changes cause the activation of the two mitogen-activated protein kinases (MAPKs), extracellular signal–regulated kinase (ERK) 1 and 2 (Gerfen et al., 2002), which are involved in various types of synaptic plasticity.

Increased phosphorylation of ERK 1/2 and overexpression of ΔFosB in striatum were found to be correlated with LID development (Pavon et al., 2006).

ERK 1/2 phosphorylation is triggered by DA- and cAMP–regulated phosphoprotein Mr 32,000 (DARPP-32). Thus D1R coupling to $G_{\alpha olf}$ proteins and adenylyl cyclase is enhanced in the dyskinetic brain, which results in an increased activity of cAMP-related protein kinase A (PKA) and phosphorylation of downstream effectors such as DARPP-32 (Picconi et al., 2003).

This signaling cascade causes the activation of mTORC1 which is responsible for several forms of synaptic plasticity related to LID (Santini et al., 2009).

Rhes (Ras homolog enriched in striatum) is a striatal protein that, like Rheb, directly binds mTORC1 and activates the complex. Moreover, L-DOPA-induced mTOR activation and LID are substantially reduced in Rhes deleted mice (Subramaniam et al., 2012).

Other important events regulated by the ERK signaling cascade during LID are the phosphorylation of histone H3 (Santini et al., 2009) and the deacetylation of histone H4 (Nicholas et al., 2008). Since chromatin remodelling such as via histone deacetylation and/or phosphorylation, plays a critical role in gene expression and nuclear reprogramming, it is likely that the abnormal and sustained ERK activation caused by D1R sensitization and DARPP-32 overactivity could modify the protein synthesis patterns in MSNs.

The basal ganglia

The basal ganglia (BG) are a complex network of nuclei in the forebrain which play critical roles in motor control. These structures receive major inputs from wide areas of the neocortex and project, through the thalamus, back to prefrontal, premotor and motor cortex.

The main role of the BG consists in elaborating a broad range of complex signals, and then in conveying them to areas of the cortex involved in motor control or cognitive function.

The BG encompasse the striatum, SN, globus pallidus (GP) and subthalamic nucleus (STN).

The **striatum**, the main input nucleus of the BG, is usually anatomically divided into three different areas: caudate nucleus and putamen (dorsal striatum) and ventral striatum which includes the nucleus accumbens (NAc).

This subdivision reflects a different functional organization. The dorsal striatum receives most of its afferents from associative and sensorimotor cortical areas, respectively. These fibers are mostly composed by glutamatergic neurons. The limbic and paralimbic cortical areas, the amygdala and the hippocampus, instead, project to the ventral striatum through cholinergic and glutamatergic pathways.

The striatum also receives important dopaminergic projections from the SNpc, GABAergic, glutamatergic and serotonergic projections from the GP, the PPN and the dorsal raphe nucleus respectively.

The main striatal neuronal population (~95%) is represented by GABAergic MSNs. The remaining 5% of neurons include aspiny interneurons, which have been classified on the basis of their morphology, protein content and electrophysiological properties as large cholinergic interneurons (ChIs), and somatostatin-, parvalbumin- and calretinin-expressing GABAergic interneurons (Kawaguchi et al., 1995).

The **SN** is divided in two functionally distinct nuclei: the SN pars reticulata (SNpr) and pars compacta (SNpc). The SNpc sends dopaminergic fibers to the striatum, and represents the main source of dorso-striatal DA. The SNpr receives GABAergic afferents from striatum and GP, glutamatergic afferents from the STN, the cerebral cortex and the peduncolo-pontine nucleus (PPN), and serotonergic innervation from the raphe nucleus. It sends GABAergic efferents mainly to the thalamus, the PPN and the superior colliculus (Lanciego et al., 2012).

The **GP**, in primates, can be subdivided into an external (GPe) and an internal (GPi) segment. In rodents, the functions of GPi are exerted by the entopeduncolar nucleus (EPN).

The GP receives major afferents from the striatum, the STN and, to a lesser extent, other structures, including the dorsal raphe nucleus, the SNpc, the thalamus and the PPN. The GPe sends projections mainly to the STN and, to a lesser extent, the striatum and SN. The GPi massively projects to the ventral and medial nucleus of the thalamus, the centromedian nucleus, and the PPN. GP neurons use GABA as a neurotransmitter (Lanciego et al., 2012).

The **STN** receives inputs from the cerebral cortex, the thalamus, the SNpc and the GPe, and projects to the striatum, the SNpr and the GP. The STN contains a large number of medium-sized neurons that use glutamate as a neurotransmitter, and a limited number of interneurons. Glutamatergic fibers originating from the STN are the only excitatory projections of the BG. The loss of nigral afferents is the main cause of the typical STN hyperactivity that is observed in parkinsonian conditions (Lanciego et al., 2012).

The direct and indirect pathway model

The BG are associated with a variety of functions such as motor control, procedural learning, routine behaviours or cognition and emotion (Stocco et al., 2010). As previously described, the BG nuclei are highly interconnected and receive afferents from many areas of the brain (Fig. 2).

The direct and indirect pathways model represents a classical, simplified scheme of neural circuits underlying motor control, and still a useful tool to interpret the parkinsonian hypokinesia and LID-associated hyperkinesia, as a consequence of the unbalance between these two functionally opposing pathways (Albin et al., 1989; Alexander et al., 1990; Smith et al., 1998).

The striato-nigral MSNs (direct pathway) project to the SNpr and GPi, preferentially express D1 receptors (D1R) and produce and release the neuropeptides dynorphin and substance P (Albin et al., 1989).

The striato-pallidal MSNs (the first step of the indirect pathway) project to the GPe, preferentially express D2 receptors (D2R), and produce and release enkephalins (Albin et al., 1989).

According to this model, cortical glutamatergic synapses activate GABAergic MSNs of the striato-nigral pathway, which exert an inhibitory action on SNpr neurons. This inhibition leads to the disinhibition of the thalamic glutamatergic neurons which project to the cortex. The activation of this circuit results in motor activation.

On the contrary, striato-pallidal MSNs inhibit the GPe, leading to disinhibition of the STN. This causes overactivation of SNpr GABAergic neurons projecting to the thalamus, and eventually movement inhibition.

Different studies identified a third pathway, called the “hyperdirect pathway”, formed by glutamatergic efferents from the cortex to the STN (Nambu et al., 1996). The cortico-STN-pallidal pathway conveys powerful excitatory inputs from the motor-related cortical areas to the pallidum, bypassing the striatum, with shorter conduction time than the inputs conveyed through the ‘direct’ and ‘indirect’ pathways. Functionally, the hyperdirect pathway suppresses motor programs (Nambu et al., 2002). Dysfunctions of the hyperdirect

pathway lead to motor and cognitive impairments such as hemiballismus or impulsivity (Temel et al., 2005).

A clear demonstration of the different role of the direct and indirect pathways has been recently provided using optogenetic approaches (Kravitz et al., 2010).

Despite its great heuristic value, the classical BG model in which the direct and indirect pathways MSNs are completely segregated has been questioned. Various studies revealed a significant proportion of D1R and D2R-coexpressing MSNs (~5-10%) in rats and monkeys (Le Moine et al., 1995; Aubert et al., 2000). Moreover, anatomical studies showed that a single striatofugal axon can arborize in both the GPi and GPe (Castle et al., 2005; Nadjar et al., 2006). Finally, a molecular cross-talk between heteromeric D1-like and D2-like DA receptors (Perreault et al., 2014), and a common endocannabinoid (eCB) signaling (Bagetta et al., 2011) have been postulated. For these reasons, this model is being continuously updated.

Even through the evident complexity of BG circuits, this simplified model remains an useful tool to understand the mechanisms underlying neurological disorders.

According to the model described above, PD results from an excessive activation of striato-pallidal pathway, causing the inhibition of thalamus and cortex.

This was easily proved through lesion and inactivation studies, showing that the inactivation of STN or GPi increases activity in motor cortex and reduces bradykinesia and tremor in parkinsonian patients (Mink et al., 1991; McIntyre et al., 2004).

In contrast to the situation in PD, the direct pathway appears to be overactive in LID, resulting in a net reduction in GPi/SNpr activity, as also clearly demonstrated by in vivo microdialysis studies showing an increase of GABA release in SNpr (Mela et al., 2007; Paolone et al., 2015; Brugnoli et al., 2016) and a reduction of GABA levels in the thalamus (Marti et al., 2012; Porras et al., 2014) after L-DOPA injection in dyskinetic animals.

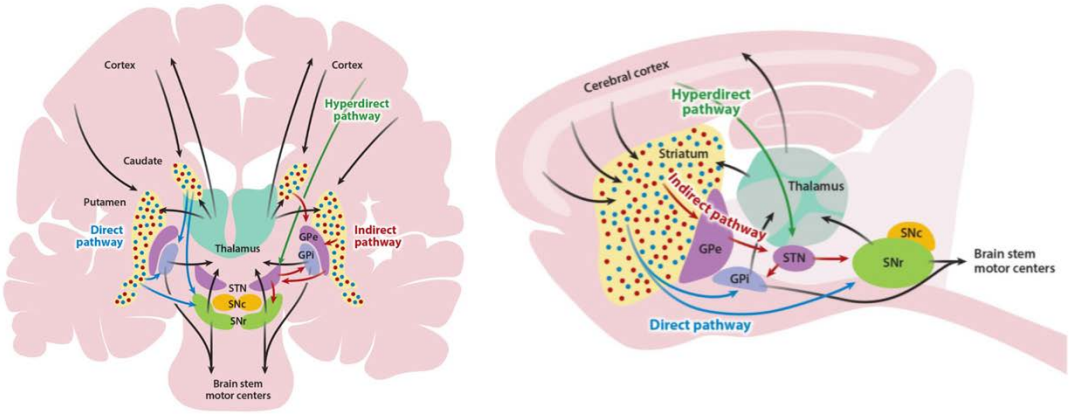

Figure 2 Simplified basal ganglia circuit diagram. Basal ganglia nuclei and their major connections in primates (left), shown in coronal view, and rodents (right), shown in sagittal view (Nelson et al., 2014).

Pharmacological treatments for LID

Despite extensive pre-clinical and clinical research focused on developing new therapy, few agents have successfully been shown to reduce dyskinesia or to successfully translate from preclinical to clinical settings.

The failure of many promising antidyskinetic therapies suggests that LID involves different neurotransmitter systems.

Amantadine, an N-methyl-D-aspartate (NMDA) receptor antagonist, is the only marketed antidyskinetic drug to date. The Movement Disorders Society stated, in an evidence-based medicine review, that amantadine was efficacious in alleviating LID (Fox et al., 2011).

The action of amantadine is to reduce peak-dose, established LID without compromising the L-DOPA antiparkinsonian benefits in the mouse (Lundblad et al., 2005; Bido et al., 2011), rat (Dekundy et al., 2007; Bido et al., 2011), non-human primate (NHP) (Blanchet et al., 1998), and PD patient (Verhangen Metman et al., 1998; Del Dotto et al., 2001). Nonselective antagonism of NMDA receptors is regarded as the mechanism whereby amantadine exerts its antidyskinetic effects (Blanchet et al., 1998).

However, amantadine is not universally effective (Sawada et al., 2010), can be poorly tolerated by some patients, and may elicit psychiatric complications (Verhangen Metman

et al., 1998). In addition, there have been suggestions of tachyphylaxis of the antidyskinetic efficacy of amantadine (Verhagen Metman et al., 1999; Thomas et al., 2004).

Eltoprazine is a potential antidyskinetic drug that showed promising results in animal models and in clinical trials. I will examine extensively this molecule in a following, specific chapter.

Another therapeutic strategy consists in employing dopaminergic agonists such as pramipexole or ropinirole along with L-DOPA in order to delay the development or the expression of LID. For instance, many clinical trials showed a delay in dyskinesia development employing ropinirole or pramipexole in the early stage of the disease (Brooks, 2000; Constantinescu et al., 2007).

Developing different pharmaceutical delivery systems for L-DOPA turned out to be a clever strategy for delaying LID expression. The use of enteral L-DOPA infusion, called Duodopa, in the treatment of advanced PD has developed over the last 20 years, showing reduction in LID and improvement in quality of life when compared to oral L-DOPA (Foltynie et al., 2013).

Recently, the possibility to deliver L-DOPA through lipid nanocarriers (Ravani et al., 2015) or chitosan coating (Cao et al., 2016) has been proposed. This strategy might represent a promising tools for reducing LID expression.

Unfortunately, some patients are unresponsive to pharmacological treatment; in these cases surgical options are sometimes available. Deep brain stimulation (DBS) of the STN and the GPi have become routine surgical methods for treating well selected PD patients with severe motor fluctuations and LID (Munhoz et al., 2014).

Contribution of pre- and post-synaptic 5-HT_{1A} and 5-HT_{1B} receptors to LID

The influence of serotonergic system in PD physiopathology is largely documented in various scientific reviews (Huot et al., 2011; Cheshire et al., 2012; Huot et al., 2013a; Bastide et al., 2015). The serotonergic system has been far less studied than the dopaminergic and glutamatergic systems in LID. Nonetheless, it is believed to play a key

role in the pathophysiology of LID, although the mechanisms underlying are largely unknown.

In fact, some studies reported an abnormal *sprouting* of striatal serotonergic axon terminals in the striatum of parkinsonian rats and NHPs as well as in PD patients (Politis et al., 2010; Rylander et al., 2010; Lee et al., 2015b). Moreover, LID was exacerbated in 6-OHDA rats following transplants of mesencephalic cells enriched with serotonergic neurons (Carlsson et al., 2007). Accordingly, administration of L-DOPA to rats with dual lesions of the medial forebrain bundle and rostral raphe nucleus did not lead to the development of AIMs (Eskow et al., 2009). Recently, the serotonin/dopamine transporter ratio (SERT/DAT) was proposed as a potential marker of disease progression and an indicator of risk for LID in PD (Lee et al., 2015b).

Treatment-induced plasticity of the striatal serotonergic innervation may thus represent a cause of altered DA dynamics. The ability of serotonergic raphe-striatal fibers to synthetize DA from L-DOPA through AADC (Arai et al., 1995), and to release synaptic DA (Berger et al., 1978) is well known.

Following degeneration of the nigro-striatal system, raphe-striatal serotonergic terminals were demonstrated to release DA, which acts as a “false neurotransmitter” in an environment devoid of the autoregulatory mechanisms (i.e. D2 autoreceptors) required for physiologic transmission. This ectopic release of DA was demonstrated to be instrumental for the development and expression of LID (Lundblad et al., 2004; Carta et al., 2007; Carta et al., 2008; Nevalainen et al., 2011).

Both serotonergic type 1A and type 1B (5-HT_{1A} and 5-HT_{1B}) receptors are coupled to a $G_{i/o}$ protein (Nichols et al., 2008) and act as autoreceptors at the somatodendritic (5-HT_{1A}) and presynaptic (5-HT_{1B}) levels. Nonetheless, these receptors can also locate postsynaptically (Glennon et al., 2000).

An in-vivo microdialysis study in 6-OHDA rats evidenced that the stimulation of 5-HT_{1A} but not 5-HT_{1B} receptors attenuated the increase in extracellular DA derived from exogenous L-DOPA (Kannari et al., 2001), supporting the hypothesis that serotonergic neurons are primarily responsible for the storage and release of DA derived from exogenous L-DOPA in the absence of dopaminergic neurons.

Two different hypotheses have been put forward to explain the antidyskinetic mechanism of serotonergic agonists: the reduction of ectopic DA release through 5-HT_{1A} and 5-HT_{1B} autoreceptors (Carta et al., 2007; Munoz et al., 2008) and the impairment of glutamatergic transmission by cortical and/or striatal 5-HT_{1A} or 5-HT_{1B} heteroreceptors (Dupre et al., 2011).

Accordingly with the second hypothesis, activation of 5-HT_{1A} receptors reduced LID expression in 6-OHDA rats along with striatal glutamate efflux (Dupre et al., 2011). Sarizotan, a non-selective 5-HT_{1A} receptor agonist, has been proven effective in reducing LID in both rodents and NHPs, at the expense of a slight reduction of the therapeutic effect of L-DOPA (Gregoire et al., 2009; Marin et al., 2009). Moreover, two phase III trials failed to show any antidyskinetic efficacy of sarizotan when compared with placebo (Muller et al., 2006; Goetz et al., 2008).

Another approach to alleviate LID and preserve on the therapeutic effect of L-DOPA might be to combine subthreshold doses of 5-HT_{1A} and 5-HT_{1B} receptor agonists, which was demonstrated to provide a synergistic effect and a reduction of LID in the MPTP-lesioned macaque (Munoz et al., 2008).

Eltoprazine, a 5-HT_{1A} and 5-HT_{1B} receptor mixed agonist developed as anti-aggressive drug, has been proven effective in reducing LID in 6-OHDA rats and MPTP macaques (Bezard et al., 2013), although this effect was accompanied by a partial worsening of the therapeutic effect of L-DOPA.

In a dose-finding phase I/IIa study, eltoprazine showed a favourable risk-to-benefit ratio and pharmacokinetic profile in patients with PD (Svenningsson et al., 2015).

Recently, eltoprazine was shown to normalize D1R-dependent cAMP/PKA and ERK/mTORC overactive signaling, associated with LID expression (Ghiglieri et al., 2016), suggesting a reduction of the hypersensitivity of the direct pathway.

Rhes/mTORC1 signaling cascade and LID

Rhes is a 266 amino acid GTP-binding protein that, among the family of small G-proteins, shares 62% identity with Dexras1, a mouse dexamethasone-inducible Ras-like protein (Falk et al., 1999).

Rhes is predominantly expressed in dorsal striatum, and less intensely in the ventral striatum (nucleus accumbens), olfactory tubercle, piriform cortex, and in the dentate gyrus of hippocampus (Spano et al., 2004; Ghiglieri et al., 2015). Recently, Rhes was also detected in TH-positive neurons of SNpc and VTA of the mouse midbrain (Pinna et al., 2016). Interestingly, Rhes ectopic expression in cerebellum was proven to exacerbate motor deficits in HD mice (Swarnkar et al., 2016).

Rhes is the product of a thyroid hormone-regulated gene during brain development (Vargiu et al., 2001), in fact neonatal hypothyroidism causes a dramatic decrease in Rhes mRNA, which is reversed by repeated T4 administration (Falk et al., 1999). But when hypothyroidism was induced in adult rats, Rhes mRNA expression was unaffected (Vargiu et al., 2001).

During adulthood, Rhes expression is positively regulated by striatal DA (Harrison et al., 2013). Accordingly, Rhes protein in whole striatum resulted significantly decreased by 6-OHDA lesion (Harrison et al., 2008).

The involvement of Rhes along the signalling pathway downstream the D1R has been observed in different studies. In striatum, Rhes is mainly localized in GABAergic MSNs, where it is involved in the modulation of sensorimotor gating and DA receptor-dependent signaling and behaviours (Errico et al., 2008; Ghiglieri et al., 2015; Sciamanna et al., 2015; Shahani et al., 2016; Vitucci et al., 2016).

The interest towards Rhes protein rose significantly when its involvement in Huntington's disease (HD), an inherited motor disorder that results in the death of striatal cells, was proven (Subramaniam et al., 2009). Rhes acts as a specific striatal E3 ligase (Subramaniam et al., 2010) able to bind and SUMOylate mutant huntingtin, thereby inhibiting its aggregation and increasing its cytotoxicity (Subramaniam et al., 2009). Furthermore, this pathogenic modification causes the inhibition of Rhes binding to mTORC1, removing its trophic effects on striatal cells (Subramaniam et al., 2011; Lee et al., 2015a).

The binding of Rhes to mTORC1 is also instrumental to LID (Subramaniam et al., 2012). Indeed, D1R coupling to $G_{\alpha olf}$ proteins and adenylyl cyclase is enhanced in the dyskinetic brain, which results in an increased activity of cAMP-related PKA and phosphorylation of downstream effectors such as DARPP-32 (Nishi et al., 1997). DARRP-32 phosphorylation at Thr-34 residue promotes ERK 1/2 signaling (Pavon et al., 2006) which, in turn, activates mTORC1, ultimately responsible for LID onset in mice and rats (Santini et al., 2009; Decressac et al., 2013).

ERK activates mTORC1 by maintaining Rheb in the active state. Until the discovery that Rhes is capable to bind and activate mTORC1 (Subramaniam & Snyder, 2011; Subramaniam et al., 2012), Rheb was believed to be the only striatal mTORC1 activator.

Interestingly, in line with Rhes ability to bind and activate mTORC1 signaling, which is known to be instrumental to LID development (Santini et al., 2009), Rhes knockout ($Rhes^{-/-}$) mice displayed significantly less dyskinesia than wild-type littermates during L-DOPA treatment (Subramaniam et al., 2012).

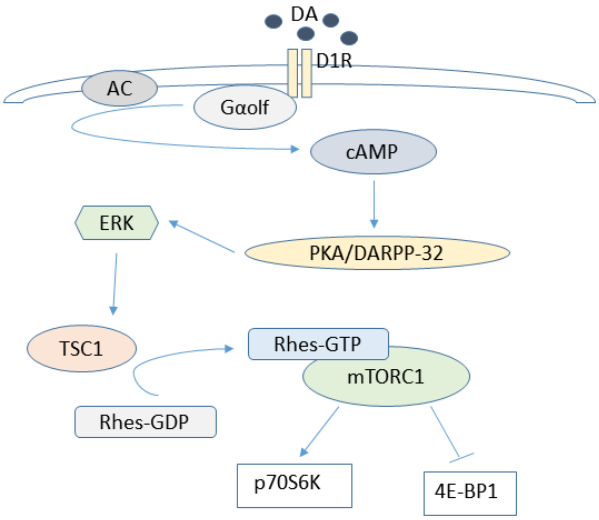

Figure 3 D1R signaling cascade. D1R coupling to $G_{\alpha olf}$ proteins and adenylyl cyclase is activated by DA, causing an increased activity of cAMP-related protein kinase A (PKA) and phosphorylation of downstream effector DARPP-32. DARRP-32 phosphorylation promotes ERK signaling which, in turn, activates mTORC1, maintaining Rhes in the active state through tuberous sclerosis protein (TSC1) phosphorylation. mTORC1 promotes the phosphorylation of the ribosomal protein S6 kinase beta-1 (p70S6K) and inhibits the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) which are responsible of cell growth, protein synthesis and metabolism.

Involvement of striatal cholinergic interneurons in LID

ChIs represent only 1-2% of neuronal population in striatum. However these giant (20-50 μm) aspiny neurons are the main source of ACh in striatum, which plays a key role in BG functions (Cicchetti et al., 2000). Thus, ChIs integrate synaptic inputs over relatively large regions and act as association interneurons (Kawaguchi et al., 1995).

ACh exerts its effects by stimulating muscarinic (M1-M5) (Caufield et al., 1998) and nicotinic (Lukas et al., 1999) receptors.

M1-like receptors (M1, M3 and M5 subtypes) are coupled to $G_{q/11}$ that stimulates the membrane-bound phospholipase C-β, which, in turn, cleaves phosphoinositol-2 into two second messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG).

M2-like receptors (M2 and M4 subtypes) are coupled to $G_{i/0}$, which inhibits adenylate cyclase activity, decreasing the production of cAMP from ATP.

The nicotinic ACh receptors (nAChR) are cation channels containing α and/or β subunits that elicit fast excitatory effects both at the presynaptic and postsynaptic level.

ChIs, also known as TANs (tonically active neurons), show a regular tonic firing (Apicella et al., 1991), and are involved in the control of motor activity, learning and reward. The pacemaking activity of ChIs provides far enough ACh levels to tonically activate muscarinic and nicotinic receptors in the striatum. This basal level of cholinergic activity is modulated by the action of acetylcholinesterase (AChE) and by the negative feedback operated by muscarinic autoreceptors.

Anticholinergics are the oldest class of medications used to treat PD, and are still employed in order to alleviate some of the troublesome symptoms of the disease, in particular the involuntary resting tremor. They can be used as monotherapy early in the course of the disease, and act synergistically with L-DOPA in more advanced stages (Olanow et al., 2001).

The classical hypothesis underlying the therapeutic effect of anticholinergic drugs is based on the striatal ACh/DA balance concept (Pisani et al., 2007). The cellular mechanism underlying the elevation of ACh release in PD was believed to involve the loss of an inhibitory control operated by D2Rs expressed on cholinergic neurons (Mackenzie et al., 1989; DeBoer et al., 1996; Ikarashi et al., 1997).This theory has become more dynamic

with the incorporation of recent physiological and anatomical breakthroughs (Aosaki et al., 2010). In fact, DA depletion has been proposed to trigger a reduction in the efficacy of the inhibitory M4 autoreceptors, which ultimately causes an increase of ACh release. This adaptive change has been attributed to an up-regulation of a GTPase protein, i.e. regulator of G protein signaling type 4 (RGS4), with a subsequent decrease in coupling of M4 receptors to K^+ and Cav_2 Ca^{2+} channels (Ding et al., 2006).

The influence of striatal ChIs on the impaired neuronal plasticity after DA depletion was well established (DeBoer et al., 1996). Recently, both pharmacological and optogenetic inhibition of ChIs has been reported to improve motor impairment in 6-OHDA mice (Ztaou et al., 2016). The same result was achieved through pharmacological blockade of postsynaptic M4R (Ztaou et al., 2016).

Alterations in cholinergic signalling and its functional interplay with the dopaminergic, GABAergic and glutamatergic systems in the striatum are also implicated in LID.

It is interesting to observe that the ablation of striatal ChIs is beneficial in both PD and LID (Won et al., 2014; Ztaou et al., 2016). Recently, an optogenetic study in 6-OHDA hemilesioned mice demonstrated that the firing rates of cholinergic interneurons, and perhaps, the levels of ACh attained, shape the ChIs modulation. In fact, short pulse optical stimulation of ChIs caused LID expression, which could be acutely blocked by mAChRs antagonism, while longer pulse stimulation inhibited LID expression due to nAChRs desensitization (Bordia et al., 2016).

Consistent with this study, various studies showed that both nAChRs agonists and antagonists reduced LID in rodent and in NHP models of PD (Quik et al., 2007; Bordia et al., 2008; Zhang et al., 2014). Furthermore, nAChRs antagonists reduce LID possibly by altering basal firing rate and DA-dependent excitation of cholinergic interneurons via alterations in ERK signaling (Ding et al., 2011).

The role of muscarinic receptors in LID is far less clear, likely due to the poor selectivity of the pharmacological tools available. In fact, it has been shown that a non-selective muscarinic antagonist, dyciclomine, attenuates LID in mice (Ding et al., 2011). Moreover, the ability of a positive M4R allosteric modulator (PAM) in reducing LID in rodents and NHPs models was recently reported (Shen et al., 2015). The underlying mechanism seems to be related to postsynaptic activation of M4Rs on D1R expressing MSNs, and the consequent reduction of the hyperactivity of direct pathway MSNs (Shen et al., 2015).

Nonetheless, another study proved that the activation of M4R causes the enhancement of direct pathway excitability, through activation of Ca^{2+} voltage channels (Hernandez-Flores et al., 2015), a finding difficult to reconcile with the proposed idea that blockade of this receptor could inhibit LID (Shen et al., 2015)

All these data suggest a complex modulation of MSNs by ChIs; further investigations are needed to elucidate all players involved.

AIM OF THE STUDY

The main purpose of my research project was to investigate the neurochemical changes induced at the BG circuitry level by new antidyskinetic agents, and specifically to investigate whether they inhibit the activity of striato-nigral MSNs (i.e. the direct pathway) in vivo. To this aim, I adopted an in vivo, combined behavioural and neurochemical approach in validated rodent models of LID, which was based on the use of in vivo microdialysis in freely moving, dyskinetic rats or mice: one microdialysis probe was implanted in the dopamine-depleted striatum and another in ipsilateral SNpr. GABA and glutamate were monitored in both areas concomitantly with abnormal involuntary movements (AIMs) expression. In fact, Morari group proposed that the rise of SNpr GABA release during AIMs appearance is a neurochemical marker of striato-nigral MSNs activation.

I focused on three different targets, which appear promising in LID therapy: the serotonergic 5-HT_{1A} and 5-HT_{1B} receptors, the mTORC1/Rhes complex and, lastly, the cholinergic muscarinic M1R and M4R.

In the first part, I focused my attention on the antidyskinetic effect of eltoprazine, a 5-HT_{1A} and 5-HT_{1B} mixed agonist reported to attenuate dyskinesia in rats and NHPs (Carta et al., 2007). Specifically, I investigated in dyskinetic rats whether eltoprazine could exert its action on direct pathway MSNs. Moreover, I tested the hypothesis of the "ectopic DA release" (Carta et al., 2007), which posits that 5-HT_{1A} agonists should attenuate dyskinesia via inhibition of L-DOPA-induced striatal DA release.

In the second part, I investigated the role of mTORC1 and Rhes, which are along the non-canonical D1 signaling cascade, in the development and expression of LID. Previous studies pointed out the antidyskinetic effect resulting from the pharmacological blockade of mTORC1 or the genetic deletion of Rhes, a striatal upstream regulator of mTORC1. I investigated whether these two strategies could also result in inhibition of the activity of direct pathway MSNs. To accomplish this aim, I performed in vivo microdialysis in Rhes knockout ($Rhes^{-/-}$) mice and wild-type littermates, and in wild-type in mice subacutely treated with rapamycin (an mTORC1 inhibitor).

In the third part of my project, I attempted to elucidate the influence of striatal cholinergic interneurons in LID. Cholinergic interneurons represent a very small percentage of striatal neuronal population, but are largely interconnected with direct and indirect pathway MSNs and modulate their activity. Previous studies proved the antidyskinetic effect of aspecific

muscarinic antagonism in vivo (Ding et al., 2011), but the lack of selective tools did not permit the identification of the receptor involved.

To bypass the selectivity issue related to available muscarinic ligands, I performed reverse microdialysis in dyskinetic rats, perfusing preferential M1R and M4R antagonists directly into the striatum at selective concentrations, simultaneously administering systemic L-DOPA. I focused my attention on M1R and M4R because they are highly expressed on striato-nigral MSNs and for recent evidence showing their involvement in LID (Shen et al., 2015; Bordia et al., 2016).

MATERIALS AND METHODS

Animals

All animals used in the study were housed in humidity and temperature controlled environment under a 12-h light/dark cycle with food and water available *ad libitum*. The experimental protocols were approved by the Italian Ministry of Health (license 170-2013-B). Adequate measures were taken to minimize animal pain and discomfort and to limit the number of animals used.

Rats

Young adult male Sprague-Dawley rats (150 g; 12-13 weeks) used in part 1 and part 3 were purchased from Harlan Italy (now Envigo) (S. Pietro al Natisone, Italy; part 1) and from Charles River (Calco, Italy; part 3).

Mice

Young adult male C57BL/6J mice (25g; 8-10 weeks old) were purchased from Harlan Laboratories (Harlan Italy, San Pietro al Natisone). Young adult male $Rhes^{-/-}$ and $Rhes^{+/+}$ mice (10-12 weeks) described in part 2 were generated in the laboratory of Prof Usiello, at CEINGE Biotecnologie Avanzate, Naples, Italy (Spano et al., 2004). Male and female $Rhes^{+/+}$ and $Rhes^{-/-}$ mice, derived from mating of heterozygous animals were backcrossed to C57BL/6 strain for 11 generations.

Lesion of the DA system

In order to lesion the DA neurons located in SNpc, and consequently deplete the striatum of DA, different protocols were used. All lesion procedures allowed achieving a unilateral massive destruction of the nigro-striatal DA projection.

6-OHDA lesion in rats (used as animal model in part 1 and 3)

Unilateral lesion of nigro-striatal DA neurons was induced in isoflurane-anaesthetized rats (Marti et al., 2007; Bido et al., 2011; Paolone et al., 2015) by stereotactically injecting 8 μg of 6-hydroxydopamine (6-OHDA; in 4 μL of saline containing 0.02% ascorbic acid) in the right medial forebrain bundle (MFB) according to the following coordinates from bregma: AP= -4.4 mm, ML= -1.2 mm, DV= -7.8 mm below dura (Paxinos et al., 1982). Two weeks after surgery, rats were injected with amphetamine (5 mg/kg i.p., dissolved in saline) and only those rats performing > 7 ipsilateral turns/min were enrolled in the study. Indeed such behavior is associated with a striatal DA depletion >95% (Marti et al., 2007).

6-OHDA lesion in mice (used as animal model in part 2)

Unilateral lesion of nigro-striatal DA neurons was performed in isoflurane-anesthetized mice (Lundblad et al., 2004). Six micrograms of 6-OHDA free-base (in 2 μL of saline containing 0.02% ascorbic acid) were stereotactically injected into the striatum according to the following coordinates from bregma (in mm); first injection, AP +1.0, ML -2.1, DV -2.9 below dura; second injection, AP +0.3, ML +2.3, DV -2.9 below dura (Paxinos et al., 2001). Two weeks after lesion, mice were selected on the basis of their rotational behaviour and forelimb use asymmetry (bar and drag tests) (Lundblad et al., 2004; Bido et al., 2011).

LID induction and AIMs ratings

Different protocols of LID induction were used in 6-OHDA lesioned mice and rats. Rats used in part 1 and 3 received 6 mg/kg L-DOPA (plus 12 mg/kg benserazide, s.c.), once a day for 21 days (Cenci et al., 1998). C57BL/6J mice used in part 2 were injected with 12 mg/Kg L-DOPA (plus 12 mg/kg benserazide i.p.) once a day, for 10 days (Bido et al., 2011).

Quantification of L-DOPA-induced AIMs was carried out as originally described by MA Cenci and collaborators (Cenci et al., 1998; Lee et al., 2000; Lundblad et al., 2002; Winkler et al., 2002; Lundblad et al., 2004) and reported by our group (Bido et al., 2011; Marti et al., 2012; Mela et al., 2012; Bido et al., 2015; Paolone et al., 2015; Brugnoli et al., 2016). Rats and mice were observed individually for 1 min every 20 min during the 2-3 h that followed an L-DOPA injection. Dyskinetic movements were classified based on their topographic distribution into three subtypes: (i) axial AIMs, that is, twisted posture or choreiform twisting of the neck and upper body toward the side contralateral to the lesion; (ii) forelimb AIMs, that is, jerky or dystonic movements of the contralateral forelimb and/or purposeless grabbing movement of the contralateral paw; (iii) orolingual AIMs, that is, orofacial muscle twitching, empty masticatory movements and contralateral tongue protrusion. Each AIM subtype was rated on frequency and amplitude scales from 0 to 4 (Cenci, 2007b). Dyskinesia score was calculated as the product of frequency x amplitude (Cenci, 2007b) and presented either as the sum of total AIMs score in one-day session (cumulative ALO AIMs score, representing AIMs score during the development of dyskinesia) or as the total AIMs score for each time point of observation in one single session (ALO AIMs score).

Behavioural studies

Motor activity in rodents was evaluated by means of different behavioural tests specific for different motor abilities, as previously described (Marti et al., 2005; Viaro et al., 2010; Bido et al., 2011). The different tests are useful to evaluate different motor parameters such as akinesia and bradykinesia under static or dynamic conditions. Akinesia appears as an abnormal absence or poverty of movements, which is associated in hemi-lesioned mice and rats to the loss of the ability to move the forepaw when placed on blocks of different heights. Bradykinesia refers to slowness of movement and, in particular, to difficulty in adjusting the correct body position, e.g. of forepaws, during backward dragging. The battery of tests described below was previously validated to assess motor deficits in parkinsonian animals (Bido et al., 2011; Paolone et al., 2015; Brugnoli et al., 2016).

Bar test

This test, also known as the catalepsy test (Sanberg et al., 1988), measures the ability of the animal to respond to an externally imposed static posture. Each rodent was placed gently on a table and the right and left forepaws were placed alternatively on blocks of increasing heights (1.5, 3 and 6 cm for mice and 3, 6 and 9 cm for rats). The immobility time (in sec) of each forepaw on the blocks was recorded (cut-off time 20 sec per step, 60 sec maximum). Akinesia was calculated as total time spent on the blocks by each forepaw.

Drag test

The test (modification of the "wheelbarrow" test) (Schallert et al., 1979), measures the ability of the animal to balance its body posture using forelimbs in response to an externally imposed dynamic stimulus (backward dragging) (Marti et al., 2005). Each rodent was gently lifted by the tail (allowing the forepaws on the table) and dragged backwards at a constant speed (about 20 cm/sec) for a fixed distance (100 cm). The number of touches made by each forepaw was counted by two separate observers (mean between the two forepaws).

Rotarod test

This test analyses the ability of the rodents to run on a rotating cylinder (diameter 8 cm) and provides information on different motor parameters such as coordination, gait, balance, muscle tone and motivation to run (Rozas et al., 1997). The fixed-speed rotarod test was employed according to a previously described protocol (Marti et al., 2005; Viaro et al., 2010). Briefly, animals were tested at stepwise increasing speeds (stepwise increase by 5 rpm from 5 to 40 rpm, 180 sec each) and time spent on the rod calculated (in sec). In parts 1 and 2, in order to explore whether the antidyskinetic effect was not due to primary motor inhibition, the rotarod performance was evaluated, both OFF and

ON L-DOPA, using a fixed-speed protocol as described (Marti et al., 2012; Paolone et al., 2015).

The three tests just described were performed in the same sequence (bar, drag and rotarod).

In vivo microdialysis

In the present work, microdialysis was used in freely moving, dyskinetic animals to simultaneously monitor GABA and glutamate release in striatum and SNpr (Bido et al., 2011; Marti et al., 2012; Mela et al., 2012; Paolone et al., 2015; Brugnoli et al., 2016), and DA release in striatum (Paolone et al., 2015). Briefly, two microdialysis probes of concentric design were stereotactically implanted under isoflurane anesthesia (1.5% in air) into the lesioned striatum and ipsilateral SNpr (3 mm and 1 mm dialysing membranes, respectively, for rats; 2 mm and 1 mm dialysisng membrane, respectively, for mice;), according to the following coordinates from bregma and the dural surface (mm): mouse dorsolateral striatum, AP +0.6, ML +2.0, DV -3.3, mouse SNpr, AP -3.3, ML -1.25, DV - 4.6; rat dorsolateral striatum, AP +0.6, ML +2.0, DV -2.0, rat SNpr, AP -5.5, ML -2.2, DV -8.0.

Twenty-four hours after surgery, probes were perfused with a modified Ringer solution ($CaCl_2$ 1.2 mmol/L, KCl 2.7 mmol/L, NaCl 148 mmol/L and $MgCl_2$ 0.85 mmol/L) at a flow rate of 2.1 μL/min (mouse) or 3 μL/min (rat). After 6 h rinsing, samples were collected (every 15 or 20 min depending on the study) for a total of 3-4 h. At least three baseline samples were collected before administration of L-DOPA, drugs or saline. In the combination studies, eltoprazine was administered 20 min before L-DOPA, muscarinic antagonists were perfused 40 min before L-DOPA.

At the end of experiment, animals were sacrificed and the correct placement of the probes was verified histologically.

Endogenous glutamate and GABA analysis

Glutamate and GABA levels in the dialysate were measured by HPLC coupled with fluorometric detection as previously described (Marti et al., 2007). Thirty microliters of ophthaldialdehyde/mercaptoethanol reagent were added to aliquots of samples (30 µL collected from rats or 28 µL from mice) and 50 µL of the mixture was automatically injected (Triathlon autosampler; Spark Holland, Emmen, the Netherlands) onto a 5-C18 Hypersil ODS analytical column (3 mm inner diameter, 10 cm length; Thermo-Fisher, USA) perfused at a flow rate of 0.48 mL/min (Jasco quaternary gradient pump PU-2089 PLUS; Jasco, Tokyo, Japan) with a mobile phase containing 0.1 M sodium acetate, 10% methanol and 2.2% tetrahydrofuran (pH 6.5). Glutamate and GABA were detected by means of a fluorescence spectrophotometer FP-2020 Plus (Jasco, Tokyo, Japan) with the excitation and the emission wavelengths set at 370 and 450 nm respectively. The limits of detection for glutamate and GABA were ~1 and ~0.5 nM, respectively, and their retention times were ~3.5 and ~18.0 min, respectively.

Endogenous DA analysis

DA was measured by HPLC coupled with electrochemical detection. Twenty microliter samples were automatically injected (AS 100 autosampler; Antec Leyden, Leiden, the Netherlands) onto an ALF-115 C18 analytical column (1 mm inner diameter, 15 cm length; Antec Leyden, Leiden, the Netherlands) perfused at a flow rate of 60 µL/min (LC 100 pump; Antec Leyden, Leiden, the Netherlands) with a mobile phase containing 50 mM phosphoric acid, 50 mM citric acid, 8 mM NaCl, 0.1 mM EDTA, and 10% methanol; pH 3.25). Detection was performed at 35˚C with an electrochemical detector (DECADE II, Antec Leyden, Leiden, The Netherlands) set at a potential of 590 mV against an Ag/AgCl reference electrode. The limit of detection for DA was ~200 pM.

Western blot analysis

Animals were sacrificed and striata rapidly dissected out (<90 sec), frozen on dry ice and stored at -80°C until analysis. Tissues were homogenized in lysis buffer (RIPA buffer, protease inhibitor cocktail and phosphatase inhibitor cocktail) and centrifuged at 13,000 rpm at 4°C for 15 min. Supernatants were collected and protein levels were quantified using the bicinchoninic acid protein assay kit (Thermo Scientific). Twenty micrograms of protein per sample were separated on a 4-12% gradient polyacrylamide precast gels (Bolt® 4-12% Bis-Tris Plus Gels, Life Technologies) in a Bolt® Mini Gel Tank apparatus (Life Technologies). Proteins were then transferred onto polyvinyl difluoride membrane, blocked for 60 min with 5% non-fat dry milk in 0.1% Tween20 Tris-buffered saline, and incubated overnight at 4°C with anti-Thr202/Tyr204-phosphorylated ERK1/2 (pERK) rabbit monoclonal antibody (Merck Millipore, cat. #05-797R, 1:1000) or with anti-ERK1/2 (totERK) rabbit polyclonal antibody (Merck Millipore cat. #06-182, 1:25000). Membranes were washed, then incubated 1 hr at room temperature with horseradish peroxidase-linked secondary antibodies (Merck Millipore, cat. #12-348, 1:5000). Immunoreactivity was visualized by enhanced chemiluminescence (ECL) detection kit (Perkin Elmer), images were acquired using the ChemiDoc MP System quantified using the ImageLab Software (Bio-Rad). Membranes were then stripped and re-probed with rabbit monoclonal anti-tubulin antibody (Merck Millipore, cat. #04-1117, 1:50000). Data were analyzed by densitometry, and the optical density of specific pERK or totERK bands was normalized to the corresponding tubulin levels.

Statistical analysis

Motor performance was expressed as time (in seconds) on bar or rod (bar and rotarodtests), and number of steps (drag test). AIMs rating was expressed as ALO score (frequency x amplitude). In microdialysis studies, GABA, glutamate and DA release has been expressed as percentage ± SEM of basal values (calculated as mean of the two samples before treatment). In Figure legends (or in Results section), basal dialysate levels of neurotransmitters were also given as absolute values (in nM). Statistical

analysis was performed by parametric one-way ANOVA or two-way repeated measure (RM) ANOVA, as appropriate. ALO AIMs data were analysed by non-parametric ANOVA followed by the Dunn's test, or by the Mann-Whitney test when only two groups were compared. Microdialysis data were analysed as AUC values using parametric one-way ANOVA followed by the Newman-Keuls test or by the Student's t-test when only two groups were compared. P values <0.05 were considered to be statistically significant.

Drugs

L-Dopa methyl ester and benserazide hydrochloride were purchased from Sigma-Aldrich (S. Louis MO, USA), 6-OHDA hydrobromide, D-amphetamine sulfate, telenzepine, PD-102807 and tropicamide from Tocris (Bristol, UK), eltoprazine from Santa Cruz Biotechnology (Dallas, TX, USA), and rapamycin from LC Laboratories (Woburn, MA, USA). Rapamycin was dissolved in a solution of 5% dimethyl sulfoxide (DMSO), 5% PEG-400 and 5% Tween-20, and was systemically administered at an injection volume of 2 mL/Kg body weight. Telenzepine, PD-102807 and tropicamide were dissolved in Ringer for local perfusion through the microdialysis probe. All the other drugs were dissolved in saline (6-OHDA in ascorbate-saline) and administered systemically at the volume of 1.0 ml/Kg body weight.

RESULTS

PART 1

Chronic Eltoprazine Prevented LID Development

L-DOPA-treated rats developed severe dyskinesia (ALO score >100) that was already maximal 1 week after treatment onset (Fig. 1.1A). Eltoprazine (0.3 mg/kg) attenuated LID development, although it did not abolish AIM appearance (Fig. 1.1A-B). Eltoprazine reduced LID without affecting locomotor AIMs (Fig. 1.1C). Furthermore, AIMs analysis during chronic treatment (Fig. 1.1D, E) showed that eltoprazine was effective since its first administration, without altering the time course of AIMs expression. To confirm that the effect of eltoprazine was not owing to primary hypolocomotive effect, rotarod performance on L-DOPA was monitored on days 6, 13, and 19 (Fig. 1.1F). As previously reported (Marti et al., 2012), animals treated with L-DOPA alone showed impaired rotarod performance at peak dyskinesia. Conversely, eltoprazine-treated rats maintained normal performance ON L-DOPA, even showing a trend to improvement with respect to baseline values.

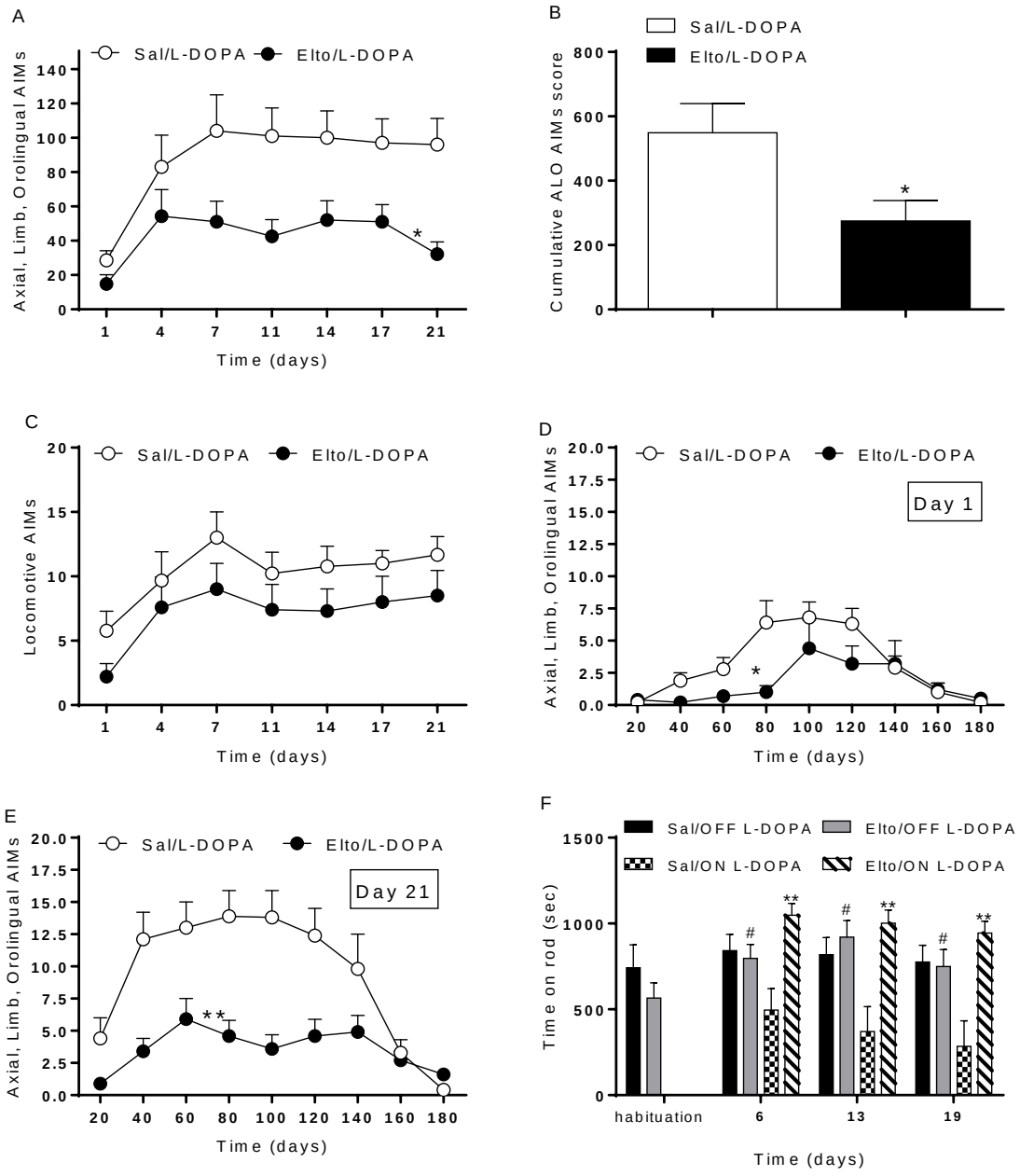

*Figure 1.1 Chronic eltoprazine attenuated LID development without compromising motor function. Panels A and C show ALO AIMs and locomotive AIMs development, respectively, during L-DOPA priming. Panel B represents the cumulative AIMs after 21 day-treatment. Panels D and E show AIMs score during the first and the last day of chronic treatment, respectively. Panel F shows rotarod performance ON/OFF L-DOPA. Data are expressed as mean ± SEM of 10 (L-DOPA-alone group) or 11 (L-DOPA/eltoprazine group) rats. Statistical analysis was performed using the Mann-Whitney test on cumulative ALO AIMs score (A, B, D, E) or RM-ANOVA followed by the Student t-test, two tailed for unpaired data (F). *p<0.05; **p<0.01; different from Sal/L-DOPA or Sal/ON L-DOPA; #p<0.05, different from Sal/OFF L-DOPA.*

Chronic Eltoprazine Prevented LID-Associated Neurotransmitter Changes in Striatum and SNpr

Acute L-DOPA challenge in L-DOPA-primed dyskinetic rats undergoing microdialysis caused AIMs appearance of the same pattern and intensity as those observed in untethered rats not subjected to surgery. Conversely, AIMs severity in rats chronically treated with eltoprazine was dramatically reduced (Fig. 1.2A-B).

Consistently, chronic eltoprazine affected the ability of L-DOPA to stimulate amino acid release in SNpr and striatum. Basal GABA and glutamate levels in the SNpr of L-DOPA-primed dyskinetic rats were 18.59 ± 3.98 and 14.65 ± 3.75 nM, respectively, and were elevated by acute L-DOPA challenge (Fig 1.2C, D). Chronic eltoprazine did not change basal nigral GABA and glutamate levels (20.2 ± 6.1 and 16.6 ± 2.8 nM, respectively). Nonetheless, L-DOPA was unable to evoke nigral amino acid release in eltoprazine-treated rats.

Likewise, acute L-DOPA challenge in rats chronically treated with L-DOPA elevated striatal GABA (Fig. 1.2E) and glutamate (Fig. 2F) levels (14.2 ± 1.5 and 31.4 ± 4.2 nM at baseline, respectively). Chronic eltoprazine did not affect basal striatal GABA (14.5 ± 3.1 nM) or glutamate (17.8 ± 3.5 nM) levels. However, L-DOPA challenge in rats chronically treated with L-DOPA plus eltoprazine only elevated GABA without altering glutamate levels.

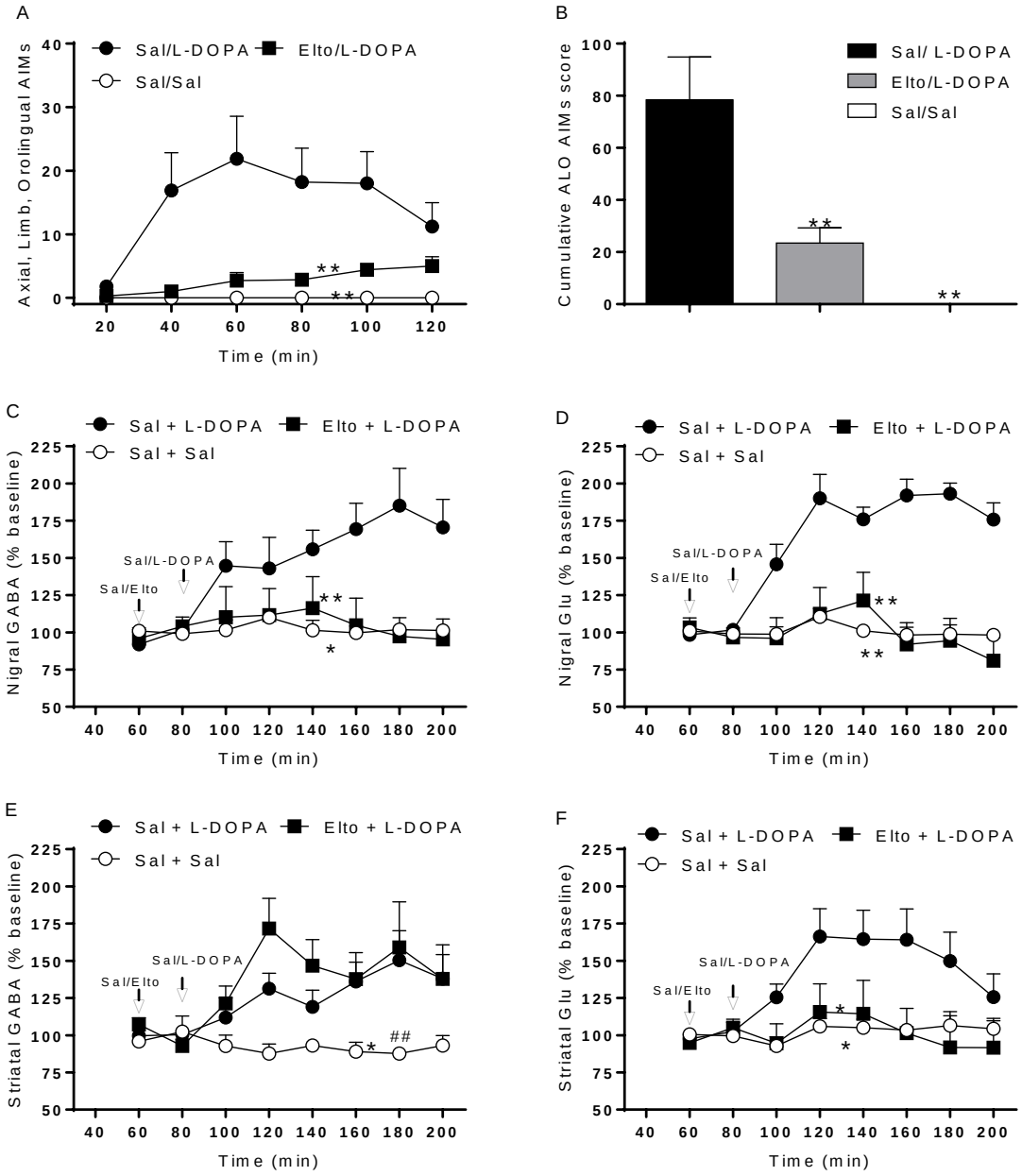

*Figure 1.2 Chronic eltoprazine prevented the rise of nigral and striatal amino acids levels associated with LID. GABA, and glutamate (Glu) were monitored simultaneously with limb, axial, and orolingual abnormal involuntary movements (AIMs; A and B) in the SNpr (C and D) and dopamine-depleted striatum (E and F) of rats chronically treated with L-DOPA alone or in combination with eltoprazine (0.3 mg/kg). Data are expressed as mean ± SEM of 8 rats per group, are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment). Statistical analysis was performed on cumulative ALO AIMs score using non-parametric ANOVA followed by the Dunn's test (A and B) or on AUC data using parametric ANOVA followed by the Newman-Keuls test (C-F). *p<0.05; **p<0.01; different from Sal/L-DOPA; #p<0.05, significantly different from Elto/Sal.*

Chronic Eltoprazine Attenuated LID-Associated Elevation of Striatal pERK

To confirm that chronic eltoprazine interferes with the process of brain sensitization to L-DOPA, striatal pERK levels were measured (Fig. 1.3). pERK elevation upon acute challenge with L-DOPA was 40% reduced in rats chronically treated with eltoprazine with respect to controls. This effect was not owing to reduction of ERK expression given that total ERK levels were not affected by eltoprazine (Fig. 1.3).

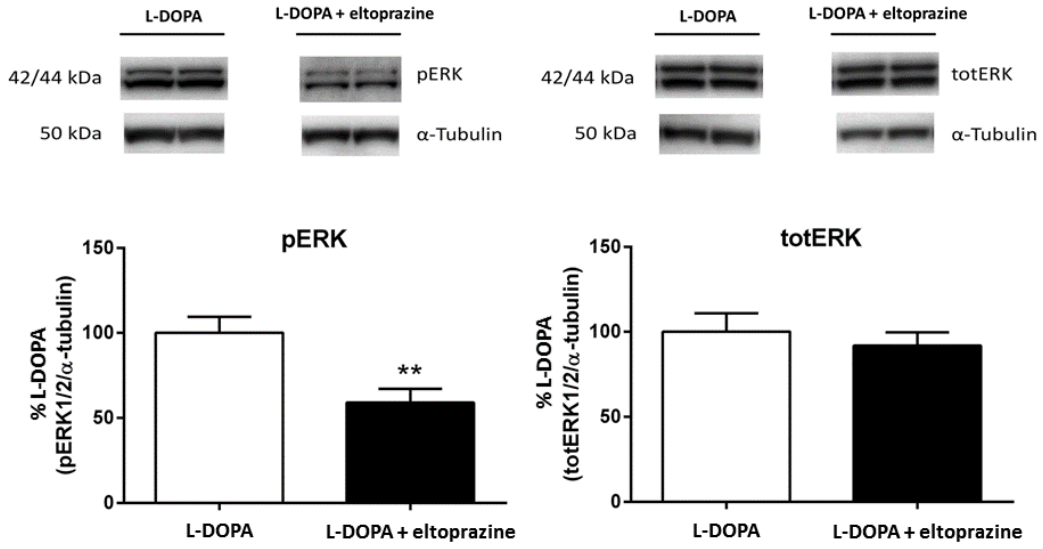

*Figure 1.3 Chronic eltoprazine reduced striatal levels of pERK. Western blot representative images (upper panel) and quantification (lower panel) of pERK (left) and tot ERK (right). Striata were collected from rats involved in the chronic study and subjected to microdialysis. Data are expressed as mean percentage ± SEM of the L-DOPA group (10 rats/group). Statistical analysis was performed using the Student t-test, two-tailed for unpaired data. **p<0.01, significantly different from L-DOPA.*

Acute Eltoprazine Prevented LID Expression and the Associated Rise in Nigral and Striatal Amino Acids, but Not Striatal DA

To assess whether eltoprazine exerts a symptomatic antidyskinetic effect by attenuating striato-nigral MSN overactivation, microdialysis was performed in dyskinetic rats acutely challenged with eltoprazine. Basal GABA and glutamate levels were 6.1 ± 1.9 and 25.5 ± 3.6 nM, respectively, in SNpr, and 8.9 ± 2.4 and 19.3 ± 3.3 nM, respectively, in striatum. Acute eltoprazine (0.3 mg/kg) abolished LID expression in animals performing microdialysis (Fig. 1.4A-B) and prevented the accompanying elevation of nigral GABA

(Fig. 1.4C) and glutamate (Fig. 1.4D) levels, without per se affecting amino acid release. Conversely, eltoprazine inhibited L-DOPA-induced striatal glutamate release (Fig. 1.4F), leaving GABA release unaffected (Fig. 1.4E).

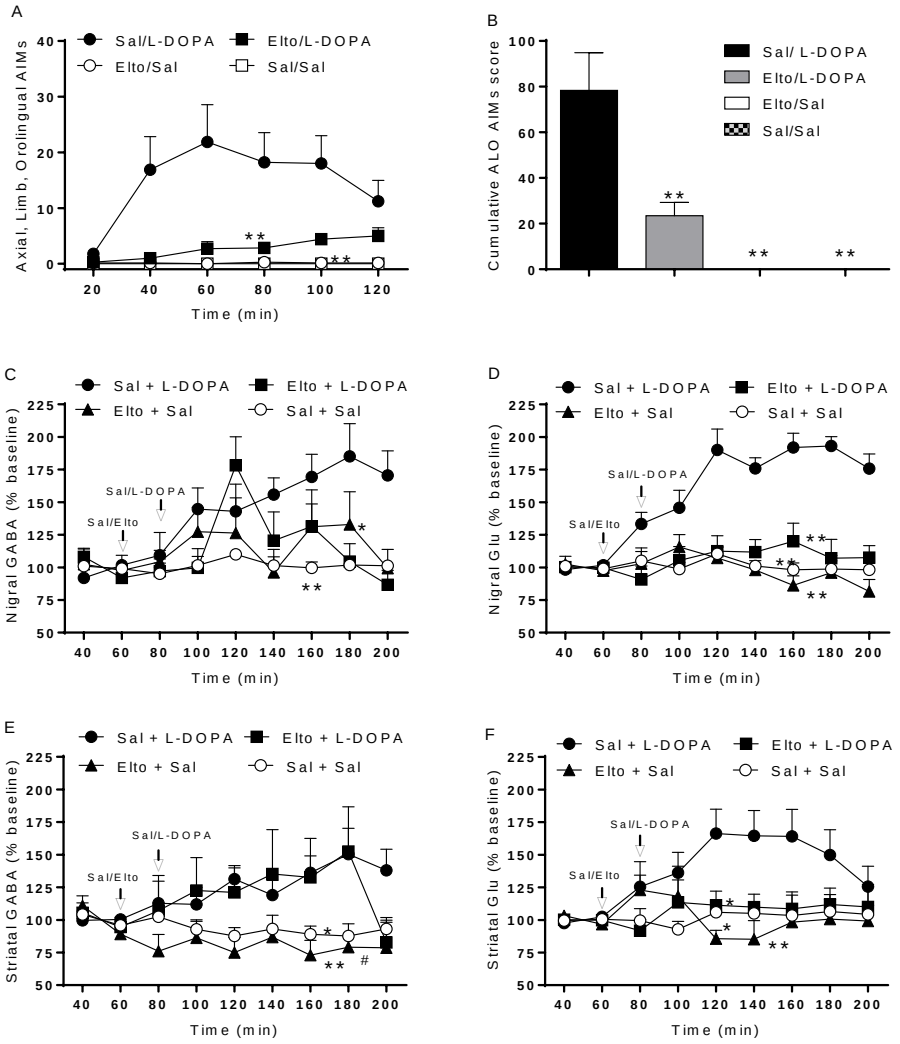

*Figure 1.4 Acute eltoprazine prevented LID expression and the accompanying rise in nigral amino acid levels. GABA and glutamate (Glu) were monitored simultaneously with limb, axial, and orolingual abnormal involuntary movements (AIMs; A and B) in SNpr (C and D) and dopamine-depleted striatum (E and F) of L-DOPA-primed dyskinetic rats acutely challenged with L-DOPA alone or in combination with eltoprazine (0.3 mg/kg). Data are expressed as mean ± SEM of 8 rats, are expressed as percentage of basal pre-treatment levels (calculated as the mean of the two samples preceding the treatment). Statistical analysis was performed on cumulative ALO AIMs score using non-parametric ANOVA followed by the Dunn's test (A and B) or on AUC data using parametric ANOVA followed by the Newman-Keuls test (C-F).*p<0.05; **p<0.01; different from Sal/L-DOPA; #p<0.05, significantly different from Elto/Sal.*

To investigate whether eltoprazine (0.3 mg/kg) reduces AIM expression through inhibition of ectopic DA release (Fig. 1.5), DA levels were measured in dyskinetic rats acutely challenged with eltoprazine. DA levels were 85% reduced in lesioned (1.4 ± 0.2 nM;

n=16), compared to unlesioned (4.6 ± 0.9 nM; n=21) striatum. L-DOPA caused AIM appearance (Fig. 1.5A-B) and simultaneously elevated DA release in both hemispheres. Eltoprazine reduced LID expression without compromising the L-DOPA ability of releasing DA in lesioned or unlesioned striata (Fig. 1.5C).

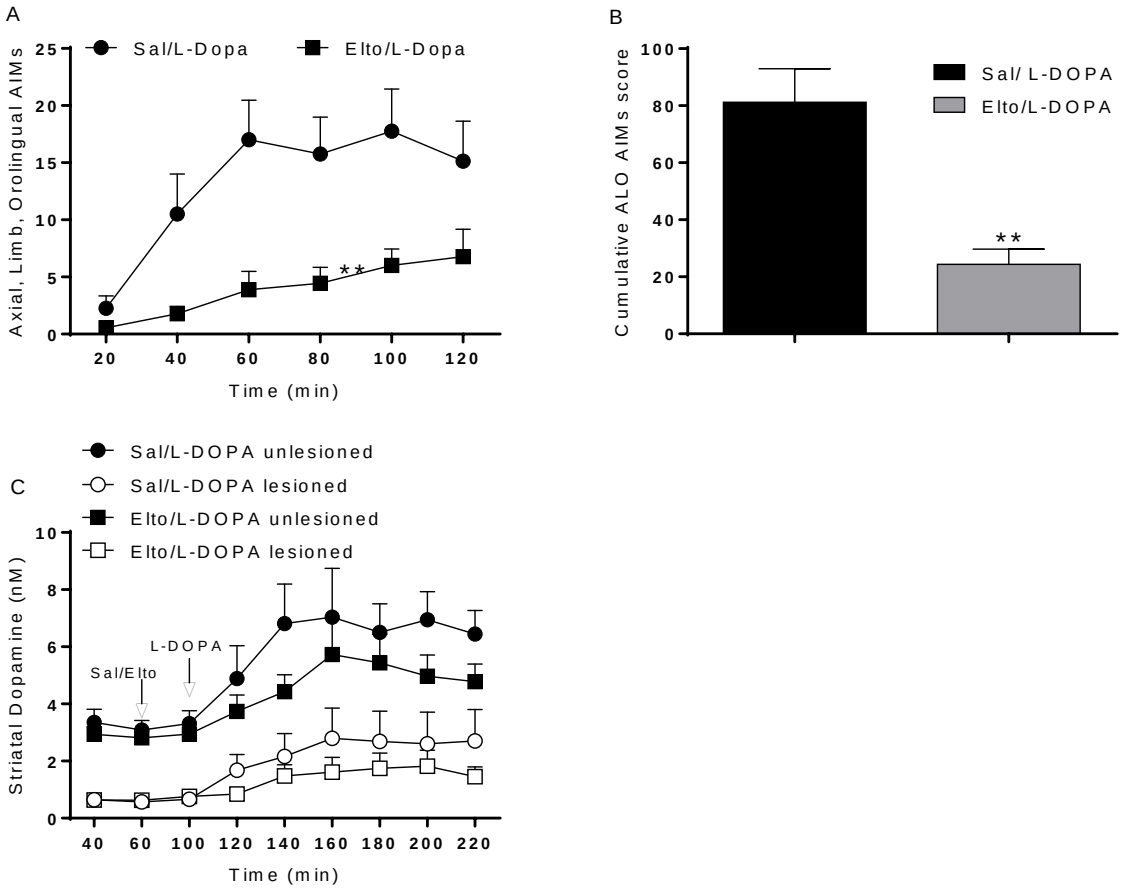

*Figure 1.5 Acute eltoprazine prevented LID expression, but not the accompanying rise of striatal dopamine levels. Dopamine was monitored simultaneously with limb, axial, and orolingual abnormal involuntary movements (AIMs; A and B) in both the dopamine-depleted and unlesioned striatum (C) of L-DOPA-primed dyskinetic rats, challenged with L-DOPA alone or in combination with eltoprazine (0.3 mg/kg). Data are means ± SEM of 8–12 rats, and are expressed as absolute values. Statistical analysis was performed on cumulative ALO AIMs score using the Mann-Whitney test. **p<0.01; different from Sal/L-DOPA.*

PART 2

Genetic deletion of Rhes prevents LID development without causing primary hypolocomotion

To confirm the involvement of Rhes in LID (Subramaniam et al., 2012), $Rhes^{-/-}$ and $Rhes^{+/+}$ mice were hemilesioned with 6-OHDA and chronically treated with L-DOPA.

Mice were submitted to behavioural tests in order to evaluate differences in motor phenotype before and after 6-OHDA lesion. No differences between genotypes were observed in motor activity in the bar, drag, rotarod and open field tests before 6-OHDA (Fig. 2.1 and Table 1). Unilateral intrastriatal injections of 6-OHDA caused a significant increase of immobility time and a reduction of stepping activity selectively at the contralateral forepaw, along with reduction of rotarod performance, in both genotypes (Table 1). Motor performances in the bar and rotarod tests after 6-OHDA were similar in $Rhes^{+/+}$ and $Rhes^{-/-}$ mice. Conversely, $Rhes^{-/-}$ mice performed worse than $Rhes^{+/+}$ mice in the drag test, stepping activity at the contralateral paw being lower in $Rhes^{-/-}$ than $Rhes^{+/+}$ mice (Table 1).

	6-OHDA	Bar test (s)		Drag test (number of steps)		Rotarod (s)
		ipsilateral	contralateral	ipsilateral	contralateral	
$Rhes^{+/+}$	before	7.3±0.6	7.2±0.6	16.4±1.1	14.7±1.2	1017±49.8
	after	5.1±1.1	10.9±1.5**	17.2±4.3	8.8±2.2**	618±154.7**
$Rhes^{-/-}$	before	9.1±1.4	8.5±0.9	15.5±0.8	14.6±0.8	1134.6±40.4
	after	5.9±0.2	9.7±0.1**	19.5±1.4	4.7±0.8*#	711.6±190.9**

*Table 1 Effects of 6-OHDA lesion on motor activity of $Rhes^{+/+}$ and $Rhes^{-/-}$ mice. Data are expressed as immobility time (in seconds; bar test), number of steps (drag test) and time on rod (in seconds; rotarod test). In the bar and drag tests, motor activity was measured separately at the ipsilateral and contralateral forepaw. Data are means of ± SEM of 7–11 animals. Statistical analysis was performed using the Student t-test. *p<0.05, **p< 0.01 versus ipsilateral forepaw; #p<0.05 versus $Rhes^{+/+}$.*

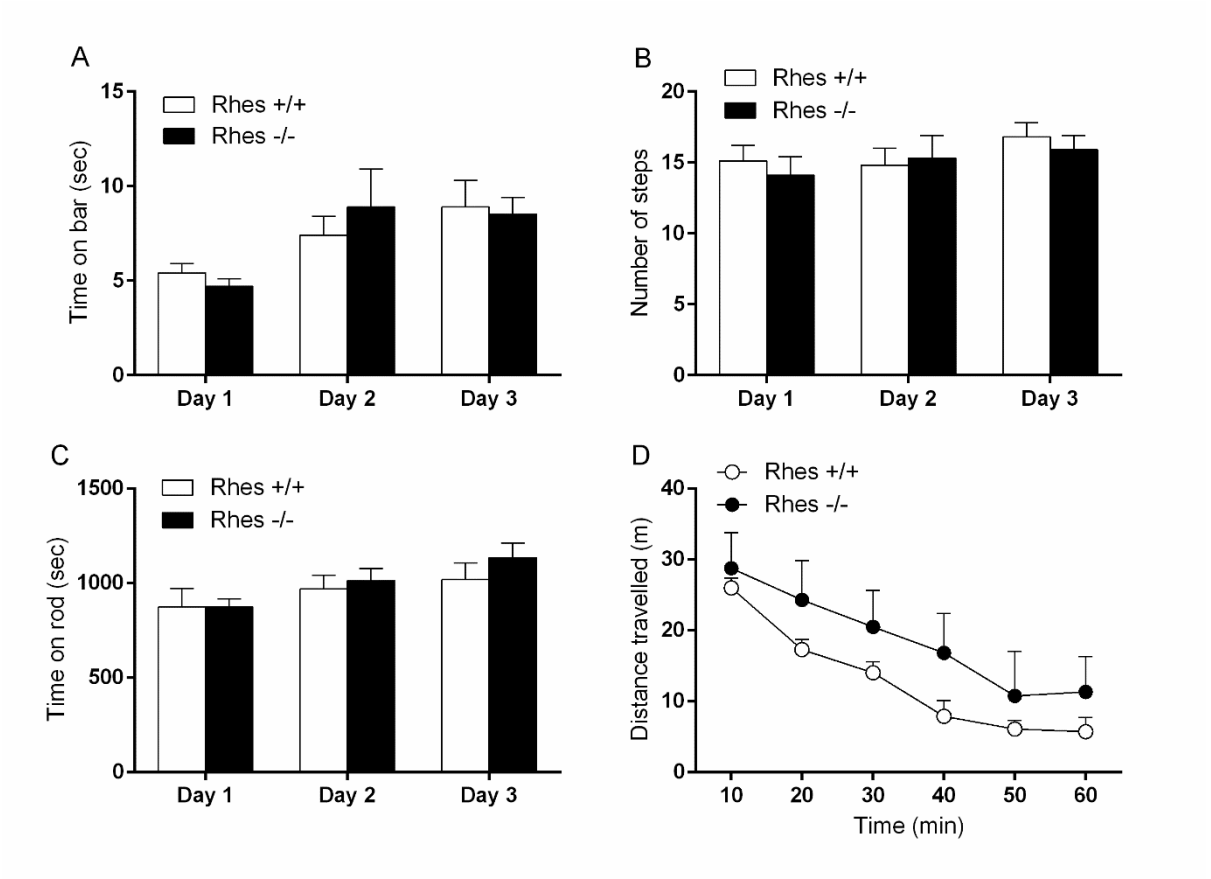

Figure 2.1. Characterisation of motor phenotype of Rhes$^{+/+}$ and Rhes$^{-/-}$ mice. Motor tests were performed using the bar test (A), drag test (B), rotarod test (C) and open field test (D). Data are expressed as absolute values (sec, number of steps, m) and are mean ± SEM of 9-10 animals per group.

As expected, chronic L-DOPA induced AIMs development in hemiparkinsonian Rhes$^{+/+}$ mice. AIMs appearance was gradual and progressive, reaching a plateau at the fourth day of treatment (Fig. 2.2A). AIMs also developed in Rhes$^{-/-}$ mice (Fig. 2.2A), although their severity was greatly (62%) attenuated compared to Rhes$^{+/+}$ mice (Fig. 2.2B). Time-course of AIMs was qualitatively similar in both genotypes, since AIMs appeared 20 min after L-dopa administration and declined two hours later (Fig. 2.2.C).

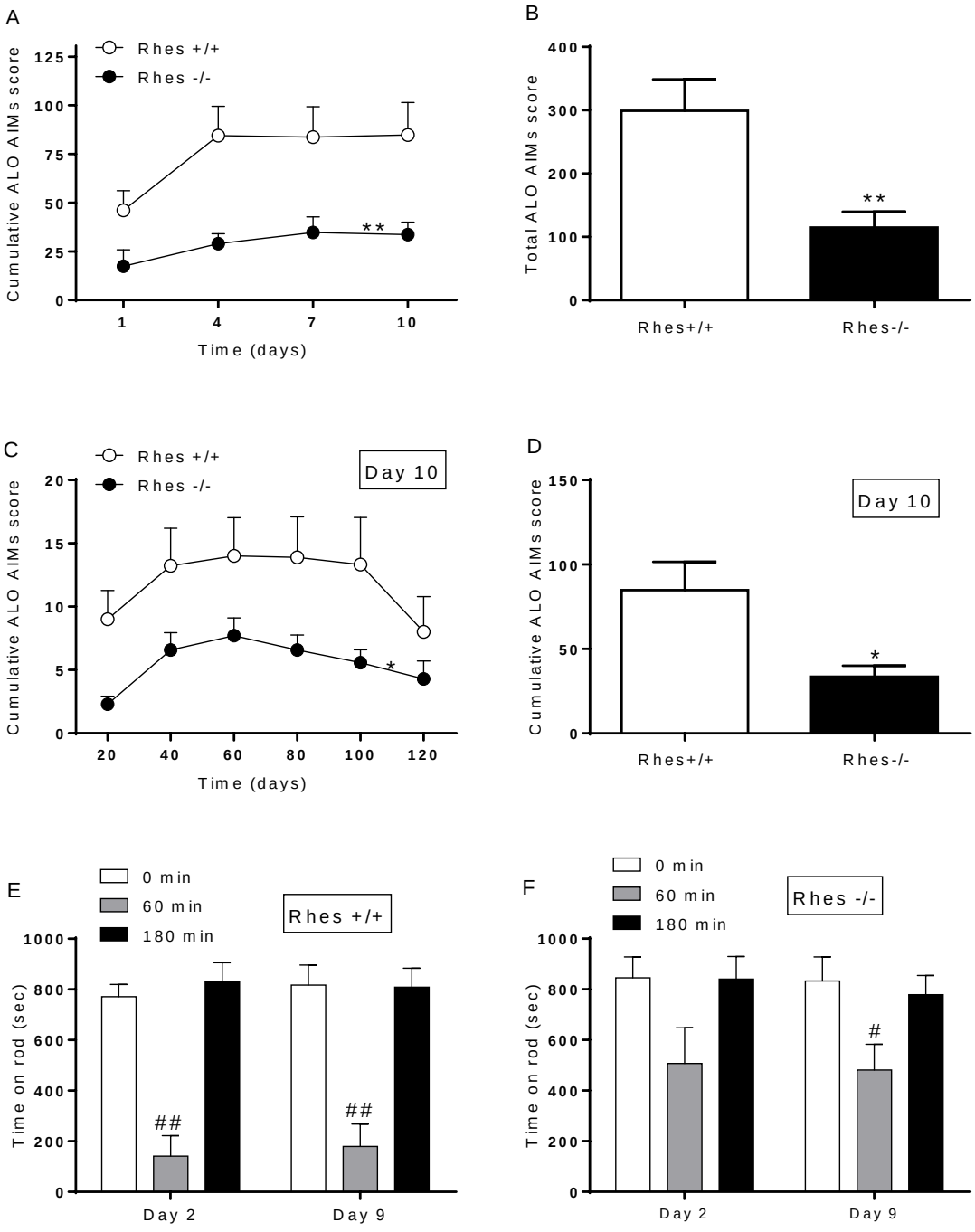

*Figure 2.2. 6-OHDA-hemilesioned Rhes$^{-/-}$ mice developed less dyskinesia than Rhes$^{+/+}$ mice upon chronic treatment with L-DOPA. Axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were rated at days 1, 4, 7 and 10 (A) and total ALO AIMs score calculated (B). The time-course of AIMs expression following L-DOPA challenge at day 10 is shown (C) and total ALO AIMS score (D). Rotarod activity was impaired in both genotypes 60 min after L-DOPA challenge, although more severely in Rhes$^{+/+}$ (E) than Rhes$^{-/-}$ (F) mice. Data are means ± SEM of 9 Rhes$^{+/+}$ and 7 Rhes$^{-/-}$ mice per group. Data were analyzed using the Mann-Whitney test on cumulative ALO AIMs score (A–D) or repeated measure ANOVA followed by the Newman–Keuls test for multiple comparisons on time on rod (E and F). *p<0.05, **<0.01 different from Rhes$^{+/+}$. #p<0.05, ##p<0.01 different from baseline (0 min).*

To investigate whether LID reduction was associated with motor impairment, we monitored mice rotarod activity 60 min and 3 h after L-DOPA administration. Rhes$^{+/+}$ mice (Fig. 2.2E) showed motor impairment 60 min after L-DOPA both at day 2 and day 9. Instead, Rhes$^{-/-}$ mice (Fig. 2.2F) showed mild motor impairment only at day 9, although a trend for motor inhibition was also observed at day 2.

Genetic deletion of Rhes attenuated L-DOPA-induced AIMs appearance along with the rise in nigral amino acids

To investigate the mechanisms underlying the reduced dyskinetic response of Rhes$^{-/-}$ mice, and specifically to examine whether LID attenuation was accompanied by a reduction of neurochemical markers of LID (Mela et al., 2007; Bido et al., 2011; Marti et al., 2012; Mela et al., 2012; Bido et al., 2015), microdialysis was performed in Rhes$^{+/+}$ and Rhes$^{-/-}$ mice at the end of chronic treatment with L-DOPA. In the microdialysis setting, LID severity after L-DOPA challenge was 65% attenuated in Rhes$^{-/-}$ mice compared to controls (Fig. 2.3A-B). The different responses to L-DOPA of Rhes$^{+/+}$ and Rhes$^{-/-}$ mice was associated with different neurochemical profiles. Rhes$^{+/+}$ and Rhes$^{-/-}$ dyskinetic mice did not differ for basal dialysate levels of striatal GABA (8.1 ± 0.6 nM and 10.5 ± 0.4 nM, respectively) and glutamate (73.2 ± 0.9 nM and 73.5 ± 1.8 nM), or nigral GABA (9.1 ± 0.3 nM and 9.9 ± 0.4 nM) and glutamate (52.5 ± 2.7 nM and 58.4 ± 2.5 nM). However, Rhes$^{-/-}$ mice did not show the increase of GABA levels in SNpr which was observed after L-DOPA administration in Rhes$^{+/+}$ mice (Fig. 2.3C). A trend for a reduced response of nigral glutamate in Rhes$^{-/-}$ mice was also observed (Fig. 2.3D). Different from SNpr, the responses of striatal GABA (Fig. 2.3E) and glutamate (Fig. 2.3F) to L-DOPA were similar between genotypes.

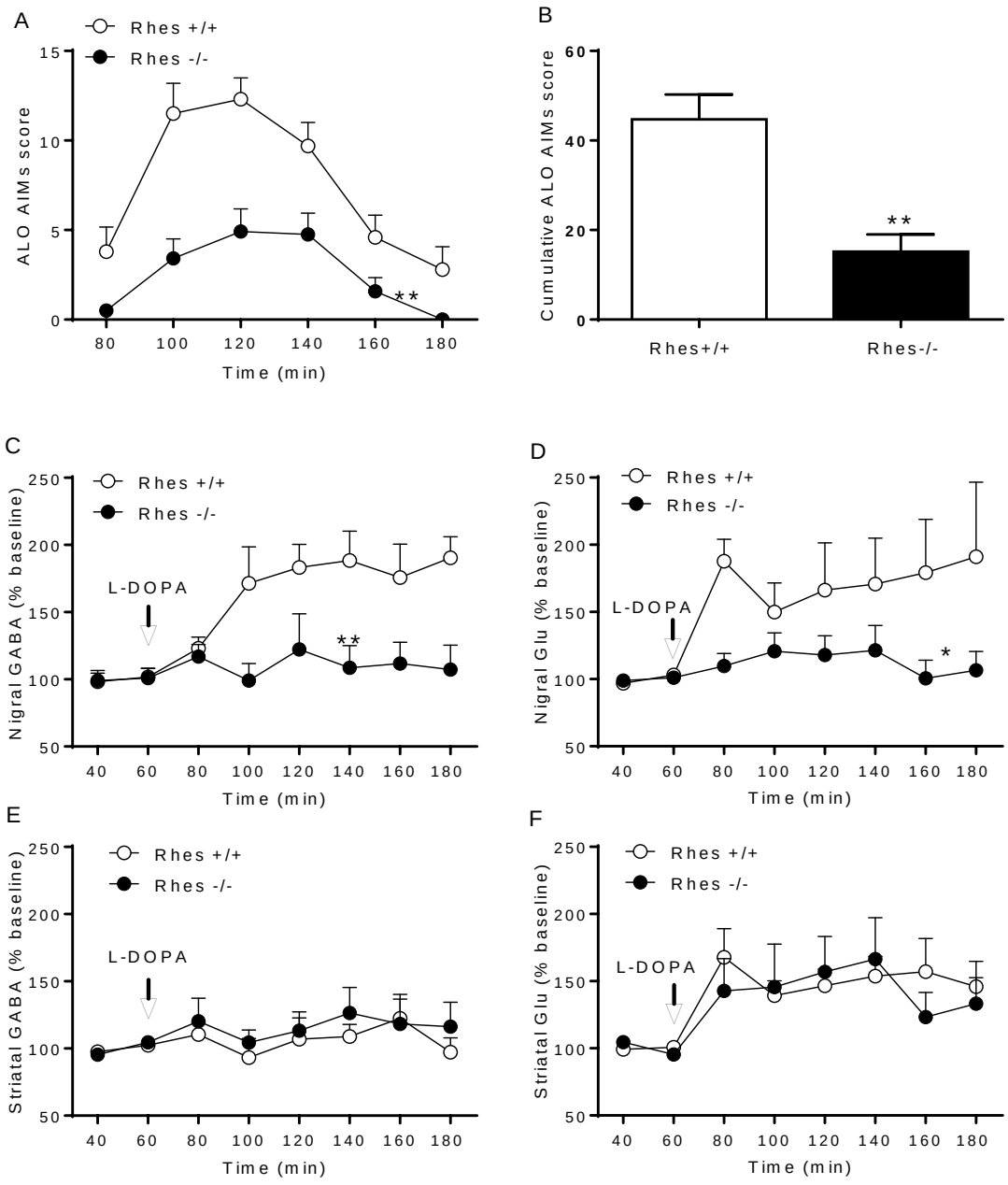

*Figure 2.3 L-DOPA challenge in dyskinetic Rhes$^{-/-}$ mice failed to evoke GABA and glutamate (Glu) release in SNpr. 6-OHDA hemilesioned Rhes$^{+/+}$ and Rhes$^{-/-}$ mice were chronically treated with L-DOPA, then subjected to dual probe microdialysis in dorsolateral striatum and ipsilateral SNpr. During the microdialysis session, mice were challenged with L-DOPA, and axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were rated over a 2-h observation period (A), and total ALO AIMs score calculated (B). GABA and glutamate levels were measured in SNpr (C–D) and striatum (E–F). Data are means± SEM of 6–10 determinations for Rhes$^{+/+}$ and 7–10 determinations for Rhes$^{-/-}$ mice. Data were analysed as cumulative ALO score using non-parametric ANOVA followed by the Mann–Whitney (A and B) and AUC data using parametric ANOVA followed by the Student's t-test (C-D). *p<0.05, **p<0.01 different from Rhes$^{+/+}$.*

Chronic rapamycin reduced LID and its neurochemical correlates in 6-OHDA hemilesioned mice

So far, no specific pharmacological inhibitors of Rhes have been developed. However, since Rhes binds and activates mTORC1 (Subramaniam et al., 2012), we investigated whether administration of rapamycin could entirely replicate the pattern of behavioural and neurochemical changes induced by genetic deletion of Rhes.

Rapamycin (5 mg/kg), given simultaneously with L-DOPA to 6-OHDA hemilesioned mice, attenuated LID development by 54% overall (Fig. 2.4A, B).

The effect of rapamycin was not owing to primary hypolocomotive effect, since control dyskinetic mice showed rotarod impairment 60 min after L-DOPA administration at both day 2 and day 9 during L-DOPA treatment (Fig. 2.4E-F), while rapamycin-treated mice did not show any worsening of rotarod performance (Fig. 2.4E-F).

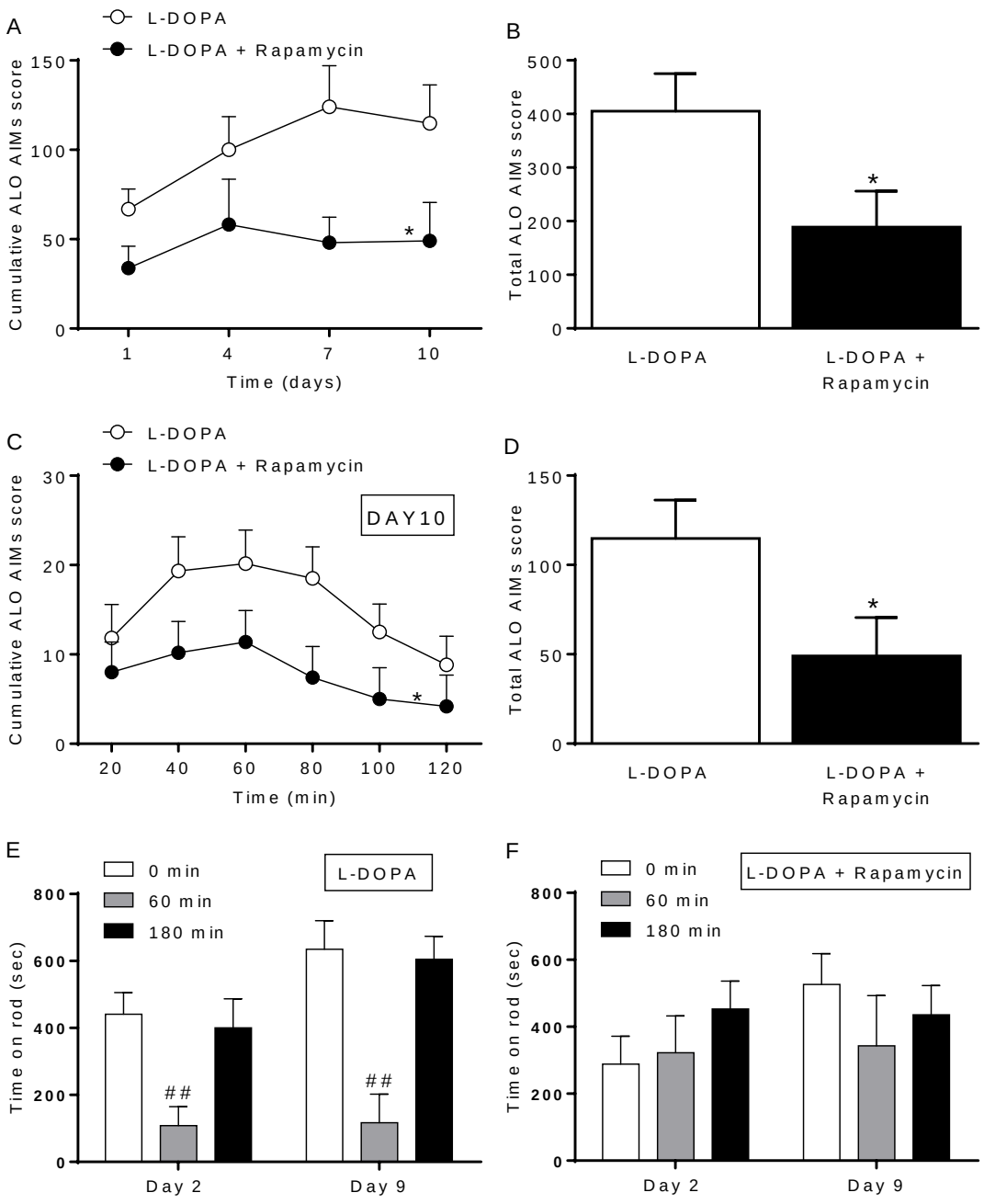

*Figure 2.4 Rapamycin attenuated dyskinesia development without compromising motor activity. 6-OHDA-hemilesioned mice were subjected to chronic treatment with L-DOPA alone or in combination with rapamycin. Axial, limb and orolingual (collectively ALO) abnormal involuntary movements (AIMs) were rated at days 1, 4, 7 and 10 (A), and total ALO AIMs score calculated (B). The time-course of AIMs expression following L-DOPA challenge at day 10 (C) and the cumulative analysis (D) are shown. Rotarod activity was impaired in L-DOPA (E) but not L-DOPA + rapamycin (F) treated mice. Data are means ± SEM of 5–6 mice per group. Data were analysed using the Mann–Whitney test on cumulative ALO AIMs score (A-D) or repeated measure ANOVA followed by the Newman–Keuls test on time on rod (D–E). *p<0.05 different from L-DOPA. ##p<0.01 different from baseline (0 min).*

Microdialysis revealed that L-DOPA failed to evoke GABA (Fig. 2.5A) and glutamate (Fig. 2.5B) release in the SNpr of rapamycin-treated mice. L-DOPA was ineffective in

modulating striatal GABA release in either treatment group (Fig. 2.5C). However, it elevated striatal glutamate release in control but not rapamycin-treated mice (Fig. 2.5D).

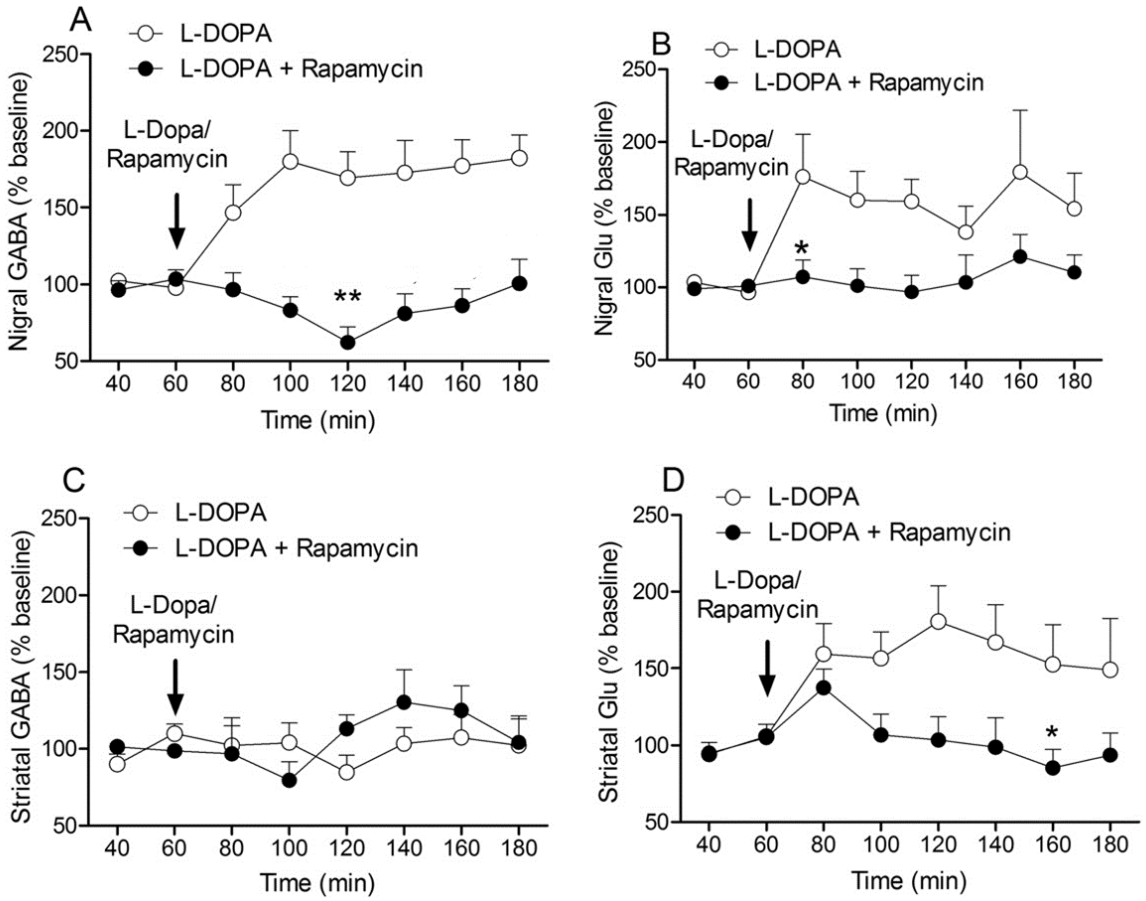

*Figure 2.5 L-DOPA challenge in rapamycin-treated dyskinetic mice failed to evoke GABA and glutamate (Glu) levels in substantia nigra reticulata (SNpr), as well as glutamate levels in striatum. 6-OHDA-hemilesioned mice were chronically treated with L-DOPA, then subjected to dual probe microdialysis in dorsolateral striatum and ipsilateral SNpr. GABA and glutamate levels were monitored in SNpr (A–B) and striatum (C–D). Data are means ± SEM of 6–10 determinations for the L-DOPA group, and 7–12 determinations for the L-DOPA + rapamycin group. Data were analysed by parametric ANOVA followed by the Student t-test. *p<0.05, **p<0.01 different from L-DOPA.*

PART 3

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

In order to evaluate whether M1Rs influence LID and its neurochemical markers expression, the M1R preferential antagonist telenzepine was perfused at the concentration of 100 nM through the probe implanted in striatum, 40 minutes before systemic (i.p.) L-DOPA administration. AIMs were monitored every 20 minutes along with sample collection.

This antagonist concentration was chosen based on the reported affinity of telenzepine for M1R (0.94 nM), and selectivity over other muscarinic antagonists (~20-fold for M2R; Ki 17.8 nM) (Christofi et al., 1991), considering an in vitro recovery of about 10% under the present microdialysis conditions (Mela et al., 2012).

As expected, L-DOPA alone caused a gradual expression of dyskinesia, which reached a maximum after 80 minutes (Fig. 3.1A). Pre-treatment with telenzepine reduced LID expression by ~40% (Fig. 3.1B).

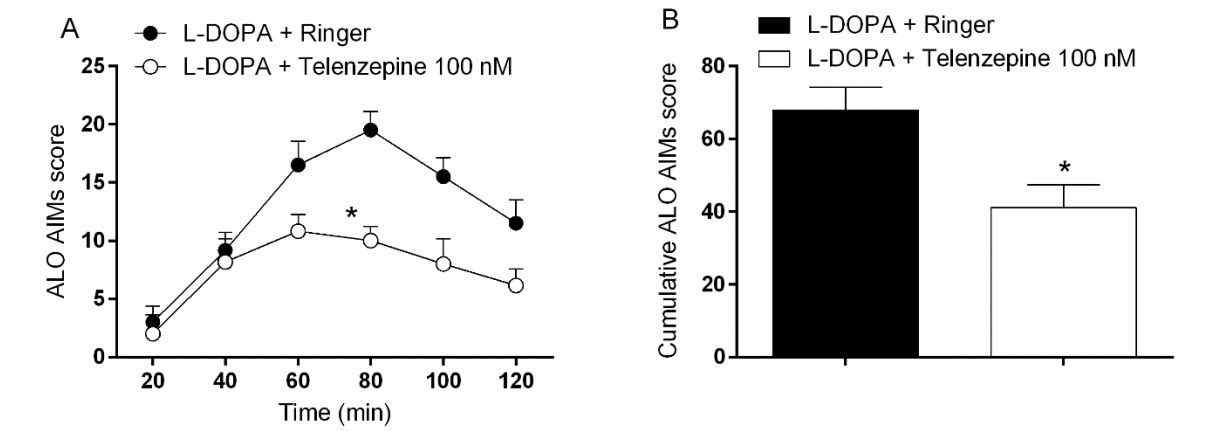

*Figure 3.1. Telenzepine attenuated LID expression in rats undergoing microdialysis. Dyskinetic rats underwent surgery for microdialysis probe implantation, and were challenged with L-DOPA alone (6 mg/kg plus 12 mg/kg benserazide, i.p.) or in combination with telenzepine (100 nM through the probe; 40 min in advance). ALO AIMs were scored every 20 min over 120 min after L-DOPA administration (A) then cumulative ALO AIMs calculated (B). Data are means ± SEM of 8-10 rats per group. Data were statistically analyzed as cumulative ALO AIMs score using the Mann-Whitney test. *p<0.05 different from control (L-DOPA+ringer).*

Neurochemical analysis evidenced that basal GABA and glutamate levels (nM) were 21.62 ± 4.06 and 43.89 ± 4.71 in striatum, and 18.13 ± 2.98 and 46.15 ± 5.48 in SNpr, respectively.

LID expression was accompanied by the rise of amino acids in SNpr, and glutamate in striatum, whereas striatal GABA release remained unaffected (Fig. 3.2A).

Intrastriatal perfusion with telenzepine inhibited L-DOPA-induced glutamate release in striatum (Fig. 3.2B), and GABA in SNpr (Fig. 3.2C), but it did not reduce the L-DOPA-induced glutamate in SNpr (Fig. 3.2D).

No significant changes in neurotransmitter release were detected in rats acutely perfused with telenzepine alone (Fig. 3.2).

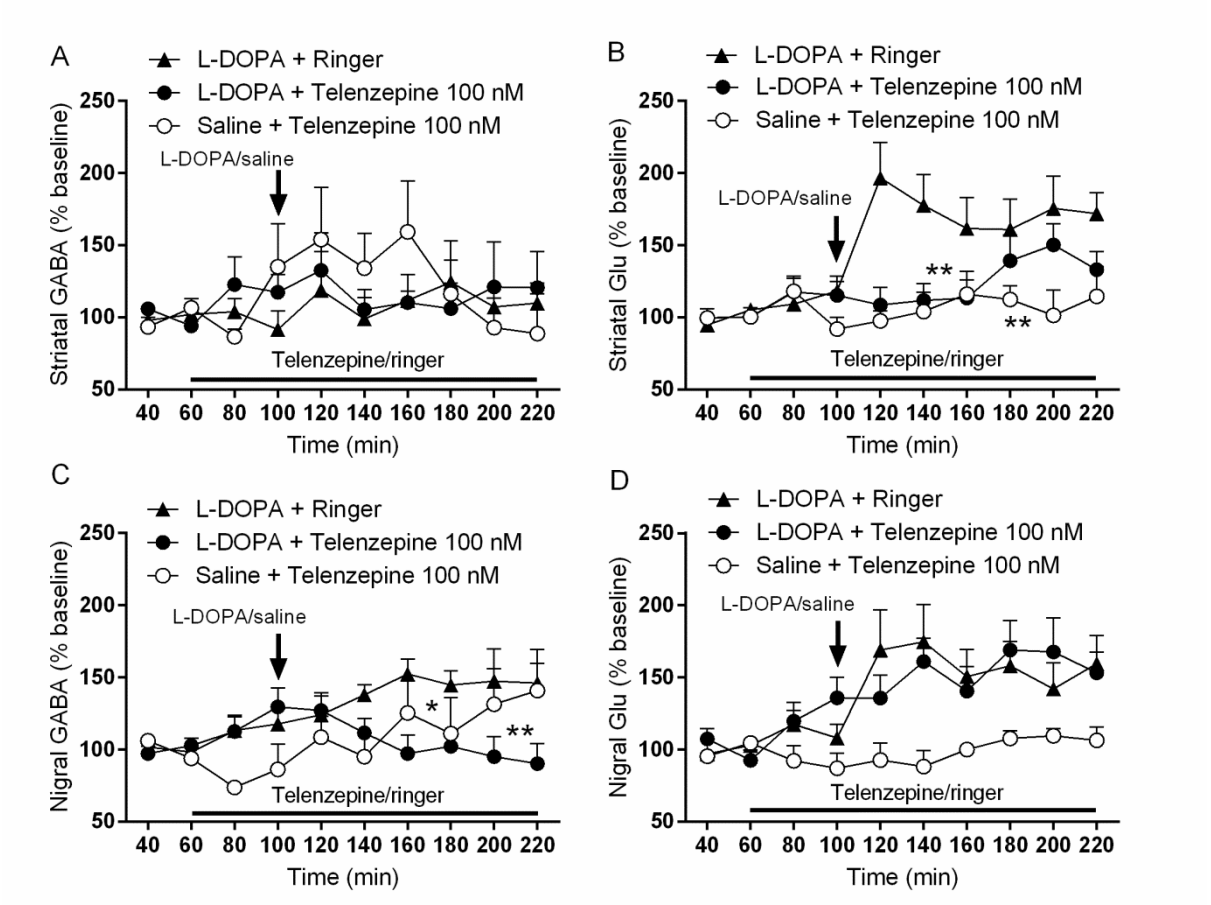

*Figure 3.2. Neurochemical effects of telenzepine in dyskinetic rats undergoing microdialysis. Dyskinetic rats were implanted with one probe in the lesioned striatum (A, B) and another in ipsilateral SNpr (C, D). Twenty-four hours later, rats received an acute challenge with L-DOPA alone (6 mg/kg plus 12 mg/kg benserazide, i.p.) or in combination with telenzepine (100 nM; through the probe, 40 min in advance). A group treated with telenzepine alone was also included. GABA (A, C) and glutamate (B, D) levels were monitored in striatum and SNpr, every 20 min for 120 min after L-Dopa administration. Data are means ± SEM of 8 determinations per group. Statistical analysis was performed on AUC values using ANOVA followed by the Newman-Keuls test. **p<0.01, *p<0.05 different from control (L-DOPA + Ringer).*

PD-102807 and tropicamide striatal perfusion reduced LID expression following a similar neurochemical profile

In order to evaluate whether M4Rs influence over LID and its neurochemical markers expression, the M4R preferential antagonists PD-102807 and tropicamide were perfused through the probe implanted in striatum at the concentrations of 3 µM and 100 nM respectively, 40 minutes before systemic (i.p.) L-DOPA administration. AIMs were monitored every 20 minutes along with sample collection.

Antagonists concentrations were chosen based on the same criteria adopted for telenzepine. PD-102807 affinity values for M4, M3, M2, M1 receptors are 91, 950, 3441, and 6559 nM, respectively (Bohme et al., 2002). Tropicamide affinity values for M4, M3, M2 and M5 receptors are 3.5, 30, 37 and 64 nM, respectively (Lazareno et al., 1993).

Both PD-102807 (Fig 3.3A-B) and tropicamide (Fig. 3.3C-D) perfusion inhibited LID expression, by~60% and ~50%, respectively.

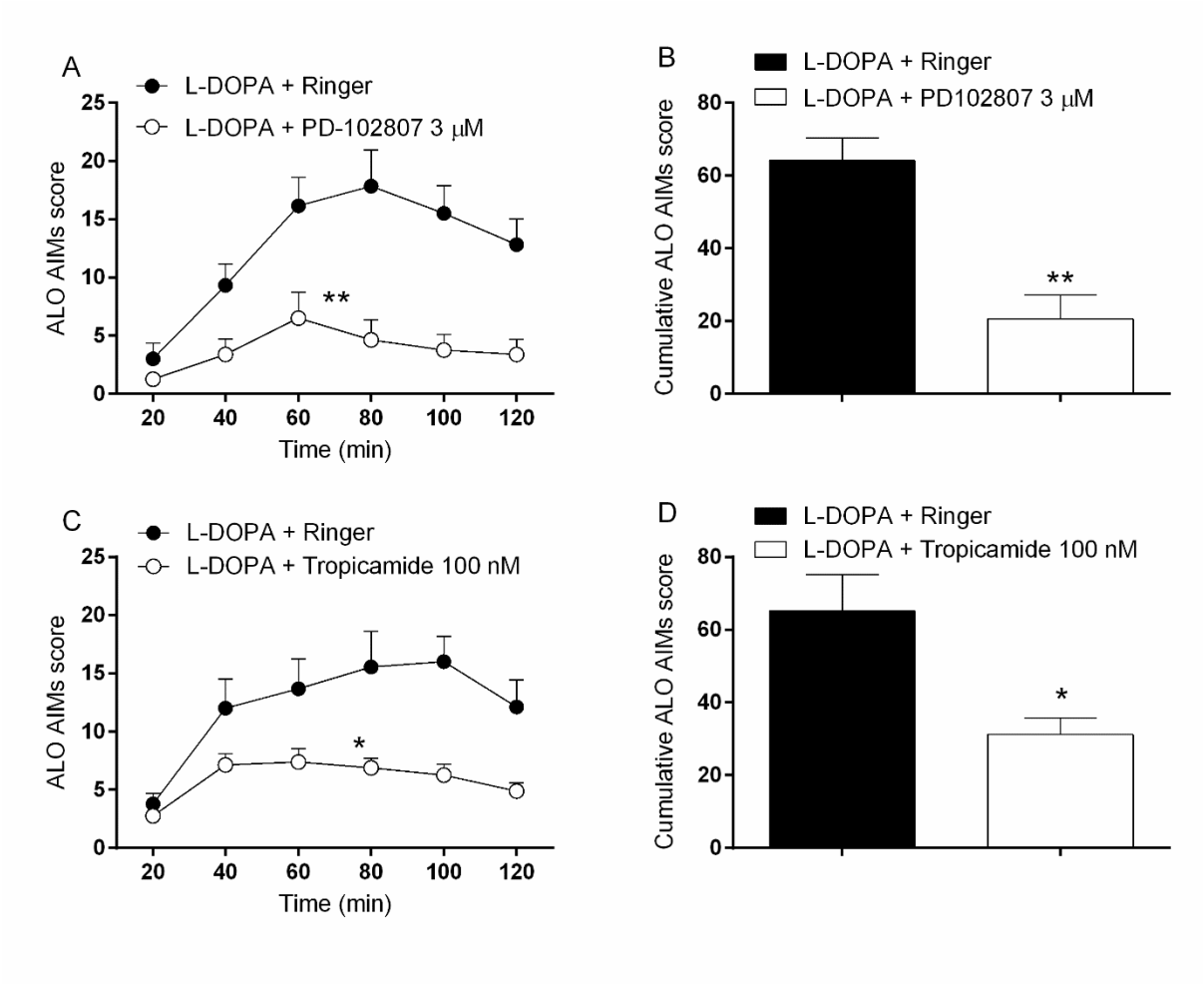

*Figure 3.3. Both PD-102807 and tropicamide alleviated LID expression in rats undergoing microdialysis. Dyskinetic rats underwent surgery for microdialysis probe implantation, and were challenged with L-DOPA alone or in combination with PD-102807 (3 μM through the probe; 40 min in advance) or tropicamide (100 nM through the probe; 40 min in advance). ALO AIMs were scored every 20 min over 120 min after L-DOPA administration (A) then cumulative ALO AIMs calculated (B). Data are means ± SEM of 8-10 rats per group. Data were statistically analyzed on cumulative ALO AIMs score using the Mann-Whitney test. *p<0.05, **p<0.01, different from control (L-DOPA + Ringer).*

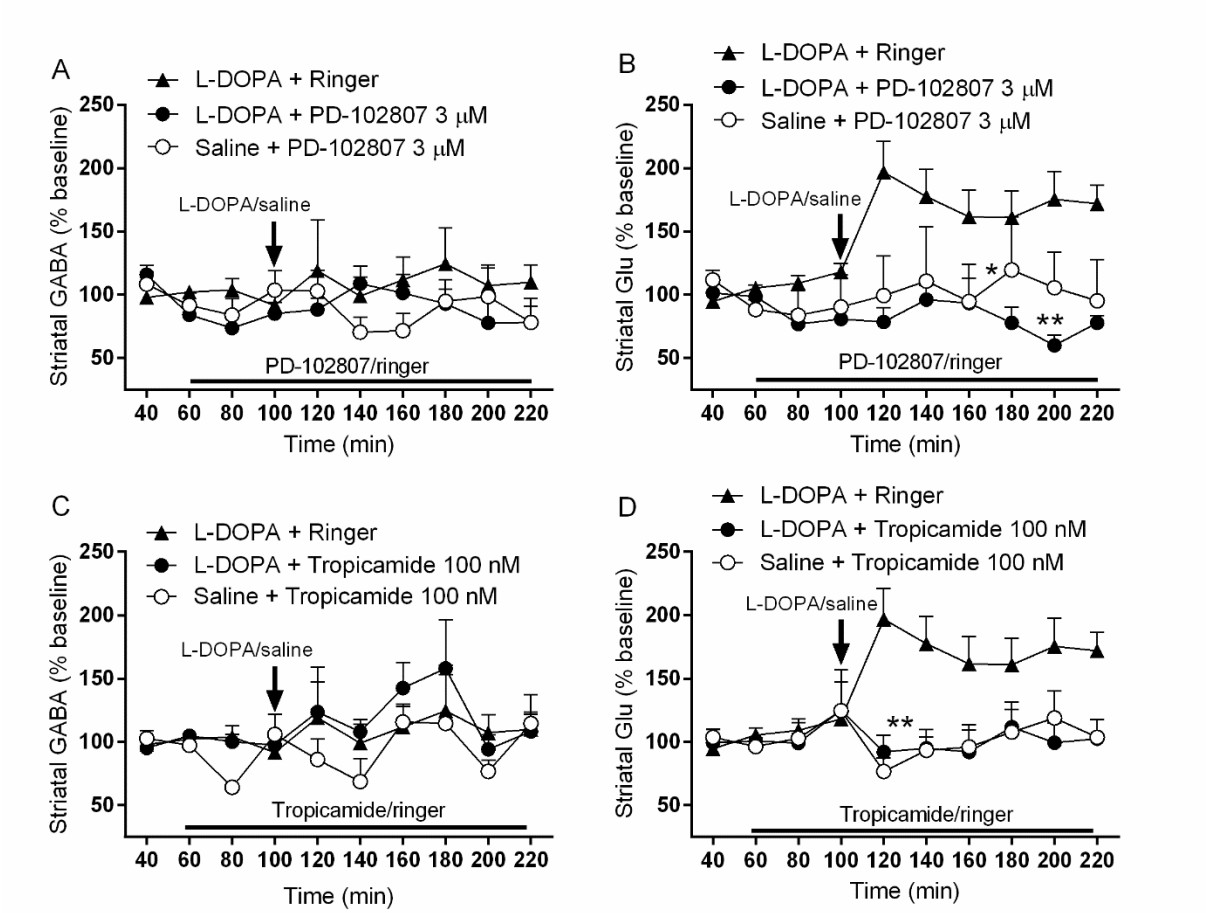

*Figure 3.4. Striatal neurochemical effects of PD-102807 and tropicamide in dyskinetic rats undergoing microdialysis. Twenty-four hours after surgery, rats received an acute challenge with L-DOPA alone (6 mg/kg plus 12 mg/kg benserazide, i.p.) or in combination with PD-102807 (3 μM through the probe; 40 min in advance) or tropicamide (100 nM through the probe; 40 min in advance). Groups treated with PD-102807 or tropicamide alone were also included. GABA (A, C) and glutamate (Glu) (B, D) levels were monitored in striatum, every 20 min for 120 min after L-DOPA administration. Basal striatal GABA and glutamate levels were 21.62 ± 4.06 and 43.89 ± 4.71 nM, respectively. Data are means ± SEM of 8 determinations per group. Statistical analysis was performed on AUC values using ANOVA followed by the Newman-Keuls test. *p<0.05, **p<0.01, different from control (L-DOPA + Ringer).*

In addition, both compounds shared the same neurochemical patterns, inhibiting the L-DOPA-induced rise of glutamate in striatum (Fig. 3.4B, D), and the L-DOPA-induced rise of GABA and glutamate in SNpr (Fig. 3.5).

Neither antagonist alone significantly affected neurotransmitters release in striatum and SNpr.

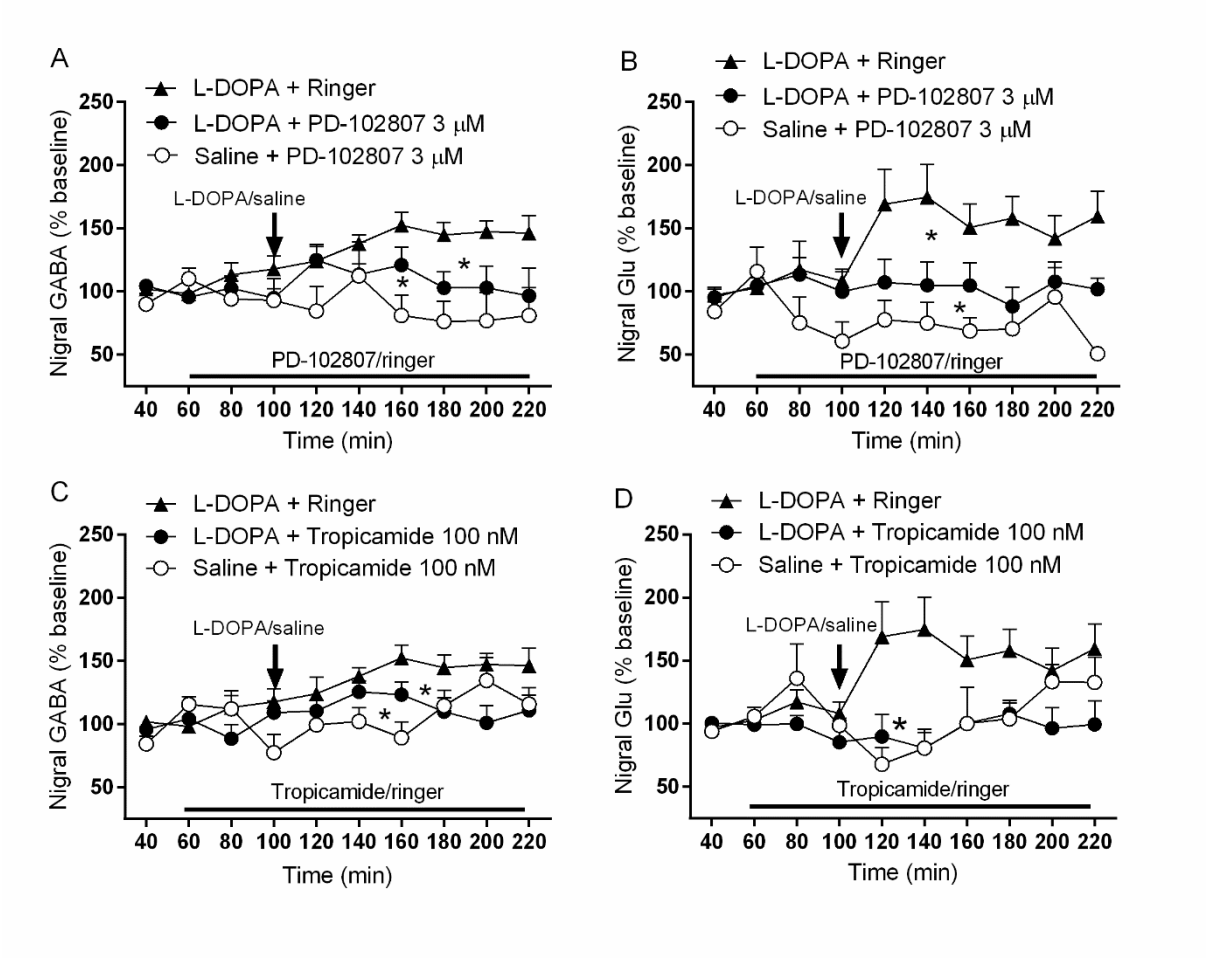

*Figure 3.5. Nigral neurochemical effects of PD-102807 and tropicamide in dyskinetic rats undergoing microdialysis. Twenty-four hours after surgery, rats received an acute challenge with L-DOPA alone (6 mg/kg plus 12 mg/kg benserazide, i.p.) or in combination with PD-102807 (3 μM through the probe; 40 min in advance) or tropicamide (100 nM through the probe; 40 min in advance). Groups treated with PD-102807 or tropicamide alone were also included. GABA (A, C) and glutamate (Glu) (B, D) levels were monitored in SNpr, every 20 min for 120 min after L-DOPA administration. Basal nigral GABA and glutamate levels were 18.13 ± 2.94 and 46.15 ± 5.44 nM, respectively. Data are means ± SEM of 8 determinations per group. Statistical analysis was performed on AUC values using ANOVA followed by the Newman-Keuls test. *p<0.05, **p<0.01, different from control (L-DOPA + Ringer).*

DISCUSSION

Part 1

The present study confirms previous evidence that eltoprazine attenuates both LID development and expression in 6-OHDA hemilesioned rats (Bezard et al., 2013), although, at variance with that study, eltoprazine did not compromise motor activity ON L-DOPA, suggesting that its antidyskinetic action was not owing to primary motor inhibition or attenuation of L-DOPA therapeutic effect.

Several studies performed over the years using in vivo microdialysis in awake, freely moving rats or mice revealed that the increase in nigral GABA release associated with AIMs appearance can be considered a marker of LID and, in particular, of L-DOPA-induced striato-nigral MSNs activation. We previously reported that the elevation of nigral GABA release does not occur in those 6-OHDA hemilesioned rats that do not develop AIMs under chronic treatment with L-DOPA, i.e. non dyskinetic rats, suggesting the elevation of nigral GABA correlates with AIMs appearance (Mela et al., 2007). Consistently, compounds that acutely inhibit AIMs expression such as amantadine (Bido et al., 2011), the mGlu5 receptor negative allosteric modulator MTEP (Mela et al., 2007) or the opioid-like neuropeptide nociceptin/orphanin FQ (Marti et al., 2012) also prevent the elevation of nigral GABA. This is also true for amantadine in the dyskinetic mouse (Bido et al., 2011). In this model, we also reported that genetic removal of RasGRF1 (but not RasGRF2), which is upstream of the non-canonical D1 pathway, abolishes LID expression and the accompanying rise of nigral GABA (Bido et al., 2015). We also proved both in rats and mice, that the elevation of GABA release associated with AIMs appearance selectively occurs in the SNpr, the GP being unaffected. This suggests that the striato-nigral “direct” but not the striato-pallidal “indirect” pathway is activated during AIMs expression, at least in these rodent models of peak-dose dyskinesia (Mela et al., 2007; Bido et al., 2015). Accordingly, the rise of nigral GABA levels associated with AIMs expression in dyskinetic rats is abolished by selective inhibition of striatal D1 but not D2 receptors (Mela et al., 2012). As a further, indirect, evidence of striato-nigral MSNs activation, GABA release in the motor thalamus is reduced during AIMs expression, both in rats (Marti et al., 2012) and nonhuman primates (Porras et al., 2014). In fact, striato-nigral MSNs activation

is expected to reduce the activity of nigro-thalamic GABA neurons, i.e. the basal ganglia output (Albin et al., 1989; Alexander & Crutcher, 1990).

The finding that eltoprazine-treated rats did not show the increase of nigral GABA release associated with LID appearance suggests that chronic eltoprazine interferes with the process of striato-nigral MSNs sensitization to L-DOPA. To confirm this view, we provided evidence that chronic eltoprazine reduced the levels of striatal pERK, which represents a biochemical correlate of non-canonical D1 receptor pathway activation in striato-nigral MSNs (Santini et al., 2009). This was more convincingly proven by a thorough biochemical study in dyskinetic rats, showing that chronic eltoprazine reduced pERK and mTOR phosphorylation, normalized PKA activity and rebalanced glutamate receptor alterations at the post-synaptic membrane (Ghiglieri et al., 2016). These data clearly indicate that eltoprazine interferes with the signaling cascade affected by LID in striato-nigral MSNs.

The antidyskinetic mechanism of serotonergic agonists has been explained by two different hypotheses (Huot et al., 2011): the reduction of ectopic DA release through 5-HT_{1A} and 5-HT_{1B} autoreceptors (Carta et al., 2007; Munoz et al., 2008) and the impairment of glutamatergic transmission by cortical and/or striatal 5-HT_{1A} or 5- HT_{1B} heteroreceptors (Dupre et al., 2011).

In support of the former hypothesis, sprouting of raphe-striatal serotonergic terminals has been observed in dyskinetic rats (Politis et al., 2010; Rylander et al., 2010). Moreover, 8-OHDPAT, a 5-HT_{1A} receptor agonist, reduced striatal DA release in L-DOPA-primed dyskinetic (Lindgren et al., 2010; Nahimi et al., 2012) 6-OHDA hemilesioned rats, although this was also found in L-DOPA unprimed rats (Kannari et al., 2001).

In support of the latter hypothesis, activation of 5-HT_{1A} heteroceptors on cortico-striatal neurons (Dupre et al., 2011; Ostock et al., 2011) impaired striatal glutamate release and palliated LID. Moreover, activation of postsynaptic 5-HT_{1A} resulted in reduction of apomorphine-induced dyskinesia although this effect was milder than that exerted on LID (Dupre et al., 2007; Munoz et al., 2008).

In our study, a low dose of eltoprazine was found effective in inhibiting LID expression and the associated release of striatal glutamate, without affecting striatal DA release. This is consistent with the hypothesis that the antidyskinetic effect of eltoprazine is

accomplished via inhibition of cortico-striatal glutamate pathway (Dupre et al., 2011; Ostock et al., 2011).

This was a bit surprising since eltoprazine, like 8-OHDPAT, inhibits serotonergic neuron activity through 5-HT_{1A} receptors in dorsal raphe slices in vitro (Millan et al., 1994). However, in vivo eltoprazine provides combined stimulation of 5-HT_{1A} and 5-HT_{1B} receptors, producing maximal LID reduction (Carta et al., 2007; Munoz et al., 2008). Therefore, the antidyskinetic mechanisms recruited by combined stimulation of 5-HT_{1A} and 5-HT_{1B} receptors might be different from those evoked by stimulation of 5-HT_{1A} receptor only.

In fact, 5-HT_{1B} receptor agonists inhibit LID (Jackson et al., 2004; Zhang et al., 2008; Jaunarajs et al., 2009) without affecting striatal 5-HT or DA release (Kannari et al., 2001; De Groote et al., 2003), suggesting they act by interacting with up-regulated postsynaptic 5-HT_{1B} receptors on striato-nigral MSNs (Zhang et al., 2008; Riahi et al., 2013).

Accordingly, it was reported (Lindgren et al., 2010) that combinations of low doses of 5-HT_{1A} and 5-HT_{1B} agonists were as effective as combinations of medium doses in reducing LID, although the latter produced a 2-fold larger inhibition of striatal DA release than the former.

Finally, it was proven that 5-HT_{1A} agonists are capable to discriminate between presynaptic and postsynaptic receptors (Newman-Tancredi et al., 2009). It could be possible that low doses of eltoprazine selectively target postsynaptic 5-HT_{1A} receptors on cortical areas, without interacting with autoreceptors on raphe neurons.

The fact that eltoprazine 0.3 mg/Kg did not reduce striatal DA release does not rule out the possibility that higher dose do, and therefore we can speculate that, at higher doses, eltoprazine can act both through dopaminergic and non-dopaminergic mechanisms.

The final question is whether eltoprazine could provide clinical advantages over pure 5-HT_{1A} or 5-HT_{1B} agonists. Some preclinical studies have observed that the antidyskinetic effect of 5-HT_{1A} agonists is associated with a reduction of L-DOPA antiparkinsonian efficacy (Iravani et al., 2006; Dekundy et al., 2007; Gregoire et al., 2009). This was correlated with an excessive 5-HT_{1A} receptor stimulation leading to the “serotonergic syndrome” (Iravani et al., 2006; Lindenbach et al., 2015) or with blockade of postsynaptic D2 receptors (Millan et al., 1994). However, in a dose-finding study in humans, eltoprazine

mildly reduced dyskinesia without compromising the antiparkinsonian efficacy of L-DOPA (Svenningsson et al., 2015). This is encouraging since this study, albeit involving a small number of patients, had a crossover design with intraindividual control for placebo. In fact, when the 5-HT_{1A} agonists sarizotan was compared to placebo (Goetz et al., 2007), it showed negligible, if any, efficacy, in line with the finding that placebo produced antidyskinetic effects (Goetz et al., 2008).

The greater selectivity of eltoprazine, which does not bind D2 receptors (Millan et al., 1994), and its mechanism of action relying on the synergistic interaction between 5-HT_{1A} and 5-HT_{1B} receptors, may explain the better clinical profile of this compound in comparison with pure, and relatively unspecific, 5-HT_{1A} agonists. Nonetheless, it is not clear why eltoprazine (and other serotonergic agonists) are dramatically effective in rodents and nonhuman primates, but exert only mild effects in humans (15% maximal LID reduction, (Svenningsson et al., 2015). Several reasons may account for this discrepancy, among which a different role played by the serotonergic system in LID in humans with respect to other species and, more in general, the greater complexity of the human disease compared to experimental models.

In conclusion, the present study confirms the antidyskinetic effect of eltoprazine (Bezard et al., 2013; Svenningsson et al., 2015; Ghiglieri et al., 2016) and shows that its action relies on inhibition of L-DOPA-induced overactivation of striato-nigral MSNs (Mela et al., 2007; Marti et al., 2012; Mela et al., 2012). Neurochemical data suggest that $5HT_{1A}$ and $5HT_{1B}$ heteroreceptors are likely involved in this process.

Part 2

Rhes is a GTP-binding protein, largely expressed in striatum (Falk et al., 1999), where it can be detected in both striato-nigral and striato-pallidal MSNs (Errico et al., 2008; Ghiglieri et al., 2015; Sciamanna et al., 2015) as well as in cholinergic interneurons (Sciamanna et al., 2015).

Rhes exerts its action on receptor signaling through GPCR (Vargiu et al., 2004), and, in particular, it inhibits D1R-mediated cAMP accumulation (Harrison et al., 2011; Ghiglieri

et al., 2015) and reduces motor activation induced by amphetamine (Shahani et al., 2016; Vitucci et al., 2016).

However, these effects are not likely to subserve the antidyskinetic effect obtained by genetic removal of Rhes since an enhanced activity along the D1R signaling cascade would worsen and not impair LID (Errico et al., 2008; Ghiglieri et al., 2015).Therefore, the antidyskinetic effect can be related to the ability of Rhes to activate mTORC1, a downstream effector of D1R signaling cascade (Subramaniam et al., 2012). Indeed, in agreement with previous studies (Santini et al., 2009; Subramaniam et al., 2012) we reported 50% LID reduction in both $Rhes^{-/-}$ and rapamycin-treated mice.

In both cases, the antidyskinetic effect was accompanied by inhibition of the LID-associated nigral GABA and glutamate rise. Previous in vivo microdialysis studies in rats and mice have shown that the rise in nigral GABA associated with LID appearance is a neurochemical marker of striato-nigral MSNs activation in LID (thoroughly discussed in Section 1). Therefore, our neurochemical data provide in vivo evidence for the involvement of mTORC1 upregulation in striato-nigral MSNs in LID (Santini et al., 2009; Santini et al., 2010).

Moreover, confirming previous findings in dyskinetic rats (Dupre et al., 2011; Paolone et al., 2015), we found that LID is associated with an increase of striatal glutamate release. However, while both Rhes deletion and rapamycin inhibited the rise of GABA and glutamate in SNpr, only rapamycin prevented glutamate release in striatum, possibly suggesting that pharmacological blockade of mTORC1 and genetic deletion of Rhes do not have the same impact on BG circuitry.

The inhibition of striatal glutamate was associated with LID attenuation (Dupre et al., 2011; Ostock et al., 2011; Paolone et al., 2015), suggesting the activation of cortico-striatal glutamatergic transmission concurrently with LID appearance (Ahmed et al., 2011; Sgambato-Faure & Cenci, 2012; Bastide et al., 2015). Therefore, the antidyskinetic mechanism of rapamycin, which is associated with the prevention of striatal glutamate release, could be explained by inhibition of cortical mTORC1. Indeed, mTORC1 influences glutamate-related transmission and synaptic plasticity (Costa-Mattioli et al., 2009; Li et al., 2010; Weston et al., 2012). On the other hand, Rhes might not share this action due to its preferential striatal localization.

Interestingly, the lack of striatal glutamate inhibition came along with poor rotarod performance ON L-DOPA. Indeed, $Rhes^{-/-}$ mice, despite being less dyskinetic than rapamycin-treated mice, had a poor rotarod performance ON L-DOPA, showing the motor impairment typical of dyskinetic animals (Marti et al., 2012; Paolone et al., 2015), whereas rapamycin-treated mice did not.

This is at variance with a previous study showing no differences between $Rhes^{+/+}$ and $Rhes^{-/-}$ mice in forelimb usage in the cylinder test (Subramaniam et al., 2012). Such discrepancy might be explained by the fact that the rotarod test is more challenging than the cylinder test, or any other stepping test, and, therefore, perhaps more efficient in unravelling motor deficits ON L-DOPA. Indeed, when we performed the drag test in $Rhes^{+/+}$ and $Rhes^{-/-}$ mice ON L-DOPA we could not observe any difference in motor performance between genotypes (data not shown).

Thus, rotarod data seem to suggest that Rhes deletion, differently from rapamycin, attenuates LID but at the same time slightly compromises motor function. This could be due to a primary hypolocomotive effect or a reduction of the therapeutic effect of L-DOPA. Since we and others (Errico et al., 2008; Ghiglieri et al., 2015) showed that Rhes deletion did not cause primary hypolocomotion (but see (Pinna et al., 2016)), the possibility that Rhes interferes with the therapeutic response to L-DOPA should be considered.

For instance, we can speculate that lack of striatal glutamate inhibition might result in activation of the striato-pallidal MSNs, thereby reducing the therapeutic effect of L-DOPA. Furthermore, it has been reported that Rhes deletion causes the abnormal increase in firing rate of ChIs in response to D2R stimulation (Sciamanna et al., 2015). Since Ach activates M1R on striato-pallidal MSNs (Galarraga et al., 1999), it might cause the facilitation of indirect pathway. All these data prompt further investigations on the impact of Rhes deletion on the therapeutic effect of L-DOPA.

From a translational perspective, findings in preclinical models of PD related to the involvement of mTOR signaling in LID (Santini et al., 2009; Decressac & Bjorklund, 2013) have not been paralleled by post mortem studies in PD patients. Therefore, whether mTORC1 pathway is dysregulated in PD and LID is presently unknown. However, an impaired mTORC1 activity has been found in the brain of patients with Huntington's disease (Lee et al., 2015a), which encourages studies also in the human dyskinetic brain.

In conclusion we proved that Rhes deletion and mTORC1 blockade attenuated LID development in mice and prevented the LID-associated rise in GABA and glutamate release in SNpr, the basal ganglia output, confirming the pathogenic role of dysfunctional striatal mTORC1 activation along the aberrant D1 signaling cascade in MSNs (Santini et al., 2009; Santini et al., 2010). These data also confirm Rhes as an attractive target in LID therapy, prompting to the identification of pharmacological Rhes inhibitors as novel antidyskinetic drugs.

Part 3

The contribution of striatal ChIs to striatal circuitry and motor behaviour has long been recognized (for review see (Di Chiara et al., 1994; Calabresi et al., 2000; Pisani et al., 2007). Striatal ChIs profusely innervate both striato-pallidal and striato-nigral MSNs and modulate their activity directly and indirectly (Di Chiara et al., 1994; Galarraga et al., 1999). Muscarinic and nicotinic receptor subtypes are unevenly distributed on the different populations of striatal neurons and nerve terminals. Striato-pallidal MSNs mainly express M1Rs, while striato-nigral MSNs express both M1Rs and M4Rs (Calabresi et al., 1992; Hersch et al., 1994). A small subset of striatal neurons also express M3Rs (Hersch et al., 1994).

ChIs control MSNs activity also via presynaptic regulation of glutamate and DA release. A morphological studies showed that all mAChRs subtypes can be found as heteroreceptors in striatum (Hersch et al., 1994), predicting that they can be differently localized on cortico-striatal (M1R, M2R and M3R) (Sugita et al., 1991), thalamo-striatal (M2R and M3R) and subthalamo-striatal (M3R or M4R) terminals. Moreover, M5R is the only muscarinic subtype present on nigral DA neurons and nerve terminals (Vilaro et al., 1990; Weiner et al., 1990). Finally, both Gi/o coupled inhibitory M2R and M4R act as autoreceptors on striatal ChIs. Within this frame, the contribution of nicotinic receptors should also be acknowledged. Main nicotinic receptors in CNS are α7 homomers and β2-subunit containing heteromers, most commonly α4β2 and α6β2 receptors (Lukas et al., 1999). In the basal ganglia, the most frequent subtypes are α4β2 and α6β2 receptors (Lukas et al., 1999). These are usually presynaptically located, for instance on DA and glutamate

terminals, although nicotinic receptors can also be located postsynaptically. For instance, the role of nicotinic receptors located on striatal GABA interneurons in the cholinergic-inhibition of striatal MSNs has been recognized (Faust et al., 2016).

Recent studies proved the involvement of striatal ChIs in LID expression (Ding et al., 2011; Won et al., 2014; Bordia et al., 2016). Since striato-nigral MSNs play a crucial role in LID we focused on the role on M1Rs and M4Rs in a rat model of LID, using muscarinic preferential antagonists in combination with L-DOPA. As previously described (Method section), muscarinic antagonists were perfused locally through the microdialysis probe implanted in DA-depleted striatum, while L-DOPA was given systemically. This reverse microdialysis approach had the two-fold advantage of i) selectively targeting striatal mAChRs, ii) circumventing the issue of the poor selectivity of mAChRs antagonists.

In fact, local perfusion in the striatum, particularly in the dorsolateral sensori-motor portion, allows targeting only those receptors surrounding the probe. Therefore, although we cannot tell how far away from the probe the compounds can diffuse, we can certainly state that only striatal receptors were hit. Moreover, by perfusing the compounds through the probe, we can set their concentrations in the dialysate so to reach extracellular levels close to the affinity values for the receptors we intend to target. We are aware that these values are approximate since we just know the standard in vitro recovery of the probe (~10%) and do not know whether in vivo recovery will be the same. Nonetheless, this approach allows a more stringent control over extracellular level of the compounds administered, definitely more accurate that if the compounds were given systemically. We have validated this approach in a couple of studies. For instance, we proved that reverse dialysis of the D1 receptor antagonist SCH23390 in the DA-depleted striatum of dyskinetic rats was able to attenuate LID expression and prevent the accompanying rise of amino acids release in the ipsilateral SNpr, confirming the role of striatal D1-expressing MSNs in LID expression (Mela et al., 2012). Moreover, reverse dialysis of a delta opioid receptor antagonist in the SNpr, but not GP or striatum, was able to prevent the antiakinetic action (and its neurochemical correlates) of the delta opioid receptor agonist SNC-80 in 6-OHDA hemilesioned rats, pointing to intranigral delta receptors as the transducers of the antiparkinsonian action of delta opioid receptor agonists (Mabrouk et al., 2008).

Our data show that the inhibition of either striatal M1Rs or M4Rs alleviates LID expression along with the LID-associated rise of nigral GABA. Since several lines of evidence (reviewed and discussed in Part I) support the view that nigral GABA release is a

marker of striato-nigral MSNs activation in LID, these data suggest that both M1R and M4R stimulation contributes to LID expression and to the underlying activation of the direct striato-nigral pathway.

Our data are in line with studies showing that deletion of ChIs or blockade of muscarinic receptors via non-selective antagonists alleviate LID (Ding et al., 2011; Won et al., 2014; Bordia et al., 2016). However, they are in sharp contrast with a study showing that systemic administration of a M4R PAM (VU-0476406) attenuates LID expression in mice and NHPs, thereby suggesting that stimulation rather than blockade of M4R is beneficial for dyskinesia (Shen et al., 2015). Differences in animal species, drugs used and technical approach might explain this discrepancy. In fact, we should consider that we have specifically targeted striatal M4Rs via reverse microdialysis, using non-selective compounds.

Nonetheless, further studies are needed to clarify the role of M4R in the modulation of striatal circuitry. Indeed, although the role of M1Rs is generally agreed upon, that of M4Rs remains less explored and quite controversial. Thus, it is no surprise that M1Rs contribute to LID since these receptors are known to increase the excitability of striato-nigral MSNs (and striato-pallidal MSNs as well). Indeed, activation of $G_{q/11}$ coupled somatodendritic M1Rs on striatal MSNs determines the closure of Kir2 and Kv7 K^+ channels, and the reduction of the opening of Cav2 Ca^{2+} channels that activate those K^+ conductances. This causes MSNs to modestly depolarize and to become more sensitive to the glutamatergic inputs coming from the cerebral cortex (Goldberg et al., 2012).

Conversely, different and sometimes opposite actions have been attributed to postsynaptic M4Rs located on striato-nigral MSNs. Recently, an in-vitro study on acutely dissociated neurons has reported that M4Rs enhance striato-nigral MSNs excitability via opening of Cav1 Ca^{2+} channels (Hernandez-Flores et al., 2015). The specificity of this effect was verified using nicardipine, specific for L-type Ca^{2+} channels, and a snake toxin (mamba-toxin 3) highly specific for M4Rs (Hernandez-Flores et al., 2015). The conclusions of this study are in line with an in vitro study in atrial cells where M4Rs increase L-type Ca^{2+} conductances via pertussis toxin sensitive mechanisms (Pemberton et al., 1995).

Opposite to these studies, electrophysiology in the rat superior cervical ganglion has unravelled that M4Rs can inhibit N-type Ca^{2+} currents (Beech et al., 1991; Bernheim et al., 1991; Beech et al., 1992; Bernheim et al., 1992; Mathie et al., 1992; Howe et al., 1995). An

inhibitory action of M4R has also been reported in striatal neurons using a M4R positive allosteric modulator (VU-10010) (Shen et al., 2015). In this study, M4Rs increase LTD, inhibit D1-receptor induced LTP, and also attenuate NMDA-induced Ca^{2+} currents (Shen et al., 2015). In addition, a cross talk between M4R and D1R has been revealed in striato-nigral MSNs, where either positive or negative interaction can be induced changing the temporal order of receptor stimulation (Hernandez-Flores et al., 2015).

Therefore, the M4R are in a position to modulate the striato-nigral MSNs through multiple mechanisms that might be differently activated in the models used. To complicate the picture further, the role of ChIs on LID may vary depending on their activation state. Indeed, an optogenetic study in mice (Bordia et al., 2016) has shown that short pulses of laser stimulation of ChIs facilitate LID appearance, via muscarinic receptor stimulation, whether longer pulses inhibit it, via nAChRs desensitization (Bordia et al., 2016). We might therefore speculate that inhibition of M4R autoreceptors on ChIs further elevate the levels of striatal ACh, leading to the desensitization of nAChRs. To test this hypothesis, we could perfuse appropriate concentrations of a nAChRs antagonist along with a M4R antagonist in the striatum.

Striatal glutamate release may be considered another neurochemical marker of LID (Paolone et al., 2015; Brugnoli et al., 2016). In fact it is well known that dyskinesia is correlated with deep changes in cortico-striatal glutamatergic transmission, both at the presynaptic and postsynaptic levels (for reviews see (Chase et al., 2003; Picconi et al., 2003; Picconi et al., 2004; Sgambato-Faure & Cenci, 2012; Bastide et al., 2015). In keeping with previous microdialysis studies (Dupre et al., 2011; Ostock et al., 2011; Paolone et al., 2015; Brugnoli et al., 2016), elevation of striatal glutamate release accompanied LID appearance. Although no study has thoroughly investigated the neurobiological substrates of this neurochemical response so far, the rise of striatal glutamate is thought to reflect the increase of glutamate release from cortico-striatal terminals which is associated with motor activation (Sgambato-Faure & Cenci, 2012). In fact, pyramidal glutamate neurons project to the striatum and are the driving force of striatofugal neurons. In support of this theory, altered synaptic plasticity, namely loss of synaptic depotentiation, has been observed at cortico-striatal terminals (Picconi et al., 2003). Moreover, inhibition of pyramidal neurons (with 5-HT_{1A} agonist) attenuated both the rise of striatal glutamate and the expression of LID (Ostock et al., 2011). Therefore, the finding that telenzepine and M4R preferential antagonists inhibited the LID-associated

nigral GABA along with striatal glutamate release is consistent with the view that striatal M1R and M4R prevents overactivation of nigro-striatal MSNs and, therefore, the cortico-basal ganglia-thalamo-cortical loop. This is consistent with the effects of eltoprazine in dyskinetic rats (Paolone et al., 2015) and rapamycin in dyskinetic mice (Brugnoli et al., 2016) (Part 2).

Although exerting similar effects on nigral GABA and striatal glutamate, however, telenzepine and PD-102807 or tropicamide differed in their ability to modulate nigral glutamate release. In fact, in contrast to telenzepine, M4R preferential antagonists prevented the L-DOPA-induced nigral glutamate release, suggesting that M1Rs and M4Rs differentially regulate striatal circuitry. As a tentative explanation, it has been shown that both telenzepine and tropicamide improve parkinsonian motor deficits in mice, although telenzepine would act along the indirect pathway whereas tropicamide along the direct one (Ztaou et al., 2016).

In conclusion, using reverse dialysis we evidenced that endogenous ACh overactivates striato-nigral MSNs in LID, through striatal M1Rs and M4Rs. Even though our experimental approach employing reverse dialysis in combination with L-DOPA was previously validated (Mela et al., 2012), the selectivity of M4R antagonists used in the present study still remains an issue. Data need to be replicated using more selective pharmacological or genetic tools, such as D1-M4R knockout mice (Ztaou et al., 2016).

CONCLUSIONS

In vivo microdialysis combined with behavioural scoring allowed revealing the neurochemical fingerprints of LID in rats and mice, and investigating the impact of antidyskinetic treatments on BG circuitry. Despite LID is a complex phenomenon, involving several neurotransmitter systems, we were able to show that LID is associated with common neurochemical changes in rats and mice such as the rise of nigral GABA and glutamate, as well as the rise of striatal glutamate, suggesting LID is sustained by similar pathological mechanisms in both species.

Different antidyskinetic compounds or strategies were analysed in this study, which are targeted on 5-HT_{1A}/5-HT_{1B} receptors, the Rhes/mTORC1 complex, and striatal M1Rs and M4Rs. We showed the antidyskinetic efficacy of the mixed 5-HT_{1A}/5-HT_{1B} agonist eltoprazine, and the mTORC1 inhibitor rapamycin, further suggesting that blockade of striatal M1Rs and M4Rs also palliates LID. Apart from the role of striatal M4Rs, which needs to be further explored, these behavioural data are in line with the literature. In addition, we showed that all these treatments, despite involving different targets, modified in similar way the LID-associated changes in BG circuitry, essentially preventing the rise in nigral GABA and striatal glutamate release. These data endorse the view that rise in nigral GABA and striatal glutamate release can be considered neurochemical correlates of LID, further indicating that all these antidyskinetic strategies impinge on a common final pathway or mechanisms of action. Further studies are needed to thoroughly investigate the source and physiopathological role of the LID-induced rise of striatal striatal glutamate. Conversely, also taking into account previous microdialysis studies and their physiopathological relevance, the present microdialysis study strongly indicates that the antidyskinetic strategies investigated share the ability of preventing LID along with the overactivation of striato-nigral MSNs, providing an in vivo evidence for the crucial role of the direct pathway in this motor disorder.

ABBREVIATIONS

PD = Parkinson’s disease
LID = Levodopa-induced dyskinesia
DA = dopamine
L-DOPA = 3,4-dihydroxy-L-phenylalanine
6-OHDA = 6-hydroxydopamine
MFB = medial forebrain bundle
AIMs = abnormal involuntary movements
BG = basal ganglia
MSNs = medium spiny neurons
GABA = γ-aminobutyric acid
GLU = glutamate
SNpc = substantia nigra pars compacta
SNpr = substantia nigra pars reticulate
GP = globus pallidus
STN = subthalamus
PKA = protein kinase A
mTORC1 = mammaliam target of rapamycin complex 1
Rhes = Ras homolog enriched in the striatum
ERK 1/2 = extracellular signal–regulated kinase
ACh = acetylcholine
ChIs = cholinergic interneurons
mAChRs = muscarinic receptors
nAChRs = nicotinic receptors

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