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MOLECULAR BASIS OF L-DOPA-INDUCED DYSKINESIA: STUDIES ON STRIATAL SIGNALING

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Cover: Triple labeling of medium spiny neurons in the striatum of a 6-OHDA-lesioned *Drd2*-EGFP transgenic mouse treated with L-DOPA. Blue immunofluorescence corresponds to DARPP-32, red to phospho-ERK and green to D2-receptor-expressing neurons (courtesy of Dr. Emmanuel Valjent, Karolinska Institutet)

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ABSTRACT

Parkinson's disease (PD) is a neurological disorder characterized by tremor, rigidity and bradykinesia. PD is caused by selective degeneration of the dopaminergic neurons, which originate in the substantia nigra pars compacta (SNc) and project to the striatum. Parkinsonian patients are treated with L-3,4-dihydroxyphenylalanine (L-DOPA), which effectively counteracts the disease by restoring dopamine (DA) transmission in the striatum. However, the use of L-DOPA is complicated by the appearance of severe motor side effects, known as L-DOPA-induced dyskinesia (LID), which represent one of the major challenges to the existing therapy for PD. The goal of this thesis is to identify molecular mechanisms involved in LID. Work has been centered on the medium spiny neurons (MSNs) of the striatum, which are the main target of L-DOPA.
In Paper I, we examined the involvement of the DA- and cAMP-dependent phosphoprotein of 32 KDa (DARPP-32) in LID. We found that genetic inactivation of DARPP-32, which leads to attenuation of cAMP signaling in MSNs, reduced dyskinesia. We also found that, in dyskinetic mice, increased cAMP-dependent protein kinase/DARPP-32 signaling participates to the activation of the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). Increased ERK1/2 phosphorylation associated with dyskinesia was paralleled by activation of mitogen- and stress-activated kinase 1 (MSK1), phosphorylation of histone H3 and increased expression of cFos. Finally, we demonstrated that inactivation of ERK1/2, achieved using SL327 (α-[amino[(4-aminophenyl)thio]methylene]-2 (trifluoromethyl)benzeneacetonitrile), reduced LID. These results indicate that a significant proportion of the abnormal involuntary movements developed in response to chronic L-DOPA are attributable to hyper-activation, in striatal MSNs, of a signaling pathway including phosphorylation of DARPP-32, ERK1/2, MSK1 and histone H3.
In Paper II, we identified the specific population of striatal MSNs affected by LID. For this purpose, we employed mice expressing enhanced green fluorescent protein (EGFP) under the control of the promoters for the dopamine D1 receptor (D1R; *Drd1a*-EGFP mice), or the dopamine D2 receptor (D2R; *Drd2*-EGFP mice), which are expressed in striatonigral and striatopallidal MSNs, respectively. We found that, in the DA depleted striatum, L-DOPA increased phosphorylation of ERK1/2, MSK1 and histone H3 in striatonigral MSNs. The effect of L-DOPA was prevented by blockade of dopamine D1Rs. The same pattern of protein phosphorylation was observed, after repeated administration of L-DOPA, in dyskinetic mice.
The ERK signaling cascade can influence the activity of the mammalian target of rapamycin (mTOR) signaling pathway, which is involved in the regulation of mRNA translation. In Paper III we investigated the involvement of the mTOR complex I (mTORC1) in PD and LID. We found that, in the DA depleted striatum, administration of L-DOPA resulted in D1R-mediated activation of mTORC1. This response occurred selectively in striatonigral MSNs and was associated with LID. Most importantly, administration of rapamycin, an inhibitor of mTORC1, reduced LID without affecting the antiparkinsonian efficacy of L-DOPA. Thus, the mTORC1 signaling cascade may represent a novel target for anti-dyskinetic therapies.

LIST OF PUBLICATIONS

I. Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Hervé D, Greengard P, Fisone G. (2007) Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesa. *J. Neurosci*. 27: 6995-7005

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III. Santini E, Heiman M, Greengard P, Valjent E, Fisone G. (2009) Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA-induced dyskinesia. *Sci. Signal.* 2: ra36

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LIST OF ABBREVIATIONS

4E-BP	Initiation factor 4E-binding protein
6-OHDA	6-hydroxydopamine
AC	Adenylyl cyclase
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ATP	Adenosine-5′-triphosphate
BAC	Bacterial artificial chromosome
CalDAG-GEFs	Ras guanyl releasing proteins (calcium and DAG-regulated)
cAMP	Cyclic adenosine monophosphate
CK2	Casein kinase 2
CRE	cAMP response element
CREB	cAMP response element binding protein
D1R	Dopamine D1 receptor
D2R	Dopamine D2 receptor
D3R	Dopamine D3 receptor
D4R	Dopamine D4 receptor
D5R	Dopamine D5 receptor
DA	Dopamine
DAG	Diacylglycerol
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein of 32 kDa
DAT	Dopamine transporter
DRs	Dopamine receptors
EGFP	Enhanced green fluorescent protein
eIFs	Eucaryotic initiation factors
EPAC1/2	Exchange protein activated by cAMP 1/2
ERK1/2	Extracellular signal-regulated kinase 1 and 2
FKBP12	FK506 binding protein 12
FRAP1	FK506 bing protein 12-rapamycin associated protein 1
FRB domain	FKBP12-rapamycin binding domain
GABA	γ-Aminobutyric acid
GAD	Glutamate decarboxylase
GAPs	GTPase activating proteins
GDP	Guanosine diphosphate

GEFs	Guanine nucleotide exchange factors
GPe/GP	Globus pallidus pars externa
GPi/mGP	Globus pallidus pars interna
GPCRs	G protein-coupled receptors
GTP	Guanosine-5′-triphosphate
HFS	High frequency stimulation
IEGs	Immediate early genes
IRES	Internal ribosomal entry site
KO	Knock-out
L-DOPA	L-3,4-dihydroxyphenylalanine
LID	L-DOPA-induced dyskinesia
LTD	Long term depression
LTP	Long term potentiation
MAGUK	Membrane-associated guanylate kinase
MAO	Monoamine oxidases
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MFB	Medial forebrain bundle
Mnk1/2	MAPK-interacting serine/threonine kinases 1 and 2
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated protein kinase 1
MSNs	Medium spiny neurons
mTOR	Mammalian target of rapamycin
mTORC1/2	mTOR complex 1 and 2
NA	Noradrenaline
NET	Noradrenaline transporter
NMDAR	N-methyl-D-aspartic acid (NMDA) receptor
PABP	Poly(A)-binding protein
PD	Parkinson’s disease
PDK-1	3-phosphoinositide dependent protein kinase-1
PI3K	Phosphoinositide 3-kinase
PIKKs	Phosphoinositide 3-kinase-related kinases
PKA	cAMP-dependent protein kinase A
PP-1	Protein phosphatase 1

PP-2A	Protein phosphatase 2A
Ras-GRF1	Ras protein-specific guanine nucleotide-releasing factor 1
Ras-GRPs	Ras-guanyl nucleotide releasing proteins
Rheb	Ras homolog enriched in brain
ROS	Reactive oxygen species
RRA	Retrorubral area
S6K (p70S6K)	p70 ribosomal protein kinase
S6rp (S6)	S6 ribosomal protein
SL327	(α-[amino[(4-aminophenyl)thio]methylene]-2 (trifluoromethyl)benzeneacetonitrile)
SNc	Substantia nigra pars compacta
SNr	Sustantia nigra pars reticulata
STEP	Striatal-enriched tyrosine phosphatase
STN	Subthalamic nucleus
TOP	Terminal oligopyrimidine
TSC	Tuberous sclerosis complex
VTA	Ventral tegmental area
WT	Wild type

TO MY FATHER

1 INTRODUCTION

1.1 BASAL GANGLIA: FUNCTIONAL-ANATOMICAL ORGANIZATION

The term basal ganglia refers to a group of subcortical nuclei that include the striatum, globus pallidus (GP), substantia nigra (SN) and subthalamic nucleus (STN)[1]. Together, these interconnected nuclei process motor, limbic, sensory and associative information coming from virtually all areas of the cerebral cortex and return the processed information to the same cortical regions. The overall function of the basal ganglia is to control the initiation and selection of voluntary movements.

The information from cortex is preferentially received in the striatum, the main recipient nucleus of the basal ganglia. From the striatum the information is transmitted to the output nuclei, the mGP and the SNr. These output nuclei project to the ventral thalamus and then back to those cortical areas providing the initial inputs to the circuit [cortical-basal ganglia-thalamocortical loops, (Alexander et al., 1986)]. In addition, they also project to subcortical regions such as the superior colliculus, the pedunculopontine nucleus and the reticular formation (Albin et al., 1989; DeLong, 1990; Groenewegen et al., 1990; Smith and Bolam, 1990; Smith et al., 1998).

The projections within the basal ganglia circuit are inhibitory. Indeed both striatal projection neurons and mGP/SNr neurons are GABAergic. Whereas the striatal neurons are quiescent under resting conditions, the mGP/ SNr neurons have high discharge rate and tonically inhibit the targets of the basal ganglia, i.e. neurons in the ventral thalamus or subcortical regions. In this way, basal ganglia associated behaviors are produced by modulating the inhibition of mGP and SNr output signals [Figure 1, (Albin et al., 1989; Chevalier and Deniau, 1990; DeLong, 1990)].

The excitatory projections to the striatum are topographically organized so that the striatum can be divided into functionally different territories. Thus, whereas the dorsolateral striatum receives somatotopically organized sensorimotor information from motor, premotor and sensory cortical areas (Brown et al., 1998), the most

[1] The striatum is a single nucleus in rodents but is divided by the internal capsule into caudate nucleus and putamen in higher vertebrates. The GP consists of two major parts, the external (GPe) and the internal segment (GPi). In rodents, the GPe is referred to as the GP and the GPi is equivalent (in terms of inputs and outputs) to the entopeduncular nucleus (EP). As The Mouse brain in Stereotaxic Coordinates of Paxinos and Franklin (2001) uses the term medial globus pallidus (mGP) to refer to the EP, this terminology

ventromedial part of the striatum collects viscerolimbic cortical afferents. The striatal areas between these extremes receive information from higher associative cortical areas (McGeorge and Faull, 1989; Berendse et al., 1992). The above description refers in particular to the topographic organization of the corticostriatal projections; however, also the thalamic and limbic afferents match this functional-anatomical organization (Groenewegen, 2003; Voorn et al., 2004).

The striatum is not only the major recipient nucleus of the basal ganglia, but also the area in which different information are integrated and processed. Integration of information at the striatal level is produced by convergence of excitatory inputs, such as cortical, thalamic and limbic, onto striatal neurons. Because of their electrophysiological membrane properties striatal projection neurons are difficult to excite and need strong convergent excitatory inputs to become active. Therefore, each specific neuronal population is activated as a result of spatial and temporal coincidence of excitatory afferents (Flaherty and Graybiel, 1991; Pennartz et al., 1994). The excitatory inputs to the striatal projection neurons are modulated by many other inputs, including those from extrinsic afferents [e.g. dopaminergic (DAergic) transmission] and from local interneurons (e.g. cholinergic transmission) (Smith and Bolam, 1990; Kawaguchi, 1997).

1.2 NEURONAL SUBTYPES IN THE STRIATUM: FOCUS ON THE MSNs

The principal neuronal cell type of the striatum is the GABAergic medium spiny projection neuron (MSNs), which accounts for 95% of the entire striatal neuronal population. The MSNs represent both the major receiving neurons and the major projecting neurons of the striatum. The remaining striatal neurons are aspiny interneurons, important to synchronize the activity of the MSNs. These interneurons consist of a variety of morphologically and neurochemically defined types. Briefly, they can be distinguished in large aspiny neurons, which use acetylcholine as a transmitter, and medium aspiny neurons, which use GABA as a transmitter and which can be further classified based on the specific expression of parvalbumin, somatostatin and calretinin. (Kawaguchi et al., 1990; Kawaguchi et al., 1995; Kawaguchi, 1997).

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is used in this thesis (Paxinos and Franklin, 2001). Similarly, the SN consists of two sub-nuclei, the pars compacta (SNc) and the pars reticulata (SNr).

The MSNs are distributed in such a way that the striatum lacks distinct cytoarchitectural organization as compared to other brain regions i.e. the hippocampus, or the cerebral cortex. Nevertheless, the MSNs constitute two subpopulations of approximately equal number, which can be distinguished on the basis of their projection targets and the selective expression of receptors and neuropeptides (Gerfen et al., 1990).

MSNs giving rise to the striatonigral pathway make a direct synaptic contact with the neurons of the output structures of the basal ganglia, mGP and SNr, and selectively express the D1 dopamine receptor (D1R), as well as substance P and dynorphin. Upon activation produced by firing of corticostriatal glutamatergic neurons, striatal MSNs discharge and release GABA, thereby inhibiting neurons in the SNr and mGP. This reduction in firing of mGP and SNr neurons leads to disinhibition of thalamo-cortical projection neurons. In contrast, the MSNs giving rise to the indirect striatopallidal pathway make synaptic contacts with neurons of the GP and selectively express the D2 dopamine receptor (D2R) and enkephalin. GP neurons contact neurons of the STN, which in turn innervate neurons of mGP and SNr. Moreover, the GP neurons can directly contact the output neurons of the basal ganglia. Activation of the MSNs of the indirect pathway leads to inhibition of the neurons of the GP, which are also GABAergic. This, in turn, leads to increased firing of output neurons (i.e. mGP and SNr neurons) by two mechanisms. First, the loss of inhibitory input to the excitatory neurons of the STN, results in increased activity and hence increased excitation of the output neurons. Secondly, the inhibition of GP neurons has a direct disinhibitory effect on the output neurons. The increased firing of mGP and SNr neurons ultimately leads to an increased inhibition of thalamo-cortical projection neurons [Figure 1, (Smith and Bolam, 1990; Gerfen, 1992b; Bolam et al., 2000)].

The release of dopamine (DA) from midbrain dopaminergic neurons into the striatum has been proposed to play an important role in integrating functionally different streams of information that ultimately influence behavioral output (Haber et al., 2000). At the level of the MSNs, DA exerts a modulatory control on the excitatory signals such as cortical and thalamic glutamatergic signals. Indeed, the MSNs are the anatomical place where the functional interaction between glutamate and DA takes place. In particular, it seems that the dendritic spines of the MSNs are the recipient structures of both corticostriatal and nigrostriatal inputs (Bouyer et al., 1984; Freund et al., 1984; Smith et al., 1994). It has been shown that the axon

terminals of corticostriatal projections form asymmetric specialization with the heads of dendritic spines of MSNs (Somogyi et al., 1981). On the other hand, DA axons of the nigrostriatal pathway form symmetric synaptic contacts mainly with the necks of the dendritic spines of MSNs (Bouyer et al., 1984; Freund et al., 1984; Smith et al., 1994; Hanley and Bolam, 1997). Thus, coincident dopaminergic and glutamatergic inputs into the striatum are important to induce behavioral outputs, i.e. to reinforce appropriate actions and to repress irrelevant actions (Arbuthnott and Wickens, 2007).

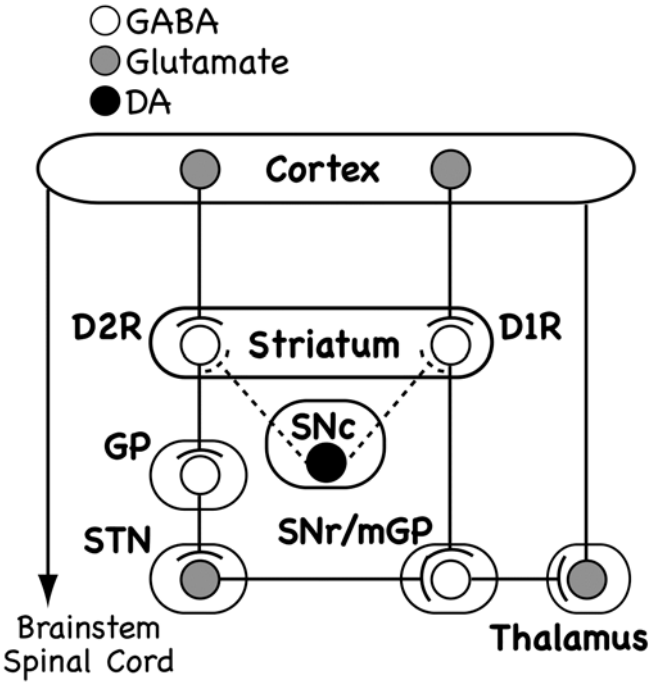

Figure 1. Diagram illustrating the general organization of the basal ganglia. The GABAergic (white) medium spiny neurons of the striatum innervate either directly, or indirectly [via GP (GPe, see footnote[1]) and STN], the SNr and mGP (GPi, see footnote[1]). These two groups of neurons are distinguished based on their ability to express D1Rs, or D2Rs (cf. text). Glutamatergic and dopaminergic neurons are shown in grey and black, respectively. DAergic projections from the SNc to the striatum (nigrostriatal pathway) are represented with dashed lines, these fibers (and the SNc DAergic neurons) degenerate selectively in Parkinson's disease. In my PhD project, I have identified alterations in signaling induced by dopamine depletion (a prominent feature of Parkinson's disease) at the level of the medium spiny neurons of the direct and indirect pathway. Furthermore, I have examined the role played by these changes in the development of L-DOPA-induced dyskinesia.

1.3 NEUROANATOMY OF THE RODENT DA SYSTEM

The neurotransmitter DA is involved in a wide range of physiological processes, such as motor behavior, cognition, emotion, food intake and endocrine regulation. Dysfunctions of the DAergic system are associated with a variety of disabling neurological diseases.

In rodents, the midbrain DA system is divided in 3 groups with distinct anatomical localization and topographical projection to the striatum and other brain regions. The A10 cell group is located in the ventral tegmental area (VTA) and projects to limbic forebrain areas, i.e. septal area, prefrontal cortex, olfactory tubercle and the nucleus accumbens (ventral striatum). The A9 and A8 cell groups are situated in the SNc and the retrorubral area (RRA), respectively, and provide all the remaining DA projections to the striatum (Smith and Kieval, 2000).

A different subdivision of midbrain DA neurons has also been suggested based on the morphology of neuronal dendrites, the expression of the calcium-binding protein, calbindin and the projection to either the patch or the matrix striatal compartments[2]. Using these markers, the striatal projecting DA neurons are distinguished in two sets, dorsal and ventral tier. Briefly, the dorsal tier DA neurons provide inputs to the striatal matrix compartment and include VTA, RRA and dorsal neurons in the SNc. While, the ventral tier DA neurons innervate the striatal patch compartments and include ventral SNc neurons and DA neurons located in the SNr (mainly in its ventral and caudal parts) (Wassef et al., 1981; Gerfen et al., 1985; Gerfen et al., 1987a; Gerfen et al., 1987b; Gerfen, 1992a; Gerfen, 1992b).

[2] The patch-matrix organization is another way to divide the striatum in functional-anatomical compartments (Gerfen, 1992a). The patches are islands of MSNs that express high levels of μ-opioid receptors, surrounded by matrix MSNs, which contain calbindin and somatostatin (Graybiel, 1990).

1.4 STRIATAL ACTIONS OF DA: FOCUS ON MSNs

1.4.1 Dopamine receptors and second messengers

In the striatum, the physiological actions of DA are mediated via its interaction with DRs expressed on MSNs[3]. DRs are a class of metabotropic receptors, characterized by seven transmembrane domains, coupled to heterotrimeric GTP binding proteins (G protein-coupled receptor, GPCRs). DRs have been divided into two different classes, D1-type (D1R and D5R) and D2-type (D2R, D3R, D4R), based on their ability to stimulate or inhibit the production of cAMP, respectively (Missale et al., 1998). D1-type receptors comprise D1Rs, which are expressed in the striatum by striatonigral MSNs and D5Rs, which are poorly expressed by striatal MSNs but are present in cholinergic interneurons. D2-type receptors comprise D2R, D3R and D4R. In the striatum, D2R are expressed by striatopallidal MSNs, nigrostriatal fibers originating from DAergic neurons situated in the SNc, cholinergic and parvalbumin-positive interneurons. D3Rs have a specific distribution in the ventral striatum but they are poorly expressed in the dorsal striatum. D4Rs are expressed in the striatum at very low levels (Kawaguchi et al., 1995; Missale et al., 1998).

The ability of D1- and D2-type receptors to exert an opposite regulation of cAMP signaling depends on their selective interaction with specific G proteins composed of different combinations of α, β and γ subunits. Binding of DA to D1R results in the activation of a G protein subunit (αs) able to stimulate the enzyme adenylyl cyclase (AC) and consequently to increase the production of cAMP [Figure 2, (Kebabian and Calne, 1979)]. Increased cAMP synthesis leads to activation of cAMP-dependent protein kinase (PKA), through dissociation of the regulatory and catalytic subunits. In its active state the catalytic subunit of PKA can bind ATP and can phosphorylate, in the cytoplasm and the nucleus, proteins that contain the appropriate consensus sequence (Fimia and Sassone-Corsi, 2001). Conversely, the interaction of dopamine with D2Rs leads to activation of G protein subunits (αi/o) that inhibit AC. Inhibition of cAMP synthesis is reflected in a reduction of the phosphorylation of downstream proteins targeted by PKA (Robinson and Caron, 1997).

[3] DRs are present also in other neuron types of the striatum (see below), for instance cholinergic interneurons express D2Rs. However, I will limit the discussion to striatal MSNs.

Striatal MSNs express high levels of a particular Gαs subunit, originally described in the olfactory epithelium, named Gαolf [Golf, (Jones and Reed, 1989; Hervé et al., 1993)] and a specific isoform of AC named AC5 (Glatt and Snyder, 1993; Mons and Cooper, 1994). Moreover MSNs are enriched in the RIIb isoform of the regulatory subunit of PKA (Cadd and McKnight, 1989). The importance of Golf, AC5 and RIIb in dopamine signaling is indicated by studies in knock out (KO) mice (Corvol et al., 2001; Lee et al., 2002; Iwamoto et al., 2003; Kim et al., 2008). For instance, the ability of dopamine to induce cAMP synthesis is inhibited in Golf KO mice (Corvol et al., 2001). Moreover, the same mice have severely impaired biochemical and behavioral responses to dopaminergic agonists and psychostimulants (Zhuang et al., 2000; Brami-Cherrier et al., 2005).

1.4.2 Physiological effects

A large proportion of the effects exerted by DA on MSNs occur by changing the way in which MSNs respond to glutamatergic signals. The binding of DA to DRs excites or inhibits MSNs by modulating the gating and the trafficking of voltage-dependent and ionotropic channels (see below). Thus, changes in membrane excitability induced by DA affect the probability that a MSN will fire an action potential in response to excitatory stimuli. It has been shown that in vivo the membrane potential of a MSN shifts between a hyperpolarized “down state”, close to the resting potential (Jiang and North, 1991), and a depolarized “up state”, close to action potential threshold (Wilson and Kawaguchi, 1996; Reynolds and Wickens, 2000). DA acting on D1Rs increases the responsiveness of striatonigral neurons to sustained release of glutamate, generating depolarized “up states”. Conversely, DA acting on D2Rs decreases the excitability of striatopallidal neurons and their response to glutamatergic input, thereby reducing depolarized “up states”.

Yet another model has been proposed to explain the role of DA as a neuromodulator of synaptic plasticity at corticostriatal synapses (Reynolds and Wickens, 2002). This model take into account the hypothesis that DA can reinforce, thus, increase the likelihood of a certain behavior being repeated, by providing a rewarding signal to the action/behavior that was performed. Thus, it has been shown that primary rewards and reward-predicting stimuli induce phasic activation of midbrain DA neurons (Schultz, 1998, 2002). At the level of corticostriatal synapses, a combination of presynaptic activity and postsynaptic depolarization in association

with a large phasic increase in DA concentration will result in potentiation of the corticostriatal synapse. In contrast, in the absence of a rewarding DAergic signal, the same combined activity will result in depression of corticostriatal synapse. (Reynolds and Wickens, 2002; Wickens et al., 2007).

In a recent study Shen et al. (2008) have shown that DA can affect corticostriatal plasticity differently in the MSNs of the direct and indirect pathway (Shen et al., 2008). They propose that, in the absence of stimuli, DAergic neurons spike autonomously to maintain striatal DA concentration at levels sufficient to keep active D2Rs, which have high affinity for DA. This will allow plasticity only in MSNs of the indirect pathway. In the MSNs of the direct pathway, D1Rs, which have lower affinity for DA, will be marginally activated, thus permitting only long term depression (LTD). However, following activation of DAergic neurons, striatal DA levels will rise transiently and will activate D1Rs, thus allowing the induction of long term potentiation (LTP) in the MSNs of the direct pathway (Shen et al., 2008).

1.5 THE DA DEPLETED STRIATUM: LESSONS FROM EXPERIMENTAL PARKINSON'S DISEASE MODELS

The main symptoms of Parkinson's disease (PD) are severe motor impairments, such as akinesia, rigidity, bradykinesia and tremor. All these symptoms are a consequence of the loss of DAergic neurons in the SNc, which is the main neuropathological feature of PD. Studies in experimental models of PD, in which DA neurons are selectively damaged by neurotoxins, such as the 6-hydroxydopamine (6-OHDA), or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (see Methodological discussion), have shown a wide range of changes affecting the striatum after DA depletion. These changes include alterations in gene expression, electrophysiological properties and morphological features of MSNs. Importantly, recent studies have shown that MSNs of the direct and indirect pathway are differentially affected by loss of DA innervations.

In the DA depleted striatum, several genes encoding proteins selectively expressed in striatopallidal MSNs are increased. These genes include *enkephalin* (Mocchetti et al., 1985; Gerfen et al., 1991; Morissette et al., 1999; Marin et al., 2007), *D2R* (Herrero et al., 1996; Betarbet and Greenamyre, 2004) and *glutamic acid decarboxylase* (*GAD67*) (Soghomonian and Chesselet, 1992; Carta et al., 2002; Katz et al., 2005). In contrast, markers of cellular activity in the striatonigral MSNs such as

mRNA expression of *preprotachykinin*, the gene precursor of the neuropeptide substance P and dynorphin are decreased after DA depletion (Gerfen et al., 1990; Herrero et al., 1996; Morissette et al., 1999; Kreitzer and Malenka, 2007; Marin et al., 2007). Whereas the level of the G-protein coupled to D1R is increased following DA depletion (Corvol, 2004), the levels of the *D1R* itself have been described as decreased or unchanged (Gerfen et al., 1990; Shinotoh et al., 1993; Turjanski et al., 1997; Hurley et al., 2001). Importantly, a decreased concentration of substance P has also been described in the SNr and GPi of PD patients (Waters et al., 1988).

DA depletion profoundly affects the electrical properties of the MSNs. Experiments performed in anaesthetized 6-OHDA-lesioned rats show that DA depletion inhibits the spontaneous activity of MSNs of the direct pathway, and enhances the activity of the MSN of the indirect pathway (Mallet et al., 2006). The subpopulation of cortical neurons that project to striatonigral MSNs show a reduction in firing rates, whereas the activity of corticostriatal neurons projecting to striatopallidal MSNs is unchanged (Mallet et al., 2006). In striatal slices, however, it was shown that the cortical terminals projecting to the MSNs of the indirect pathway are more likely to release glutamate and activate their target neurons (Kreitzer and Malenka, 2007).

DA depletion also impairs the control of several forms of synaptic plasticity in the striatum. In fact, LTD and LTP induced in MSNs by cortical high frequency stimulation (HFS) are prevented after 6-OHDA lesions (Calabresi et al., 1992a; Calabresi et al., 1992b). A recent study showed that the absence of DA differentially affects the induction of plasticity at the corticostriatal synapses of direct and indirect MSNs. In DA depleted striatum, LTP is not induced in striatonigral MSNs whereas LTD is not induced in striatopallidal MSNs (Shen et al., 2008).

DA depletion has also been found to alter the spine morphology of MSNs. In particular, loss of glutamatergic synapses and spines, and shrinkage of dendritic trees has been described in striatopallidal MSNs. In contrast, the absence of DA has no discernible morphological or physiological effects on synaptic function in striatonigral MSNs (Day et al., 2006). A recent study in MPTP treated non-human primates confirms a loss of dendritic spines, particularly in the motor region of the putamen, but does not demonstrate a differential reduction between D1- and D2-expressing MSNs (Obeso et al., 2008). Importantly, spine and glutamatergic synapse loss has also been described in post mortem striatal tissue from PD patients (McNeill et al., 1988; Zaja-Milatovic et al., 2005).

The effects of DA depletion on the activity of striatal interneurons are somewhat controversial. In vitro experiments showed that fast spiking GABAergic interneurons become less excitable after DA depletion (Fino et al., 2007). However, in vivo recordings from DA depleted rats show that these neurons do not change their firing rate in response to cortical stimulation (Cho et al., 2002). Moreover, it has been found that DA depletion is associated with increased excitability of cholinergic interneurons and inhibition of GABA interneurons. These changes may result in impaired corticostriatal transmission and reduced feed-forward intrastriatal inhibition.

To conclude, the overall effect of DA depletion appears to result in unbalanced corticostriatal transmission, which leads to enhanced activation of GABAergic striatopallidal MSNs and excessive inhibition of neurons in the GPe. Together, these findings may be directly related to the difficulty that PD patients have in initiating and selecting voluntary movements.

1.6 L-DOPA INDUCED DYSKINESIA: EVIDENCE FOR A HYPERSENSITIVITY OF THE STRIATONIGRAL PATHWAY

The primary therapeutic strategy for the treatment of PD is based on the administration of L-3,4-dihydroxyphenylalanine (L-DOPA), the direct metabolic precursor of DA (Oertel and Quinn, 1997). At the beginning of the therapy, L-DOPA is effective and improves the motor symptoms of patients with PD considerably. However, within few years, DA replacement therapy induces unwanted, debilitating, involuntary movements known as L-DOPA-induced dyskinesia (LID). It has been reported that, in PD patients, the onset of LID occurs in parallel to the rise of L-DOPA concentration in the ventricular cerebrospinal fluid (Olanow et al., 1991). Indeed, LID is usually most severe during the two hours that follow the administration of the drug and which correspond to the peak level of L-DOPA in the brain (Nutt, 1990). Epidemiological studies have pointed out that the risk of developing LID over time is dependent on many factors, including age of disease onset, disease severity (i.e., the extent of putaminal DA denervation), duration of the therapy and treatment regimen (Friedman, 1985a; Friedman, 1985b; Schrag and Quinn, 2000).

Studies with experimental models of LID (see Methodological discussion), indicate that the ability of the striatum to store, release and clear exogenous DA is greatly impaired after severe degeneration of nigrostriatal fibers. The lack of DAergic

terminals, containing the D2 autoreceptor, DA transporter (DAT) and monoamine oxidase A (MAO-A) enzyme, leads to an abnormally large increase in extracellular DA following parenteral L-DOPA administration (Wachtel and Abercrombie, 1994; Arai et al., 1995; Tanaka et al., 1999). In the absence of DAergic neurons, L-DOPA is decarboxylated and DA is released in an uncontrolled manner by serotoninergic terminals, which project from the midbrain raphe nuclei to the striatum (Arai et al., 1995; Tanaka et al., 1999). These terminals have been shown to increase following 6-OHDA lesion (Guerra et al., 1997; Maeda et al., 2005; Carta et al., 2006) and administration of agonists acting at inhibitory serotonin autoreceptors, or depletion of serotonin, strongly reduce the expression of LID (Carta et al., 2007).

The fluctuation in the levels of extracellular DA produced by L-DOPA and occurring in advanced PD causes a non-physiological condition that affects striatal MSNs. Thus, in 6-OHDA lesioned rats, LID correlates with changes in gene expression at the level of MSNs (Lee et al., 2000; Cenci; Winkler et al., 2002). Indeed, accumulating evidence indicate that aberrant responses of MSNs are an important determinant in the development of LID.

Several independent studies in 6-OHDA lesioned rodents have examined the effects of DA receptor stimulation on the expression of immediate early genes (IEGs) and late-response genes[4]. It has been shown that a single injection of L-DOPA or D1R agonists increases the expression of *FosB/ΔFosB*, in DA depleted striatum, without producing a response in the intact striatum (Berke et al., 1998; Andersson et al., 2001; Westin et al., 2007). Thus, depletion of DA caused by degeneration of nigrostriatal DAergic neurons results in an enhancement of the responsiveness of MSNs to activation of DAergic receptors. This hypersensitivity is paralleled by the hyperkinetic behavioral response induced by doses of the same treatments that would be ineffective in naïve animals.

In striatopallidal MSNs, the increase in *enkephalin* expression produced by DA denervation is reversed by continuous treatment with D2R agonists. In striatonigral MSNs a single injection with D1R agonists reverses the decreased expression of *dynorphin* that occurs after DA depletion. However, continuous administration of D1R agonists results in a further abnormal enhancement of *dynorphin* expression (Gerfen et al., 1990). A similar regulation has been shown also for IEGs (Gerfen et

[4] The induction of IEGs, most of them transcription factors i.e. c-Fos, leads to changes in gene expression of the late-response genes such as neuropeptides (Gerfen, 2000).

al., 2002). Accumulating evidence indicates that such persistent hypersensitivity of striatal D1Rs may be implicated in long-term molecular changes implicated in LID.

In rodent models of LID, a positive correlation has been reported between dyskinesia and high expression of *prodynorphin* mRNA and *FosB/ΔFosB*-related transcription factors (Cenci et al., 1998; Andersson et al., 1999; Winkler et al., 2002; Konradi et al., 2004; Sgambato-Faure et al., 2005; Pavón et al., 2006; Darmopil et al., 2009). In dyskinetic rats, FosB/ΔFosB transcription factors bind with high affinity to DNA sequences such as cAMP response elements (CRE) and sequences present in the *prodynorphin* gene promoter (Andersson et al., 2001). Moreover, intrastriatal infusion of antisense oligonucleotides for *FosB/ΔFosB* mRNA reduces the expression of prodynorphin and attenuates dyskinesia, indicating that the molecular changes mediated by FosB/ΔFosB play a role in the development of LID (Andersson et al., 1999).

Examples of IEGs that are upregulated during dyskinesia are *Zif268* and *Arc*. In particular, it has been shown that acute L-DOPA administration increases the levels of *Zif268* mRNA both in striatonigral and striatopallidal neurons following DA depletion. Repeated treatment with L-DOPA normalizes *Zif268* mRNA in the striatopallidal, but not in striatonigral MSNs (Carta et al., 2005). Similarly, the expression of *Arc*, an IEG involved in cytoskeletal rearrangement and synaptic plasticity, increases during repeated administration of L-DOPA in dynorphin positive neurons, which correspond to the MSNs of the direct pathway (see above). (Sgambato-Faure et al., 2005) Thus, prolonged upregulation of IEGs during chronic L-DOPA administration may induce persistent effects implicated in LID and changing preferentially the physiological functions of striatonigral MSNs.

Electrophysiological studies in animal models of LID and in dyskinetic patients showed that the activity of mGP/GPi and SNr is altered by changes in bursting discharge and increased amplitude in oscillatory activities (Alonso-Frech et al., 2006; Meissner et al., 2006; Kliem et al., 2007). Furthermore, a study in the rat has described a temporal correlation between the development of dyskinetic movements and a large increase in the levels of GABA in SNr (Mela et al., 2007).

Pharmacological studies in PD patients have shown that continuous infusion of L-DOPA or long-acting DA receptor agonists produced a stable motor improvement without, or with only mild, dyskinesia (Mouradian et al., 1987; Chase, 1998; de la Fuente-Fernández et al.; Nyholm and Aquilonius, 2004; Rascol et al., 2006). In

addition, therapy with selective D1 agonists produces dyskinesia similarly to L-DOPA, whereas agonists at D2 and/or D3 receptors induce little dyskinesia when given to L-DOPA naïve patient (Rascol et al., 2006). Similar results have been obtained in animal models of LID, where stimulation of D1Rs, or combined stimulation of D1Rs/D2Rs, induces dyskinesia (Delfino et al., 2004), selective D1R antagonists suppress LID (Westin et al., 2007) and D2R and/or D3R agonists do not produce dyskinesia (Lundblad et al., 2002; Delfino et al., 2004).

An overactive glutamate transmission within the basal ganglia has been proposed to play a role in LID (Chase and Oh, 2000). Together with the enhancement of D1R signaling, this phenomenon may lead to abnormal corticostriatal synaptic plasticity in MSNs. The first demonstration of altered synaptic plasticity in association with dyskinesia was obtained by Picconi et al. (2003). These authors found that, in striatal brain slices obtained from dyskinetic rats, LTP of corticostriatal synapses could not be reverted by low frequency stimulation. Such irreversible potentiation induced by L-DOPA may have an important physiological role in LID because it would interfere with the function of MSNs in the selection of movement sequences (Picconi et al., 2003).

It has been reported that altered trafficking of NMDAR subunits is involved in LID (Gardoni et al., 2006). Biochemical analyses have shown high levels of NR2A and low levels of NR2B in the striatal post-synaptic density (PSD)-enriched fraction of dyskinetic rats. This reduction appears to be caused by altered anchoring of NR2B to members of the membrane-associated guanylate kinase (MAGUK) protein family, which are present in the PSD. Importantly, dyskinesia was induced in non-dyskinetic rats by treatment with a synthetic peptide that disrupted the binding of NR2B to MAGUK protein (Gardoni et al., 2006).

In conclusion, the results presented above indicate that fluctuating extracellular DA, acting at hypersensitized striatal D1Rs, results in altered transmission at the level of MSNs and that these changes may have a role in the development of LID.

1.7 cAMP/PKA/DARPP-32 SIGNALING PATHWAY

The DA and cAMP-regulated phoshoprotein of 32 kDa (DARPP-32) is a phosphoprotein highly expressed in dopaminoceptive areas of the brain, and in particular in the striatum where it is present in both direct and indirect MSNs.

(Walaas et al., 1983 1983; Ouimet et al., 1984; Walaas and Greengard, 1984; Ouimet et al., 1998).

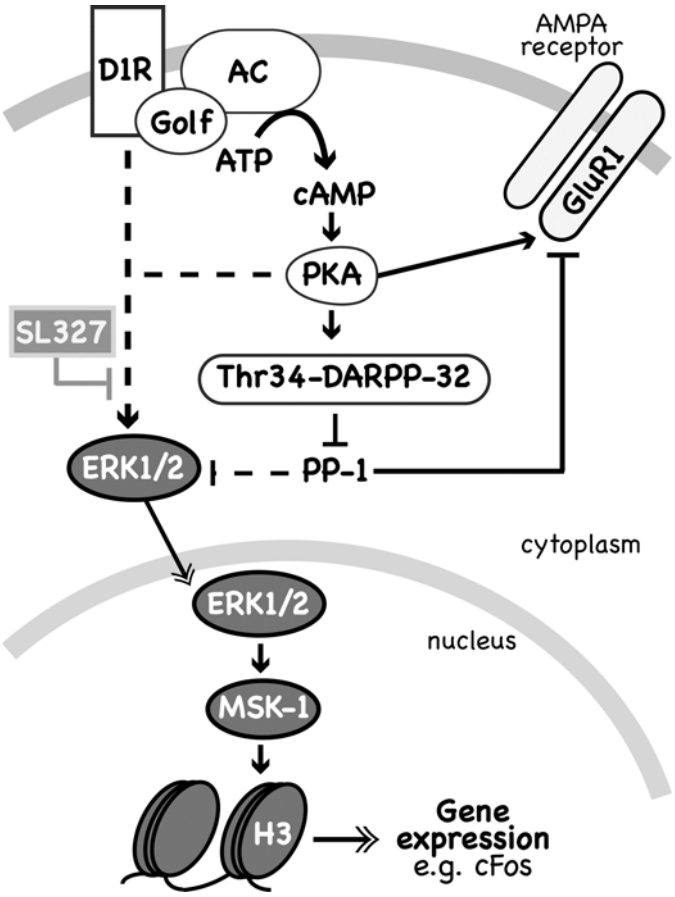

Figure 2. Schematic diagram illustrating cAMP/PKA/DARPP-32 and ERK pathway. Activation of D1Rs, which are expressed by MSNs of the direct pathway, leads to production of cAMP via Golf-mediated activation of AC. cAMP stimulates PKA, which phosphorylates GluR1 and DARPP-32. PKA-mediated phosphorylation of GluR1 is intensified by phospho-Thr34-DARPP-32 via inhibition of PP-1. In addition, stimulation of D1Rs leads to activation of ERK1/2 (cf. text). The increased phosphorylation of ERK1/2 may be facilitated by phospho-Thr-34-DARPP-32-mediated inhibition of PP-1 (cf. text). Phospho-ERK translocation to the nucleus results in phosphorylation/activation of MSK-1 and histone H3. This, in turn, leads to chromatin rearrangements and transcription of IEGs, such as *c-Fos*. SL327 is a MEK inhibitor that blocks activation of ERK1/2. Arrows indicate phosphorylation/activation, double arrows association/dissociation/translocation and blocked lines inhibition.

D1R-mediated activation of PKA leads to phosphorylation of DARPP-32 at the threonine residue in position 34. When DARPP-32 is phosphorylated at Thr34, its NH2-terminal domain interacts with the catalytic site of protein phosphatase-1 (PP-

1), thereby reducing activity (Hemmings et al., 1984; Desdouits et al., 1995; Kwon et al., 1997; Cohen, 2002). The following suppression of dephosphorylation of downstream targets regulated by PKA increases cAMP-mediated responses (Fienberg et al., 1998). Thus, in striatonigral MSNs, D1R signaling induces not only PKA-mediated phosphorylation of target proteins but also concomitant inhibition of their dephosphorylation, mediated by the interaction of phospho-Thr34-DARPP-32 with PP-1 (Figure 2). Phospho-Thr34-DARPP-32 is mainly dephosphorylated by the calcium-dependent protein phosphatase, calcineurin (protein phosphatase 2B).

DARPP-32 is also regulated by cyclin-dependent kinase 5, which phosphorylates Thr75 and converts DARPP-32 into an inhibitor of PKA. Activation of D1Rs has been shown to reduce phosphorylation of DARPP-32 on Thr75, most likely via PKA-mediated phosphorylation and activation of protein phosphatase-2A (PP-2A), which is responsible for dephosphorylation of DARPP-32 at Thr75 (Usui et al., 1998; Nishi et al., 2000; Ahn et al., 2007). In this way, activation of PP-2A further promotes D1 receptor-mediated stimulation of the cAMP pathway, by removing the inhibition exerted by phosphoThr75-DARPP-32 on PKA. Thus, DARPP-32 appears to be a bi-functional molecule in MSNs, able to act either as a protein phosphatase inhibitor or a protein kinase inhibitor, depending on whether Thr-34 or Thr-75 is phosphorylated (Greengard, 2001).

Recently, it has been shown that phosphorylation of DARPP-32 can also regulate its subcellular localization. In mice, phosphorylation catalyzed by casein kinase 2 (CK2) on Ser97, which is located in the vicinity of a nuclear export signal on DARPP-32, is necessary for the translocation of DARPP-32 from the nucleus to the cytoplasm. Activation of D1R, promotes the dephosphorylation of Ser97, via PKA-dependent activation of PP-2A. This regulation results in the nuclear accumulation of DARPP-32 and is important for the phosphorylation of nuclear targets, such as histone H3 (Stipanovich et al., 2008).

The importance of DARPP-32 in D1R-mediated transmission has been demonstrated by several studies performed with different types of genetically modified mice. DARPP-32 KO mice and DARPP-32 mutant mice lacking phosphorylation sites, such as Thr34, Thr75 and Ser97, show altered biochemical and behavioral responses to several classes of drugs that target striatal MSNs, including cocaine, amphetamine and other psychoactive drugs (Fienberg et al., 1998; Bibb et al., 2001; Lindskog et al., 2002; Svenningsson et al., 2003; Andersson et al., 2005;

Valjent et al., 2005; Borgkvist and Fisone, 2006; Zachariou et al., 2006; Zhang et al., 2006; Borgkvist et al., 2007; Stipanovich et al., 2008).

In striatopallidal MSNs, cAMP/PKA signaling is inhibited by activation of D2Rs. For instance, in experiment with striatal slices it has been shown that application of the D2R agonist, quinpirole, reduces the phosphorylation of DARPP-32 at Thr34 (Nishi et al., 1997; Bateup et al., 2008). Blocking D2Rs by systemic administration of D2R antagonists increases the levels of phospho-Thr34-DARPP-32 in the striatum (Svenningsson et al., 2000; Hakansson et al., 2006; Bateup et al., 2008). Moreover, in DARPP-32 KO mice, treatment with a D2R antagonist, results in a reduced cataleptic response (Fienberg et al., 1998).

DAergic modulation of cAMP/PKA/DARPP-32 pathway produces a variety of effects in MSNs, most of which are related to changes in neuronal excitability. For instance, D1-mediated activation of cAMP/PKA/DARPP-32 results in increased phosphorylation of the GluR1 subunit of the glutamate α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) [Figure 2, (Snyder et al., 2000)]. This effect promotes neuronal excitability by increasing AMPA channel conductance and cell surface expression (Roche et al., 1996; Banke et al., 2000; Mangiavacchi and Wolf, 2004). On the other hand, activation of D2Rs reduces the phosphorylation of GluR1 and decreases AMPAR current, while blockade of D2Rs exert the opposite effects (Cepeda et al., 1993; Hakansson et al., 2006).

Activation of cAMP/PKA/DARPP-32 has also been shown to enhance NMDAR transmission through two different mechanisms (Flores-Hernandez et al., 2002). A direct mechanism which involves PKA- and DARPP-32-dependent phosphorylation of the NMDAR at the NR1 subunit (Blank et al., 1997; Fienberg et al., 1998; Snyder et al., 1998) and an indirect mechanism, based on PKA/DARPP-32-mediated increase of L-type Ca^{2+} currents (Surmeier et al., 1995). Increased NMDAR transmission results in enhanced cytosolic Ca^{2+}, which, in association with cAMP/PKA signaling, activates the transcription factor Ca^{2+}/cAMP response element binding protein (CREB) and promotes CRE-dependent gene expression.

Stimulation of D1R inhibits voltage-dependent Na^{+} channels and requires activation of PKA and DARPP-32-mediated inhibition of PP-1 (Calabresi et al., 1987; Schiffmann et al., 1995; Surmeier et al., 1995; Cantrell et al., 1997; Schiffmann et al., 1998). This mechanism controls and coordinates neuronal excitability in response to enhanced glutamatergic input, which shifts the membrane potential of

MSNs from a hyperpolarized “down-state” to a depolarized “up-state”, close to action potential threshold (Wickens and Wilson, 1998).

The work described above indicates that DARPP-32 participates to the integration of signals mediated by DA and glutamate on striatal MSNs (Greengard, 2001). An example of the complex interaction between DA and glutamate in the striatum is the interaction between cAMP/PKA/DARPP-32 and extracellular-signal regulated kinases 1/2 (ERK1/2) signaling pathways.

1.8 ERK1/2 SIGNALING PATHWAY

ERK1/2 are serine/threonine kinases belonging to the mitogen-activated protein kinase (MAPK) family, characterized by a Thr-Glu-Tyr motif in the activation loop. Phosphorylation of the Thr and Tyr residues is required for ERK1/2 activation (Sweatt, 2004; Thomas and Huganir, 2004). Once activated, ERK1/2 phosphorylate several cytoplasmic and nuclear substrates thereby participating to the control of various processes, including neuronal plasticity, transcriptional and translational activity, modulation of ion channels and dendritic spine arborization (Sweatt, 2004; Thomas and Huganir, 2004; Kolch, 2005). The role of ERK1/2 in short and long-term neuronal responses has been addressed by using drugs that inhibits the mitogen-activated protein kinase/ERK kinase (MEK), such as SL327 [Figure 2, (Mizoguchi et al., 2004; Lu et al., 2005; Miller and Marshall, 2005; Valjent et al., 2005; Ferguson et al., 2006; Valjent et al., 2006)].

In the striatum, studies using DA releasing drugs, such as psychostimulants, showed that activation of ERK1/2 depends on D1R and NMDAR activation (Berhow et al., 1996; Valjent et al., 2000; Valjent et al., 2001; Choe et al., 2002; Salzmann et al., 2003; Valjent et al., 2004; Bertran-Gonzalez et al., 2008). In fact, activation of ERK1/2 induced by these drugs is prevented by concomitant administration of D1R, or NMDAR antagonists. (Valjent et al., 2000; Salzmann et al., 2003; Valjent et al., 2004; Zhang et al., 2004; Valjent et al., 2005). These results have an important functional implication, indicating that ERK pathway activation results from a concomitant release of DA and glutamate.

Depolarization and activation of NMDAR and L-type voltage-dependent Ca^{2+} channels induces activation of ERK signaling through increased levels of intracellular Ca^{2+} (Fiore et al., 1993; Rosen et al., 1994; Xia et al., 1996). Ca^{2+} influx activates guanine nucleotide exchange factors (GEFs), such as the brain-specific exchange

factor Ras-guanyl nucleotide releasing factor 1 (Ras-GRF1). Ras-GRF1 promotes the exchange of GDP for GTP on the small G protein Ras (Martegani et al., 1992; Shou et al., 1992; Farnsworth et al., 1995; Fasano et al., 2009), which activates protein kinases of the Raf family, leading to sequential phosphorylation and activation of MEKs and finally ERK1/2 (Thomas and Huganir, 2004; Kolch, 2005). Ca^{2+} in combination with diacylglycerol (DAG) produces a similar activation of a different family of GEFs, the Ras-guanyl nucleotide releasing proteins (Ras-GRPs, or CalDAG-GEFs), which are highly expressed in striatal MSNs (Toki et al., 2001) and which may also contribute to ERK1/2 activation [cf. Results and discussion, (Crittenden et al., 2009)].

An additional mechanism by which D1Rs can promote ERK activation is by increasing intracellular Ca^{2+} concentration through positive modulation of NMDARs, and, possibly, L-type Ca2+ channels (Surmeier et al., 1995; Dudman et al., 2003).

The activation of ERK induced by cocaine, a psychostimulant drug that increases DA release, involves D1R-mediated phosphorylation of DARPP-32 (Valjent et al., 2005). It has been proposed that phospho-Thr34-DARPP-32 promotes ERK phosphorylation via inhibition of PP-1 and reduced dephosphorylation of MEK and of the striatal-enriched protein tyrosine phosphatase (STEP), a phosphatase highly enriched in MSNs that dephosphorylates and consequently inactivates ERK (Figure 2). Increased levels of phospho-MEK result in a stimulation of kinase activity and phosphorylation of ERK. Increased levels of phospho-STEP result in decreased phosphatase activity and suppression of ERK dephosphorylation (Valjent et al., 2005).

Another mechanism by which the cAMP cascade could promote ERK signaling is via PKA-mediated phosphorylation and activation of Ras-GRF1 (Mattingly, 1999). Recent studies have investigated the role of Ras-GRF1 as a component of the ERK activation cascade and as integrator of DA and glutamate transmission at striatal level (Fasano et al., 2009). In Ras-GRF1 KO mice, the behavioral responses to cocaine are attenuated, whereas facilitation is observed in mice overexpressing Ras-GRF1 (Fasano et al., 2009). Interestingly, Ras-GRF1 is also activated by the subunits βγ of G proteins, and this effect is prevented by PP-1 (Mattingly and Macara, 1996). This observation provides a further potential mechanisms by which phospho-Thr34-DARPP-32 may promote Ras-GRF1 and ERK signaling. Increased ERK phosphorylation in response to cAMP accumulation may also occur through

activation of the exchange protein activated by cAMP 1 and 2 (EPAC1 and 2), which activate the Ras family GTPases, Rap1 and 2 (Bos, 2006).

1.8.1 Downstream targets of ERK1/2

ERK1/2 affect a variety of neuronal processes, including synaptic plasticity, through regulation of nuclear and cytoplasmic downstream targets. In particular, the ability of ERK to regulate gene transcription depends on the sequential phosphorylation of nuclear proteins, ultimately responsible for changes in protein expression. In striatal MSNs, activated ERK phosphorylates the mitogen- and stress-activated kinase 1 (MSK1), which is responsible for the phosphorylation of histone H3 (Brami-Cherrier et al., 2005; Heffron and Mandell, 2005; Valjent et al., 2006). Phosphorylation of H3 participates in chromatin remodeling and favors transcription (Brami-Cherrier et al., 2005; Kumar et al., 2005). It is possible that these changes are involved in the regulation of the expression of several genes, including *c-fos*, *fosB/ΔfosB*, *zif-268*, and *arc* [Figure 2, (Valjent et al., 2000; Salzmann et al., 2003; Ferguson and Robinson, 2004; Brami-Cherrier et al., 2005; Ferguson et al., 2006; Valjent et al., 2006)]. The potential impact of ERK signaling in the long-term responses to dopamine has been studied with the use of mice carrying genetic deletion of some of the nuclear targets of ERK1/2. For instance, MSK1 KO mice and zif-268 KO mice show behavioral impairments in response to cocaine (Brami-Cherrier et al., 2005; Valjent et al., 2006).

In addition to its role on transcriptional control, ERK1/2 can participate to translational regulation by interacting with other signaling cascades, such as the mTORC1 pathway.

1.9 mTOR SIGNALING CASCADE

Mammalian target of rapamycin [mTOR; also known as FK506 binding protein 12-rapamycin associated protein 1 (FRAP1)] is a multidomain protein and member of the family of phosphoinositide 3-kinase-related kinases (PIKKs) highly conserved from yeast to human (Martin and Blenis, 2002). mTOR is an essential gene, as revealed by the observation that mTOR KO mice die in uterus shortly after implantation, (Gangloff et al., 2004; Murakami et al., 2004).

mTOR is a critical component of two distinct multiprotein complexes, mTOR complex 1 (mTORC1) and complex 2 (mTORC2). The mTORC1 complex is the rapamycin sensitive complex and consists of mTOR, raptor (a regulatory associated protein of mTOR), LST8 (also known as GβL) and PRAS40. Rapamycin binds to the immunophilin FK506 binding protein 12 (FKBP12) to generate a highly potent and specific inhibitor of mTORC1-dependent signaling through direct binding to the FKBP12-rapamycin binding (FRB) domain of the mTOR kinase (Kim et al., 2002; Loewith et al., 2002; Jacinto and Hall, 2003; Sarbassov et al., 2004; Hoeffer et al., 2008). The mTORC1 complex signals to 4E-binding protein (4E-BP) and p70 ribosomal protein S6 kinase (p70S6K, S6K), which results in enhanced protein translation [Figure 3, (Hay and Sonenberg, 2004; Raught et al., 2004)]. The mTORC2 complex contains mTOR, rictor, LST8 and mSIN1. The mTORC2 complex responds to stimuli induced by growth factors and it is involved in the regulation of cytoskeletal organization (Hara et al., 2002; Kim et al., 2002; Loewith et al., 2002; Jacinto et al., 2004; Sarbassov et al., 2004; Sarbassov et al., 2006).

Many lines of evidence showed that activation of mTORC1 increases protein synthesis (Wang and Proud, 2006). At the level of the nervous system, it is known that consolidation and storage of long-term memories requires activation of protein translation (Kandel, 2001). Studies performed in rodents and invertebrates, revealed that different forms of synaptic plasticity that require protein synthesis, involve activation of mTORC1 signaling and are prevented by mTORC1 inhibitors, such as rapamycin (Casadio et al., 1999; Beaumont et al., 2001; Tang et al., 2002; Tischmeyer et al., 2003; Hou and Klann, 2004; Huang et al., 2004; Dash et al., 2006). Thus, the mTORC1 pathway has been studied mostly in connection to long-term memory and its pathological dysfunctions.

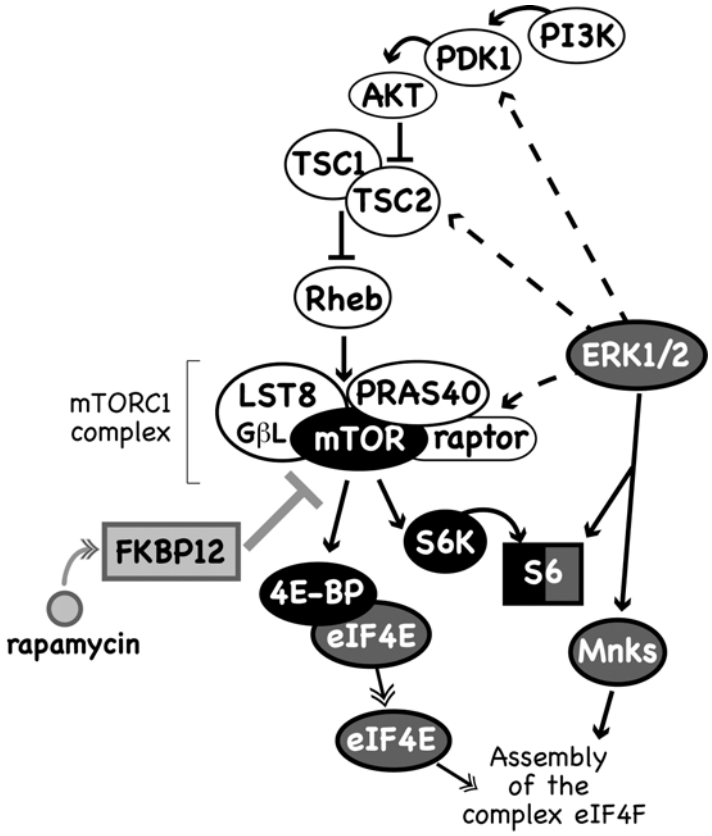

Figure 3. Schematic diagram illustrating the mTORC1 pathway. PI3K/PDK1/Akt promotes mTORC1 signaling through inhibition of the TSC complex 1 and 2 and activation of Rheb. The mTORC1 complex phosphorylates 4E-BP and S6K. Phosphorylation of 4E-BP leads to the release of sequestered eIF4E, which binds other proteins of the initiation complex eIF4F. Activation of S6K leads to phosphorylation of S6. eIF4E is phosphorylated by Mnks, which are activated by ERK1/2. Phosphorylation of eIF4E, S6K and S6 is correlated with enhanced translation initiation. ERK can also interact with the mTORC1 complex via p90RSK-mediated phosphorylation of PDK-1, TSC2 and raptor. Rapamycin bound to FKBP12 disrupts mTORC1 complex thereby preventing the initiation of cap-dependent translation. Arrows indicate phosphorylation/activation, double arrows association/dissociation/translocation and blocked lines inhibition.

In vitro and in vivo studies, mostly in the hippocampus, have described mTORC1 as a downstream target of the phosphatidylinositol-3 kinase (PI3K) signaling pathway. Activation of PI3K leads to recruitment of Akt to the membrane where it is phopshorylated and activated by the PI3K-dependent kinase [PDK1, (Sabatini, 2006)]. Akt phosphorylates and inhibits the tuberous sclerosis complex, which is a heterodimer composed of TSC1 and 2 subunits (also know as hamartin and tuberin). TSC2 exhibits GTPase-activating protein (GAP) activity towards a small G protein named Ras homologue enriched in the brain (Rheb), converting it to the inactive GDP-bound form. Akt dependent phosphorylation of TSC2 decreases its GAP

activity, resulting in the sequential activation of Rheb and mTOR (Hay and Sonenberg, 2004). In the hippocampus, it has also been described that ERK activates mTORC1 under certain conditions. This has been proposed to occur via ERK-dependent activation of p90RSK, which phosphorylates PDK1, TSC2 and raptor [Figure 3, (Frödin et al., 2000; Carrière et al., 2008; Roux and Blenis, 2004; Ma et al., 2005)]. The crosstalk between ERK, PI3K and mTORC1 pathways is important in the hippocampus, since activation of downstream targets of mTORC1 (i.e. 4E-BP and S6K) by different stimuli, such as forskolin, HFS, mGluR agonists, are partially or completely blocked by PI3K, or ERK inhibitor (Banko et al., 2004; Kelleher et al., 2004b; Tsokas et al., 2005).

Once activated, mTORC1 participates in the regulation of cap-dependent translation initiation[5] by modulating the activity of translation initiation factors (eIFs) (Dever, 2002). In addition, mTORC1 has been implicated in the regulation of other proteins, such as eEF2, involved in translation elongation (Browne and Proud, 2004).

1.9.1 Downstream targets of mTORC1: 4E-BP

4E-BP, one of the direct targets of mTORC1, modulates the formation of the eIF4F cap-binding complex. 4E-BP binds eIF4E and prevents its interaction with eIF4G (Pause et al., 1994). When not bound to 4E-BP, eIF4E participates together with eIF4G and other eIFs, to the formation of the initiation translation complex, eIF4F (Mader et al., 1995; Marcotrigiano et al., 1999). Thus, 4E-BP, by sequestering

[5] Translation initiation refers to the recruitment of the ribosome, associated to other translation factors, at the AUG start codon (the first codon always translated in the eukaryotic protein synthesis) on a mRNA. In neurons, two principal pathways implicated in initiation of protein synthesis have been described. The first pathway, termed cap-dependent translation initiation, relies on the fact that eukaryotic mRNAs are co-transcriptionally modified by attachment of an inverted, methylated guanine moiety to produce the 5´-terminal structure m^7GpppN (where N is the first transcribed nucleotide), i.e. the cap-structure. The cap-structure is an anchoring point for the cap-binding protein complex that mediates the recruitment of the small subunit of the ribosome at the extreme 5' end of the mRNA (Sonenberg et al., 2000). The second pathway uses complex secondary structure elements in the RNA called internal ribosomal entry sites (IRES) to recruit the small ribosomal subunits either directly via RNA-ribosome contacts or indirectly via initiation factors that bind the IRES and the ribosome. This pathway does not relay on the cap-structure and therefore it is called cap-independent (Stoneley and Willis, 2004). There is evidence indicating the simultaneous existence of both pathways, but the majority of the eukaryotic mRNAs seem to be translated in a cap-dependent manner.

eIF4E, prevents the formation of eIF4F complex and inhibits protein synthesis (Pause et al., 1994; Haghighat et al., 1995). The binding of 4E-BP to eIF4E is regulated by phosphorylation: unphosphorylated 4E-BP binds to eIF4E and inhibit translation whereas multiple-site phosphorylation of 4E-BP prevents their binding and allows eIF4F formation (Pause et al., 1994; Beretta et al., 1996). Phosphorylation of 4E-BP at its multiple sites occurs in an ordered, hierarchical fashion and only full phosphorylation of 4E-BP appears to block eIF4E binding (Gingras et al., 2001). The major protein kinase that phosphorylates 4E-BP is mTORC1, although the identity of all the kinases that phosphorylate each site has not been firmly established [Figure 3, (Hay and Sonenberg, 2004)].

Studies performed in hippocampal slices indicate that phosphorylation of 4E-BP and S6K (see below), correlates with increased translational activity and that this effect is blocked by the mTORC1 inhibitor, rapamycin (Zho et al., 2002; Hou and Klann, 2004; Kelleher et al., 2004a; Tsokas et al., 2005; Antion et al.). Consistent with this idea, in vivo studies have shown that proper regulation of 4E-BP is required for normal synaptic plasticity and memory. (Banko et al., 2005; Banko et al., 2007).

1.9.2 Downstream targets of mTORC1: S6K and S6

mTORC1 also directly phosphorylates and activates S6K (Cammalleri et al., 2003; Sabatini, 2006; Sancak et al., 2007; Vander Haar et al., 2007). S6K phosphorylates the ribosomal protein S6 (S6rp, S6), which is located close to the mRNA- and tRNA-binding sites on the 40S ribosomal subunit. Numerous kinases mediate the phosphorylation and activation of S6K and S6. Indeed, S6K is activated by PI3K/PDK1 and ERK pathways, whereas S6 is phoshorylated by S6K1, S6K2 and ERK1/2 directly, or indirectly through other kinases [Figure 3, (Pullen et al., 1998; Dash et al., 2004; Kelleher et al., 2004b; Kelleher et al., 2004a; Pende et al., 2004)].

At the moment, the role of S6K and S6 phosphorylation in translational regulation remains to be fully understood (Dufner and Thomas, 1999). In general, however, S6 phosphorylation correlates with increased levels of translation and in the mouse liver, conditional deletion of S6 impairs ribosome biogenesis and cell proliferation (Volarevic et al., 2000). The S6Ks and the phosphorylation of S6 have been implicated in the translational regulation of specific mRNAs, encoding for components of the translational machinery, such as poly(A)-binding protein (PABP)

and S6 itself. These mRNAs contain terminal oligopyrimidine tracts at the 5-terminal (Ruvinsky and Meyuhas, 2006).

1.9.3 Regulation of eIF4E phosphorylation

Besides being involved in the assembly of the eIF4F, the cap-binding protein eIF4E is a target for direct phosphorylation. Phosphorylation of eIF4E is stimulated by ERK and correlates with increased translation rates in serum-stimulated cells (Scheper and Proud, 2002; Scheper et al., 2002). This effect may be explained by the observation that phosphorylation of eIF4E reduces its cap-binding affinity and promotes eIF4E recycling after the ribosome has bound to mRNA.

The MAPK-interacting serine/threonine kinases 1 and 2 (Mnk1 and Mnk2), which are activated by ERK, phosphorylate eIF4E on Ser209 [Figure 3, (Pyronnet et al., 1999; Waskiewicz et al., 1999; Scheper et al., 2001)]. In mice that are deficient in both Mnk1 and Mnk2, phosphorylation of eIF4E is abolished (Ueda et al., 2004). It has been proposed that phosphorylation of eIF4E by Mnks depends on their interaction with eIF4G and, therefore, that phosphorylation of eIF4E is an indirect measure of eIF4F assembly (Pyronnet et al., 1999). Despite this evidence, Mnk1 and Mnk2 KO mice are viable and normal (Ueda et al., 2004). More recently, studies using dominant-negative MEK transgenic mice, showed that L-LTP is associated with ERK- and Mnks-dependent increase in eIF4E phosphorylation, consistent with the requirement of ERK for translational-dependent forms of synaptic plasticity and learning (Banko et al., 2004; Kelleher et al., 2004b). Furthermore, it has been demonstrated that ERK-dependent phosphorylation of eIF4E is involved in hippocampus-dependent memory formation (Kelleher et al., 2004a). Nevertheless, more direct evidence is necessary to prove that ERK dependent phosphorylation of eIF4E is a critical step in the initiation of translation.

2 SPECIFIC AIMS

The main goals of this Ph.D. project have been: 1) to identify changes in intracellular striatal signaling that could be involved in the generation of LID; 2) to identify the specific neuronal population of the striatum in which these changes occur; and 3) to examine pharmacological or genetic manipulations able to counteract these changes, for their ability to reduce LID.

The Specific Aims were:

PAPER I

- To study the involvement of cAMP/PKA/DARPP-32 and ERK1/2 pathways in the generation of LID.

PAPER II

- To identify the specific population of striatal MSNs affected by the changes in ERK1/2 signaling associated to LID.

PAPER III

- To study the regulation of the mTOR pathway induced by L-DOPA in the DA depleted striatum.
- To identify the specific population of striatal MSNs affected by the changes in mTORC1 signaling associated to LID.
- To determine the role of mTORC1 pathway in LID.

Unpublished

- To study the regulation of Golf induced by LID.
- To identify the specific population of striatal MSNs affected by the changes in the state of phosphorylation of DARPP-32 associated to LID.

3 METHODOLOGICAL DISCUSSION

Detailed descriptions of the experimental approaches of this thesis are given in the "Materials and Methods" section of the individual original articles. The purpose of this chapter is to provide a methodological overview, which is not included in these articles. In the first part, I discuss the models of PD and LID with special emphasis on those employed in these studies. In the second part, I provide a more in-depth description of the various strains of genetically modified mice utilized in this thesis.

3.1 EXPERIMENTAL MODELS OF PD

3.1.1 The MPTP-model in non-human primates

MPTP is a neurotoxin that causes acute and irreversible Parkinsonism in humans (Langston et al., 1983). The MPTP experimental model consists in systemic administration of the neurotoxin to non-human primates or mice. After systemic administration, MPTP crosses the blood-brain barrier. Once in the brain, it is converted in MPP+ within non-DAergic cells, such as glial cells and serotoninergic neurons. Thereafter, MPP+ released in the extracellular space enters DAergic neurons through DAT and acts by blocking the mitochondrial complex I. This inhibition decreases the synthesis of ATP and increases the production of reactive oxygen species (ROS) that cause cell death.

The advantage of the MPTP treated non-human primate model of PD is that it exhibits several features that closely resemble the parkinsonian state observed in patients. For instance, as in idiopathic PD, the sensitivity of DAergic neurons to MPTP-induced neurodegeneration is higher in the SNc than in VTA (Sirinathsinghji et al., 1992; Varastet et al., 1994). Furthermore, the motor symptoms that follow neurotoxin-induced DAergic cell death are similar to those observed in human parkinsonism. Thus, the animals show abnormal body posture at rest, paucity and slowness of spontaneous movements, rigidity and tremor. Importantly, these motor impairments are improved by medications that relieve parkinsonian symptoms in patients (Schneider, 1989; Boyce et al., 1990; Löschmann et al., 1992; Gomez-Mancilla and Bédard, 1993; Pearce et al., 1998; Langston et al., 2000). The

weaknesses with the non-human primate model of PD are ethical, economical and logistic restrictions, which have limited this approach to a few laboratories in the world.

3.1.2 The 6-OHDA-model in rodents

6-OHDA is widely used in rats and mice, to reproduce the loss of dopaminergic innervation to the striatum typical of PD. Because 6-OHDA does not cross the blood-brain barrier it has to be administered with intracerebral or intraventricular infusion. 6-OHDA is taken up by DAT and noradrenaline transporters (NET), it accumulates in the cytosol and generates ROS and quinines that cause cell death by oxidative stress. In order to prevent degeneration of noradrenergic (NA) neurons animal subjected to lesion with 6-OHDA are sometimes treated systemically with an inhibitor of NET, such as desipramine, which prevents uptake of the toxin into NA terminals. In the mouse model used in this thesis, 6-OHDA lesions were produced specifically in the dorsolateral striatum, which is devoid of NA fibers (Swanson and Hartman, 1975). Therefore we did not treat the animals with desipramine.

6-OHDA injected into SNc, MFB or dorsolateral striatum induces a dose-dependent degeneration of SNc DAergic neurons. Injections into the SNc or MFB cause degeneration of DAergic neurons within 24 hours (Jeon et al., 1995), while injection into the dorsal striatum produces a slower retrograde degeneration, which peaks one to three weeks after lesion (Sauer and Oertel, 1994; Przedborski et al., 1995). Even if a drawback common to all the PD models induced by neurotoxins is the rapidity of DAergic neurodegeneration compared to that occurring in Parkinsonian patients (which lasts for years), it seems that the 6-OHDA lesions obtained by intra-striatal injections can reproduce, at least in part, its progression.

Both bilateral and unilateral 6-OHDA lesions have been successfully performed in rodents. Rodents with bilateral lesions show a range of motor impairments, such as postural abnormalities at rests (Wolfarth et al., 1996), reduction of spontaneous movements (Marshall et al., 1974; Schallert et al., 1978; Schallert et al., 1979), and increased muscle resistance to passive stimuli (Schallert et al., 1978; Wolfarth et al., 1996). Muscle resistance in bilaterally lesioned rats has electromyographic features that are similar to parkinsonian rigidity (Wolfarth et al., 1996; Berardelli et al., 2001). In addition, footprint analysis shows that these animals take short steps when they walk forward, which reproduces the gait abnormalities observed in PD patients

(Schallert et al., 1978). Overall, bilateral 6-OHDA lesion shows some of the motor impairments characteristic of PD. However, since bilateral DA depletion causes severe anhedonia for food and water, the animals require intensive post-operative care. On the contrary, unilateral lesions are well tolerated by both rats and mice.

Unilateral lesioned mice and rats are the most widely used animal model of PD and are often referred to as hemiparkinsonian (Schwarting and Huston, 1996). The unilateral lesion induces asymmetries of the body posture and contralateral sensorymotor deficits. The sensorimotor asymmetry has been used to design simple and objective tests for monitoring the effects of symptomatic or restorative treatments. The most common test consists of recording the number of turns that are performed by an animal after a challenge with DA agonists (turning or rotational behavior). Administration of L-DOPA or agonists of DRs, such as apomorphine, induces rotations towards the side contralateral to the lesion, while DA releasing drugs, such as amphetamine, elicit turning behavior towards the side ipsilateral to the lesion. These behaviors reflect the sensitized response to activation of DRs, developed in the DA-depleted striatum (Ungerstedt, 1968; Schwarting and Huston, 1996). Ipsilateral rotation has been used to monitor the effects of DA-denervating lesions, neural grafts and neuroprotective treatment, while contralateral rotation has been used to assess the anti-akinetic potential of candidate antiparkinsonian drugs (Schwarting and Huston, 1996). The use of contralateral rotational behavior to screen for anti-akinetic drugs has been criticized. It has been shown, for instance, that the ability of antiparkinsonian drugs to induce contralateral rotation does not correlate with the ability of these drugs to improve motor impairments in hemiparkinsonian rats, as determined with other, more sophisticated, tests (cf. below) (Lundblad et al., 2002; Metz and Whishaw).

Careful qualitative movement analyses have revealed that unilateral 6-OHDA-lesioned rodents exhibit specific motor deficits that can be studied with a variety of behavioral tests rather than rotational behavioral (Olsson et al., 1995; Cenci et al., 2002). For instance, 6-OHDA lesioned rodents are consistently impaired in their ability to use the forelimb contralateral to the side of the lesion to adjust posture, explore, walk, groom and perform other types of spontaneous behavior (Cenci et al., 2002). These impairments are assessed in the cylinder test. The test is based on the explorative behavior that rats and mice display when placed in a novel environment (in this case, a glass cylinder). This behavior is mainly expressed by rearing toward the wall of the cylinder, using the forelimbs to support the body (Cenci et al., 2002).

A sham-lesioned animal uses both forelimbs to explore, while a 6-OHDA lesioned rodent utilizes preferentially the forelimb ipsilateral to the side of the lesion. Administration of L-DOPA causes a dose-dependent improvement of the forelimb akinesia in 6-OHDA lesioned animals (Figure 4).

In this thesis I have used the cylinder test to: 1) evaluate 6-OHDA-induced forelimb akinesia (Paper I); 2) assess the improvement of forelimb akinesia with the antiparkinsonian drug L-DOPA (Paper I); 3) evaluate the potential interfering effects of antidyskinetic treatments (Paper III) and genetic deletion (Paper I) on the therapeutic properties of L-DOPA.

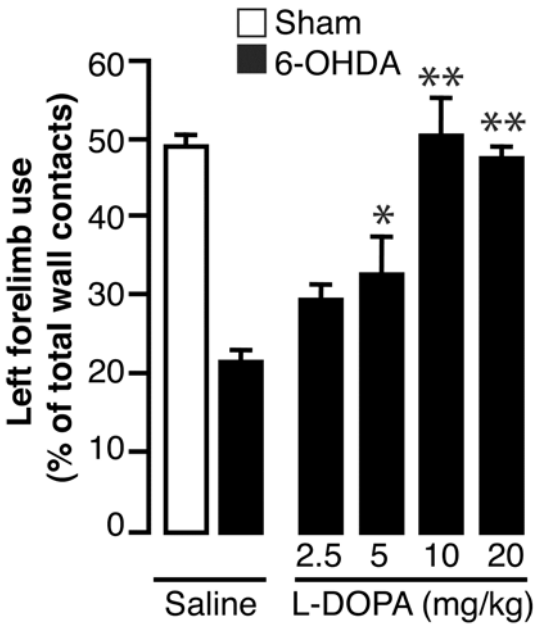

Figure 4. Effect of L-DOPA administration on 6-OHDA-induced akinesia. Forelimb use was measured in Sham-lesioned (white bar) and 6-OHDA-lesioned (black bars) mice treated with saline or different doses of L-DOPA (2.5, 5, 10, 20 mg/kg). During the cylinder test, the number of wall contacts with the right and the left forelimb performed by each mouse was counted. The use of the impaired (left) forelimb was expressed as a percentage of the total number of supporting wall contacts. L-DOPA induces a dose depend recovery of left forelimb akinesia. The data are represented as means ± SEM (n=10). *p<0.05, **p<0.01 vs. 6-OHDA-lesioned mice treated with saline, one way ANOVA, followed by Bonferroni-Dunn test.

3.2 EXPERIMENTAL MODELS OF DYSKINESIA

3.2.1 Dyskinesia in the MPTP model of PD

Chronic L-DOPA administration in MPTP treated non-human primates induces AIMs that are very similar to dyskinesia observed in humans (Langston et al., 2000). The AIMs appear when L-DOPA exerts its maximal antiparkinsonian effect (Schneider, 1989; Gomez-Mancilla and Bédard, 1993; Cenci et al., 2002) and they show the same profile of peak dose dyskinesia. Furthermore, dyskinesia interferes with normal motor behavior both in non-human primates and in parkinsonian patients

(Pearce et al., 1998; Brotchie and Fox, 1999; Hagell and Widner, 1999). The similarities are so striking that experimental AIMs in non-human primates can be evaluated using the same rating criteria as in the clinical setting (Schneider, 1989; Boyce et al., 1990; Gomez-Mancilla and Bédard, 1993; Brotchie and Fox, 1999).

3.2.2 Dyskinesia in 6-OHDA models of PD

In rodents, two principal behavioral models of LID that take advantage on movement asymmetry induced by unilateral 6-OHDA lesions, have been characterized: development of abnormal involuntary movements (AIMs) and sensitization of rotational behavior.

AIMs are involuntary movements affecting the forelimb (repetitive jerky movements), the trunk (dystonic twirls) and the orofacial musculature (jaw movements and tongue protrusion) of the side of body contralateral to the lesion. They can be quantified in amplitude and duration (Lee et al., 2000; Lundblad et al., 2002; Winkler et al., 2002) by using the same criteria applied in the clinic (Hagell and Widner, 1999). Overall, AIMs have the same time course of peak dose dyskinesia and disrupt physiological motor activities as observed both in dyskinetic patients and in non-human primate models of dyskinesia. Importantly, drugs with anti-parkinsonian properties but less potential to induce dyskinesia in human, such as amantadine, bromocriptine and ropinirole, do not induce substantial AIMs in rats. One of the criticisms that the AIMs model of LID has received is related to the lack of similarity between the choreiform manifestation of dyskinesia observed in humans and the AIMs (Chase, 1998; Langston et al., 2000; Bezard et al., 2001). According to this criticism, AIMs are similar to the stereotyped behavior observed in rodents treated with DAergic drugs. However, the two behaviors are different (Creese and Iversen, 1973; Andersson et al., 2001). The stereotyped behavior consists of purposeless repetition of otherwise normal motor activities (i.e. grooming, sniffing, licking) while AIMs are never performed during normal motor behavior (Cenci et al., 2002).

A debate has emerged as to the validity of sensitization of rotational behavior as a model of LID in rodents (Henry et al., 1998, 1999; Marin et al., 2007). In support of the validity of this model, it has been shown that a non-dyskinesiogenic treatment, such as continuous infusion of L-DOPA, fails to induce rotational sensitization (Engber et al., 1989; Nielsen and Soghomonian, 2003; Bibbiani et al., 2005). However, rotational sensitization is induced by *e.g.* bromocriptine or ropinirole that

are two examples of drugs with very low dyskinesiogenic potential (Lundblad et al., 2002). Furthermore, it has been argued that rotational behavior does not allow to clearly distinguish between dyskinetic and anti-akinetic effects of drugs (Lundblad et al., 2002; Metz and Whishaw, 2002).

It as been proposed that both sensitization of rotational behavior and AIMs represent dyskinesia, the difference between the two models is their sensitivity (Carta et al., 2006). According to this view, sensitized rotational behavior would represent a correlate of mild and delayed onset dyskinesia (such as that observed in patients and non-human primates treated with ropinirole and bromocriptine), whereas AIMs would be a marker for more intense dyskinetic movements (such as those induced by L-DOPA). Therefore, while all dyskinetic treatments seem to induce sensitization of rotational behavior only those resulting in more intense dyskinesia induce AIMs.

Recently, Pinna et al., (2006) examined the influence of the environment (hemispherical bowls and standard cages) in which the two tests (rotational behavior and AIMs, respectively) are usually performed. Rats treated chronically with L-DOPA develop sensitization of rotational behavior and AIMs when tested in hemispherical bowls (such as those normally utilized to determine rotational behavior); in contrast, the animals show only AIMs when tested in standard cages. Therefore, it appears that rectangular cages somehow reduce the expression of rotational behavior (Pinna et al., 2006). These results may explain some of the discrepancies obtained when studying the effects of high (L-DOPA) and low (ropinirole and bromocriptine) diskinesiogenic drugs using sensitization of rotational behavior (Carta et al., 2006).

It has also been pointed out that the rotational behavior induced by drugs with different dysinesiogenic properties is qualitatively different (Carta et al., 2006). Thus, more refined analyses should be performed in order to discriminate between the two kinds of rotations. For instance, ropinirole induces rotations with large diameter, which are made with all the four limbs, whereas L-DOPA induces turning with twisted posture with the animal using only the hind limbs to support the body. The distorted body posture following L-DOPA treatment is in some cases so severe that the animal falls towards the side contralateral to the lesion during the rotational behavior (Carta et al., 2006).

3.3 GENETICALLY MODIFIED MICE

A unique approach to study the molecular basis of neuronal activity consists in manipulating the genome of higher organisms, in order to identify how individual molecules operate within neural circuits, regulate brain function and control synaptic plasticity. This allows not only a direct assessment of gene function in intact animals, but also the design of useful animal models of human disease. In this context, the mouse represents an exceptional species, which is prone to targeted gene modifications, possesses the complex genome and the neuroanatomical organization of mammals and can be studied in paradigms which model a wide array of human neurological or psychiatric diseases.

3.3.1 Conventional genetic inactivation

Precise alteration of endogenous genes (gene targeting) is accomplished by homologous recombination in embryonic stem (ES) cells and has been used extensively to generate null, or knock-out (KO) mice. The derivation from mouse embryos of ES cells, which retain totipotency even after gene targeting has permitted the generation of intact animals carrying the genetic alteration. Phenotyping of these null mutant mice has provided invaluable information for the identification of key proteins involved in all functional aspects of the nervous system.

In this thesis I have been studied DARPP-32 KO mice (Paper I) and Golf$^{+/-}$ mice (Unpublished result I) in order to identify their role in LID.

3.3.1.1 DARPP-32 KO mice

Disruption of the DARPP-32 gene was performed by homologous recombination (HR) between a targeting vector and the DARPP-32 gene locus in the E14 embryonic stem cell line (ES) derived from the 129/Ola substrain (Fienberg et al., 1998). Targeted clones were injected into C57BL/6 blastocysts and chimeric offspring were crossed with C57BL/6J female mice generating F_1 hybrid mice. Backcrossing into the C57BL/6J strain was performed to obtain the N_2 through N_6 generations.

In the experiments presented in this thesis, wild type (WT) and congenic ($N_{>20}$) DARPP-32$^{-/-}$ mice were generated from the offspring of DARPP-32$^{+/+}$ x DARPP-32$^{+/+}$

and DARPP-32$^{-/-}$ x DARPP-32$^{-/-}$ mating pairs. DARPP-32$^{-/-}$ x DARPP-32$^{-/-}$ mating was performed separately for no more than two generations.

In the initial study (Fienberg et al., 1998), F_1, N_2, and N_6 mice were tested in electrophysiological, neurochemical and behavioral experiments. No differences were found in the phenotype of DARPP32$^{-/-}$ at the various levels of backcrossing. Furthermore, ablation of DARPP-32 did not produce any abnormalities in gross brain anatomy or body weight, and DARPP-32$^{-/-}$ mice appeared indistinguishable from wild-type animals.

3.3.1.2 Golf heterozygous mice

Disruption of the Golf gene (*Gnal*) was performed by homologous recombination between a targeting vector and the Golf gene locus in the embryonic stem cell line (ES) derived from the 129/Sv substrain (Belluscio et al., 1998). Targeted clones were injected into C57BL/6 blastocysts and chimeric offspring were crossed with C57BL/6J female mice generating F_1 hybrid mice. Backcrossing into the C57BL/6J strain was performed for $N_{>8}$ to obtain homozygous (Golf$^{-/-}$), heterozygous (Golf$^{+/-}$) and wild type (Golf$^{+/+}$) littermates (Corvol et al., 2007).

The WT and heterozygous ($N_{>20}$) mice studied in this thesis were generated from the offspring of Golf$^{+/+}$ x Golf$^{-/-}$ mating pairs. These studies were carried out in collaboration with Dr. Jean-Antoine Girault and Dr. Denis Hervé (INSERM, U536, Université Pierre et Marie Curie, Institut du Fer a Moulin, Paris, France). Previous studies, in Golf$^{+/-}$ mice have shown decreased biochemical and behavioral responses to acute drugs acting on DA release whereas long-term responses were preserved (Corvol et al., 2001; Corvol et al., 2007).

The conventional KO technology has limited utility in several situations. First of all, the gene of interest may be essential for development and survival, in which case gene KO leads to a lethal phenotype. The gene of interest may also share redundancy with other genes. In this case, compensatory mechanisms would render the phenotype not detectable. When a protein is expressed both in the central and peripheral nervous system, a general KO may not address with sufficient specificity the role of a gene in brain function. In addition, due to the anatomical complexity of the nervous system, the same protein may fulfill distinct functions depending on its site of expression within specific neurons and neural systems. Therefore, the general deletion of a

protein may be inadequate to understand fine molecular processes in higher brain functions. For these reasons, the next step of gene targeting technologies has been to reach spatial and temporal control of gene deletion. One popular approach to control targeted genetic inactivation in mice is the Cre-LoxP strategy, which gives the possibility to generate spatially and temporally controlled conditional mutations.

3.3.2 BAC Transgenic mice

One useful approach to study brain function is the use of transgenic mice obtained through a stable integration of foreign DNA into the mouse genome. This recent technology is based on the generation of transgenic mice using large genomic DNA clones, called bacterial artificial chromosomes (BACs). BACs are large-insert DNA clones based on the Escherichia coli (E. coli) fertility factor, plasmid F (Shizuya et al.). BACs have served as the primary genomic DNA clones to most of the genome sequence projects. Several methods have been developed to utilize HR in E. coli to modify BACs and to introduce desirable mutations, including insertion, deletion and point mutations (Yang et al., 1997; Gong et al., 2002).

Transgenic mice are usually generated by direct microinjection of BAC DNA into fertilized mouse embryos (one cell stage), followed by transfer of these embryos into recipient mothers that can carry the pregnancy to term. Transgenic founders, which have the BAC DNA stably and integrated into the genome, can then transmit the integrated BAC transgene to their offspring to establish a transgenic mouse line. Since the BAC integration is a random event, each transgenic line is unique in that the BAC transgene is integrated at distinct chromosomal locations and the lines have a different number of copies of the BAC transgene. The major advantage of using BACs for transgenic studies is that BAC transgenic constructs overcome positional effects to produce integration site-indipendent, copy number-dependent, and accurate transgene expression in vivo (Heintz, 2001).

BAC transgenic studies, particularly the Gene Expression System Atlas (GENSAT) BAC transgenic project, have demonstrated that 80%-85% over a total of around 100 different BAC transgenic lines produced accurate and reproducible transgene expression in vivo (Gong et al., 2003).

The BAC transgenic mouse lines have been used to investigate gene expression pattern and gene regulation, to label neurons of interest for in vivo imaging or electrophysiological studies, to develop Cre mouse line for conditional gene

targeting, to study neuronal circuitry mapping, to tag proteins in vivo, to model human disease cause by a dominant genetic mutation.

I will limit the discussion to the BAC transgenic mouse lines that I have used in this thesis, i.e. EGFP-transgenic mice (Paper II and III) and epitope-tagged proteins-transgenic mice (Unpublished result II).

3.3.2.1 EGFP transgenic mice: Drd1a- and Drd2-EGFP mice

The development of BAC transgenic methodology for reporter gene analysis allowed the mapping of gene expression at cellular resolution. This can be achieved by using, for instance, modified BAC vectors that carries the enhanced green fluorescent protein (EGFP) reporter gene.

In Paper II and III, we have used two different lines of BAC transgenic mice originally derived from the GENSAT project. In these mice, the promoter of the D1R (Drd1a) or D2R (Drd2) controls the expression of the EGFP reporter gene. This genetic manipulation has given us the possibility to examine the involvement of the two different populations of MSNs, striatonigral vs. striatopallidal (Gerfen et al., 1990), in the activation of the ERK (Paper II) and mTORC1 (Paper III) signaling pathways induced by L-DOPA in the DA depleted striatum.

The *Drd1a*- and *Drd2*-EGFP transgenic mice used in these experiments were obtained from breeding EGFP positive mice (hemizygous for the transgene) with a mixed Swiss-Webster-C57BL/6 background with C57BL/6 (N_4). EGFP positive and WT offspring were indistinguishable in terms of body weight, and gross anatomy, and EGFP expression did not produce abnormalities in any of the brain areas (striatum, hippocampus and cortical areas) that have been characterized so far. This is in agreement with the idea that the introduction of BAC per se does not modify the function of the MSNs in which it is expressed (Wang and Proud, 2006).

Recently, Shuen et al. (2008) questioned the use of *Drd1a*- and *Drd2*-EGFP transgenic mice to specifically identify direct and indirect MSNs. These authors crossed a *Drd2*-EGFP mouse with a BAC transgenic mouse expressing a red fluorescent protein (tdTomato) under the control of the *Drd1a*- promoter. They found that, in the resulting F_1 progeny, around 39% of MSNs did not express either fluorescent protein. This proportion increased to around 50% when *Drd1a*-EGFP mice were crossed with *Drd2*-EGFP mice (Shuen et al., 2008). These results implied

that the expression of the reporter gene was too low and that either the EGFP transgenic model is not a useful tool, or that a high number of MSNs do not express D1Rs or D2Rs. However, another recent paper using *Drd1a*-EGFP and *Drd2*-EGFP transgenic mice showed that the majority of MSNs express either D1Rs or D2Rs and that only a small percentage of MSNs express both (5%, 6%, and 7% of MSNs in the dorsal striatum, core and shell, respectively). Moreover, in *Drd1a*-EGFP/*Drd2*-EGFP double transgenic mice, EGFP labels all the striatal MSNs. Confirming the suitability of the two lines of EGFP transgenic mice for cell specific localization studies (Matamales et al., 2009).

In this thesis, both lines have been used to localize the activity of the ERK (Paper II) and mTORC1 signaling pathway (Paper III) in striatonigral vs. striatopallidal MSNs. In our studies it has also been employed an antibody to enhance the endogenous EGFP fluorescence.

3.3.2.2 Tagged mice: D1R-DARPP-32/Flag and D2R-DARPP-32/Myc mice

Epitope tags, such as Flag tag and Myc tag, can be engineered in the BAC to form fusion proteins. These tags can be inserted at the N- or C-terminal of proteins. Transgenic mice carrying tagged proteins can be used to study protein expression in a specific sub-set of neurons or to visualize their subcellular localizations. Furthermore, the epitope-tags allow immunoprecipitation of protein complexes containing the tagged protein from brain extracts.

In this thesis I made use of BAC transgenic mice that express DARPP-32 tagged with Flag and Myc in striatonigral and striatopallidal MSNs, respectively (Unpublished result II). These mice were available through a collaboration with Dr. Paul Greengard (Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, New York, NY, USA). Using these mice and a selective immunoprecipitation protocol it is possible to immunoprecipitate phosphorylated DARPP-32 from striatopallidal or striatonigral MSNs (Bateup et al., 2008). This technique was employed to analyze the state of phosphorylation of DARPP-32 specifically in MSNs of the direct and indirect pathway.

D1R-DARPP-32/Flag and D2R-DARPP-32/Myc BAC mice were generated according to the GENSAT BAC modification protocol (Gong et al., 2002). The

modified shuttle vectors for the two lines of mice have been recombined into the BAC containing the full *Drd1a*- and *Drd2*-promoter. BAC DNA from positive co-integrates was injected into pronuclei from FVB/N oocytes to generate transgenic mice (Bateup et al., 2008). The D1R-DARPP-32/Flag and D2R-DARPP-32/Myc were backcrossed ($N_{>10}$) with C57Bl/6. The mice used in this thesis were heterozygous double-transgenic mice expressing both Flag- and Myc-tagged DARPP-32 generated from the offspring of heterozygous D1R-DARPP-32/Flag x heterozygous D2R-DARPP-32/Myc mating pairs (Bateup et al., 2008).

In a recent paper (Bateup et al., 2008) the BAC double-trangenic mice has been carefully characterized by showing the selectivity of the expression of the tagged-DARPP-32 proteins in striatonigral or striatopallidal MSNs, the high specificity of the Flag and Myc immunoprecipitations, as well as the absence of signaling alterations in response to the expression of tagged DARPP-32.

4 RESULTS AND DISCUSSION

4.1 LID IS ASSOCIATED WITH INCREASED LEVELS OF GOLF (UNPUBLISHED RESULTS I)

Hypersensitivity of D1Rs has been proposed to develop in association to PD and to be involved in the abnormal responses to L-DOPA observed in dyskinesia (Guigoni et al., 2005). This hypersensitivity can be accounted for by increased levels of Golf, the G protein that couples D1Rs to activation of AC in the striatum (Corvol et al., 2004). Accordingly, it has been reported that the striata of DA-depleted rats or parkinsonian patients contain higher levels of Golf and that this leads to an enhancement of DA-stimulated cAMP production (Corvol et al., 2007). Furthermore, in MPTP-lesioned monkeys, L-DOPA-induced dyskinesia appears concomitantly with augmented coupling of striatal dopamine D1Rs to G-protein (Aubert et al., 2005).

We have investigated whether the levels of Golf are increased also in the striata of in DA depleted mice (Unpublished results I). In agreement with the previous work mentioned above, we found that 6-OHDA-lesion leads to increased Golf levels in the dorsolateral striatum (Figure 5A). We also found that repeated administration of L-DOPA (20 mg/kg for 10 days) to 6-OHDA-lesioned mice resulted in a normalization of Golf to baseline level only in mice that showed low dyskinesia (Figure 5A and B). Indeed, a positive correlation was found between severity of AIMs and increased Golf (Figure 5C). The absence of Golf down-regulation in dyskinetic mice is in line with the persistent sensitivity of D1R transmission associated to LID.

4.2 LID IS ASSOCIATED WITH PKA-MEDIATED PHOSPHORYLATION OF DARPP-32 AT THR 34 (PAPER I)

In the DA depleted striatum, activation of D1Rs results in increased production of cAMP via Golf mediated stimulation of AC, leading to activation of PKA. DARPP-32 is one of the direct targets of PKA expressed in the striatum. Thus stimulation of D1Rs is accompanied by increased phosphorylation of DARPP-32 at the PKA dependent phosphorylation site, Thr34. Using a rat model of LID Picconi et al. (2003)

demonstrated the existence of an association between dyskinesia and increased phosphorylation of DARPP-32 (Picconi et al., 2003).

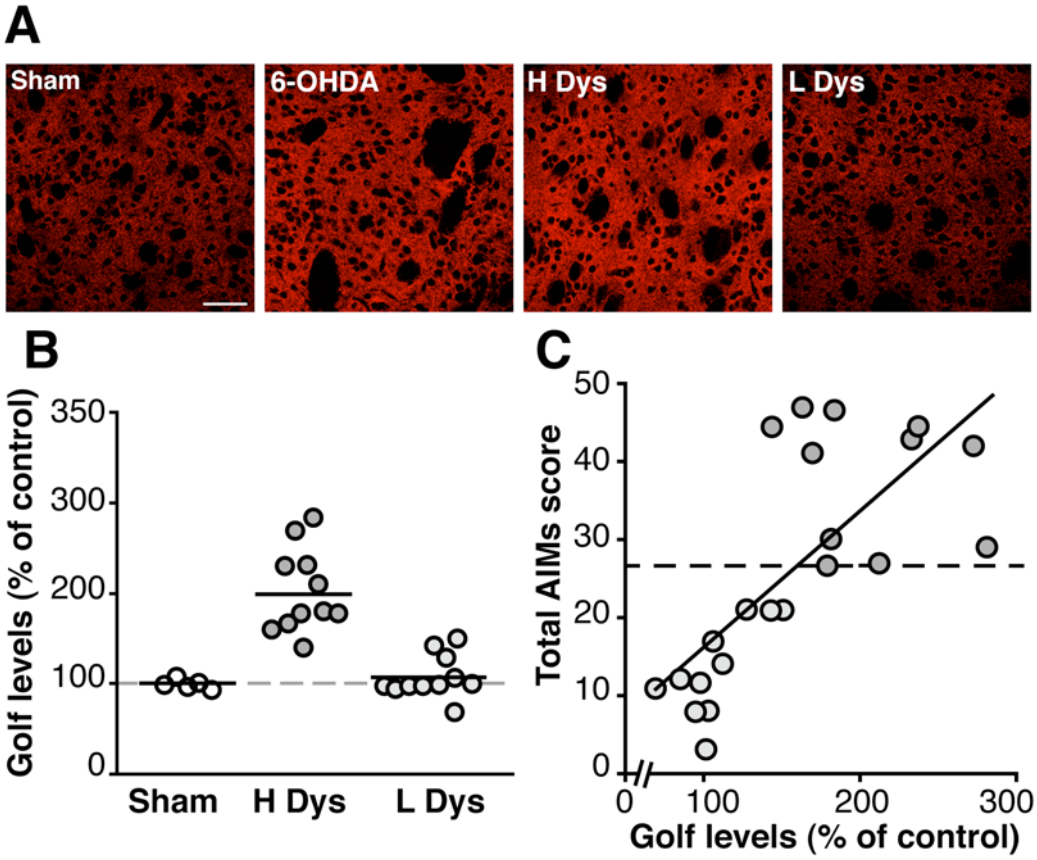

Figure 5. Increased Golf levels are associated with LID. (**A)** Immunofluorescence detection of Golf in the dorsal striatum of a sham-lesioned mouse (Sham), a 6-OHDA-lesioned mouse (6-OHDA) and 6-OHDA-lesioned mice showing high (H Dys), or low (L Dys) dyskinesia. Scale bar 40 μm. (**B)** Scatter diagram of Golf levels measured in Sham-lesioned mice (Sham) and 6-OHDA-lesioned mice showing H Dys, or L Dys. The animals were divided into high and low dyskinetic based on the median value of their total AIMs score. (**C)** Simple regression analysis showing a significant correlation between severity of AIMs and Golf levels ($R=0.78$, $p<0.001$). Broken line indicates the median value (28).

In Paper I we have investigated whether the phosphorylation of DARPP-32 was also present in a mouse model of PD and LID. Administration of L-DOPA to sham-lesioned mice did not alter the state of phosphorylation of DARPP-32 at Thr-34. In contrast, a single injection of L-DOPA (20 mg/kg) to 6-OHDA-lesioned mice produced a high increase in the phosphorylation of DARPP-32 at Thr34. Repeated administration of L-DOPA (20 mg/kg for 10 days) to 6-OHDA lesioned mice normalized this response. However, when the mice that received chronic L-DOPA were divided into highly and low dyskinetic we found that the levels of phospho-Thr34-DARPP-32 were still significantly elevated in the highly dyskinetic group. Accordingly, simple regression analysis revealed the existence of a positive

correlation between severity of AIMs and levels of phosphorylated DARPP-32. This finding indicates that, in dyskinetic mice, chronic L-DOPA fails to normalize the increase in D1R sensitivity caused by DA depletion.

4.3 LID IS ATTENUATED IN DARPP-32-NULL MICE (PAPER I)

Based on the positive correlation between severity of dyskinesia and phosphorylation of DARPP-32 and in order to understand the possible involvement of DARPP-32 in the generation of dyskinesia, we proceeded to examine LID in DARPP-32-null mice (Paper I).

Administration of L-DOPA (20 mg/kg) for 10 days induced dyskinesia in both WT and DARPP-32 KO mice. However, AIMs were significantly lower in DARPP-32 KO mice as compared to WT mice. This decrease was attributable to a significant reduction of axial, limb and orofacial (ALO) AIMs. In contrast, no difference was found in the ability of the two strains of mice to express locomotive AIMs. In this regard, it should be mention that the ALO AIMs are generally regarded as a more predictive marker of LID than locomotive AIMs (Andersson et al., 1999). Furthermore, it has been show that ALO are associated with molecular changes (e.g. increased levels of *fosB/ΔfosB* mRNA) occurring in the dorsolateral portion of the striatum, which is specifically innervated by nigrostriatal fibers (i.e. by the fibers that degenerate selectively in PD) (Andersson et al., 1999).

4.4 PHOSPORYLATION OF THR-34-DARPP-32 OCCURS IN MSNS OF STRIATONIGRAL PATHWAY (UNPUBLISHED RESULTS II)

Our results indicate that, in the striatum, enhanced phosphorylation of DARPP-32 results in reduced dyskinesia. However, DARPP-32 is expressed in all striatal MSNs. Therefore, we investigated whether the increase in DARPP-32 phosphorylation associated to LID occurred in the MSNs of the direct or in those of indirect pathway (Unpublished results II). To study DARPP-32 phosphorylation selectively in these two populations of neurons we made use of double BAC transgenic mice that express Flag-tagged- and Myc-tagged-DARPP-32 under the control of *Drd1a*- and *Drd2*-promoter, respectively. After lesion with 6-OHDA, the mice received L-DOPA (20mg/kg) for 10 days. As expected, this treatment induced severe dyskinesia in both D1R-DARPP-32 Flag and D2R-DARPP-32-Myc transgenic mice. The average score

of total AIMs in these animals was similar to that previously observed in C57BL6 mice, indicating the absence of intracellular signaling alteration in MSNs expressing tagged DARPP-32 (Bateup et al., 2008).

Western blotting analysis revealed that phospho-Thr34-DARPP-32 was significantly increased in the striatonigral MSNs of dyskinetic mice, whereas no change was found in the state of phosphorylation of DARPP-32, in striatopallidal MSNs (Figure 6).

These results indicated that, in the DA depleted striatum, continuous administration of L-DOPA induced abnormal phosphorylation of DARPP-32 specifically in striatonigral (D1R expressing) MSNs. Importantly, preliminary results obtained in conditional KO mice lacking DARPP-32 in striatonigral, or striatopallidal MSNs show that deletion of DARPP-32 in striatonigral MSNs is crucial for the generation of LID. Taken together, these data underlie the importance of direct pathway neurons in the generation of LID.

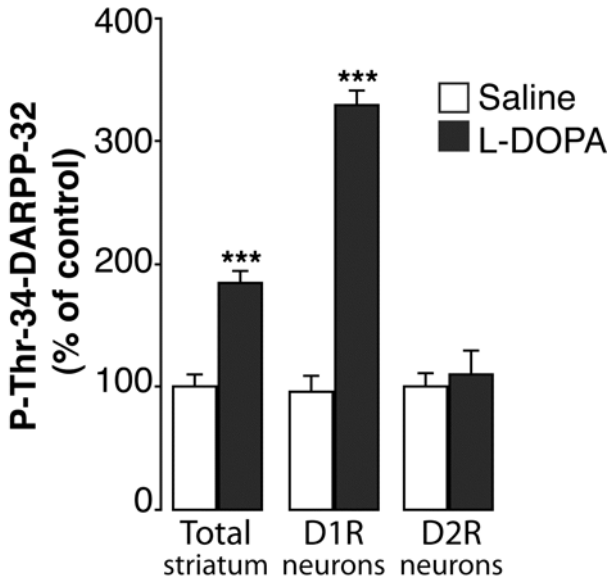

Figure 6. LID-associated phosphorylation of Thr34-DARPP-32 occurs in striatonigral MSNs. 6-OHDA-lesioned D1R/D2R-DARPP-32-Flag/Myc mice were treated for 10 days with saline (white bars) or L-DOPA (20 mg/kg; black bars) and were sacrificed 30 minutes after the last injection. DARPP-32 was immunoprecipitated from striatal extracts and phospho-Thr34-DARPP-32 was determined by Western blotting. Phospho-Thr34 data were normalized to total DARPP-32 levels expressed as percent of 6-OHDA-lesioned saline control. Left, data from endogenous non-tagged DARPP-32 (Total striatum). Center, data from Flag-tagged DARPP-32 (D1R neurons). Right, data from Myc-tagged DARPP-32 (D2R neurons). The bar graphs are a summary of the data represented as means ± SEM (n=10-20). ***$p<0.001$ vs. respective control 6-OHDA-lesioned mice treated with Saline, Student's *t*-test.

4.5 PKA-MEDIATED PHOSPHORYLATION OF GLUR1 IS ASSOCIATED WITH LID AND IS ATTENUATED IN DARPP-32-NULL MICE (PAPER I)

AMPA receptor transmission has been involved in the generation of diskinesia (Konitsiotis et al., 2000). In fact, it has been demonstrated that, in MPTP-lesioned primates, an AMPA receptor agonist increases dyskinesia, while blockade of the receptor results in a significant attenuation of LID (Konitsiotis et al., 2000).

AMPA receptors are phosphorylated by PKA at Ser845 of the GluR1 subunit (Roche et al., 1996). It has been proposed that phosphorylation at Ser845 promotes glutamate AMPA receptor transmission by increasing open AMPA channel probability (Banke et al., 2000) and surface expression (Mangiavacchi and Wolf, 2004). On the basis of the above observations and considering the increase in PKA-activity associated to LID, we decided to investigate the state of phosphorylation of GluR1 in DA depleted and dyskinetic mice (Paper I).

Administration of L-DOPA to sham-lesioned mice did not affect GluR1 phosphorylation. In contrast, a single injection of L-DOPA (20 mg/kg) to 6-OHDA-lesioned mice increased the phosphorylation of GluR1 at Ser845. This response appeared to involve specifically PKA-mediated signaling, as suggested by unaltered phosphorylation of GluR1 at Ser831, a calcium/calmodulin protein kinase II and protein kinase C dependent site (Roche et al., 1996; Mammen et al., 1997). Repeated administration decreased the ability of L-DOPA to induce PKA-mediated phosphorylation of GluR1. However, we found that the levels of phospho-Ser845-GluR1 were consistently higher in highly dyskinetic mice as compared to low dyskinetic or non-dyskinetic mice. In line with this observation, simple regression analysis revealed the existence of a positive correlation between severity of AIMs and phosphorylation of GluR1 at Ser845. These results contribute to explain previous data showing that LID is accompanied by the inability of corticostriatal synapses to undergo depotentiation (Picconi et al., 2003). Thus, in dyskinetic animals, increased phosphorylation of GluR1, which increases surface expression of AMPA receptors (Mangiavacchi and Wolf, 2004), may hamper corticostriatal depotentiation by potentiating glutamatergic transmission.

DARPP-32 is involved in PKA-mediated phosphorylation of GluR1 (Snyder et al., 2000). Therefore, we decided to examine the possible role of DARPP-32 in the increase of phospho-Ser845-GluR1 observed in dyskinetic mice (Paper I). We found that the ability of L-DOPA to induce phosphorylation of GluR1 was significantly

reduced in DARPP-32 KO mice as compared to WT mice. The mechanism responsible for the decreased levels of phospho-Ser845-GluR1 in DARPP-32 KO mice can be ascribed to the role of phosphorylated DARPP-32 in cAMP/PKA-mediated signaling. In fact, it has been shown that phospho-Thr34-DARPP-32 amplifies cAMP/PKA-dependent phosphorylation, by inhibiting PP-1-mediated dephosphorylation of downstream protein targets, including GluR1 (Snyder et al., 2000; Hakansson et al., 2006). Thus, the lack of DARPP-32-mediated amplification may result in a partial reduction of cAMP/PKA signaling, leading to reduced GluR1 phosphorylation.

4.6 LID IS ASSOCIATED WITH HYPERPHOSPHORYLATION OF ERK1/2 (PAPER I AND II)

It has been shown that, in 6-OHDA lesioned mice, chronic treatment with L-DOPA stimulates ERK1/2 phosphorylation (Pavón et al., 2006). Therefore, we examined the possibility that changes in phosphorylation of ERK1/2 were implicated in dyskinesia (Paper I). We found that, after DA depletion, administration of L-DOPA (20 mg/kg) induced a large increase in phospho-ERK1 and -ERK2 (Paper I and II). This effect was attenuated after repeated administration of L-DOPA (20mg/kg for 10 days), but remained more pronounced in highly dyskinetic mice. Simple regression analysis revealed a positive correlation between increased ERK1 and ERK2 phosphorylation and severity of dyskinesia. These results are in line with previous reports indicating that striatal DA depletion confers to a D1R agonist the ability to stimulate ERK phosphorylation (Gerfen et al., 2002; Kim et al., 2006). Using different models of LID, these results have also been confirmed by other laboratories (Westin et al., 2007; Nicholas et al., 2008; Schuster et al., 2008).

4.7 ACTIVATION OF ERK1/2 IS RESTRICTED TO STRIATONIGRAL MSNs (PAPER II)

In Paper II we investigated the specific localization of activated ERK in sriatonigral and striatopallidal MSNs. To distinguish between the two populations of striatal MSNs we made use of BAC transgenic mice expressing enhanced green fluorescent protein (EGFP) under the control of the promoter for the D1R (*Drd1a*-EGFP), or the D2R (*Drd2*-EGFP) (Gong et al., 2003; Bertran-Gonzalez et al., 2008;

Matamales et al., 2009). Immunofluorescence analyses revealed that the increased phosphorylation of ERK induced by L-DOPA (20mg/kg) in 6-OHDA-lesioned mice, occurred exclusively in striatonigral MSNs. This effect was present at 30 minutes after the administration of L-DOPA and persisted for at least 1 hour.

We proceeded by analyzing the pattern of ERK activation in dyskinetic *Drd1a*- and *Drd2*-EGFP mice. We found that, in these mice, the pattern of ERK activation was indistinguishable from that observed after acute injection of L-DOPA.

These results indicate that, following loss of DA in the basal ganglia, striatal MSNs are critically affected by changes in signal transduction properties. Importantly, our findings also demonstrate that changes in the state of phosphorylation of ERK1/2 and DARPP-32 associated to dyskinesia occur in the same group of striatal neurons, i.e. the MSNs of the direct pathway. This result is in contrast with a recent report showing the ability of L-DOPA to increase ERK in both striatonigral and striatopallidal MSNs of dyskinetic rats (Westin et al., 2007). This difference may be attributable to the different animal model used (rats vs. mice in this study).

4.8 ACTIVATION OF ERK1/2 IS INDUCED UPON STIMULATION OF D1Rs AND IS ATTENUATED IN DARPP-32-NULL MICE (PAPER I AND II)

It has been reported that, following striatal DAergic denervation, a D1R agonist acquires the ability to increase the state of phosphorylation of ERK (Gerfen et al., 2002). Moreover, data obtained in a rat model of LID suggested that the increase in ERK phosphorylation produced by L-DOPA is mediated via activation of D1Rs (Westin et al., 2007).

Based on this evidence, we investigated the role of D1Rs in L-DOPA-induced activation of ERK1/2 using our mouse model of LID (Paper II). We found that the ability of L-DOPA to increase ERK phosphorylation in the dorsal striatum was prevented by administration of the D1R antagonist SCH23390, but not by administration of the D2R antagonist, raclopride. These results, which are supported by recent evidence obtained in 6-OHDA lesioned D1R- and D2R-null mice (Darmopil et al., 2009), further support the idea that L-DOPA induces activation of ERK in the MSNs of the direct pathway (which selectively express D1Rs).

D1R-mediated activation of ERK may involve PKA-dependent phosphorylation/activation of Ras-GRF1 or activation of NMDARs (see

Introduction). We did not investigate the role NMDAR-dependent activation of ERK in LID. However, it has been reported that activation of ERK is dependent by concomitant activation of D1Rs and NMDARs (see Girault et al., 2007). Interestingly, NMDAR transmission has been involved in the generation of LID (Hallett et al., 2005) and NMDAR antagonists are effective in reducing dyskinesia in animal model of LID (Hadj Tahar et al., 2004; Wessell et al., 2004). NMDAR transmission could promote ERK activation by increasing intracellular Ca^{2+} concentration. Ca^{2+} promotes the activation of ERK by increasing the activity of GEFs proteins upstream of ERK signaling cascades, such as Ras-GRF1 and CalDAG-GEFs (see Introduction). Recent work has shown that the levels of CalDAG-GEFs proteins are changed in the striatum of dyskinetic rats (Crittenden et al., 2009) and that increased levels of CalDAG-GEFII may concur to the increase in ERK phosphorylation associated to LID (Crittenden et al., 2009).

It has been reported that dopaminomimetic drugs activate ERK1/2 through a mechanism involving PKA mediated phosphorylation of DARPP-32 (Valjent et al., 2005). In line with these observations, we found that the ability of L-DOPA to induce phosphorylation of ERK was significantly reduced in DARPP-32 KO mice (Paper I). This finding is in contrast with a recent report indicating that increased ERK phosphorylation induced by L-DOPA is maintained in 6-OHDA-lesioned DARPP-32 KO (Gerfen et al., 2008). These different results can be explained by taking into account the site of the lesion (SNc vs. striatum in this study) and the number of subjects analyzed (n=5 vs. n=19 DARPP-32 KO mice in this study). However, preliminary results obtained using conditional KO mice lacking DARPP-32 in striatonigral MSNs support the involvement of phosphoThr34-DARPP-32 in the activation of ERK produced by L-DOPA.

In conclusion, our results indicate that LID is accompanied by enhanced cAMP and ERK signaling in the striatal MSNs of the direct pathway. They also suggest that decreased activation of ERK1/2, together with reduced phosphorylation of GluR1, may represent the molecular mechanism underling the attenuated dyskinetic phenotype observed in DARPP-32 KO mice.

4.9 LID IS ASSOCIATED WITH AN INCREASED PHOSPHORYLATION OF MSK1 AND HISTONE H3 (PAPER I)

It has been shown that activation of ERK results in sequential phosphorylation of MSK1 and histone H3 (Deak et al., 1998; Brami-Cherrier et al., 2005). This in turn leads to changes in chromatin structure and transcriptional regulation (Nowak and Corces, 2004). In Paper I we investigated whether the increase in ERK phosphorylation associated to LID was accompanied by upregulation of phospho-MSK1 and phospho-histone H3. We studied the phosphorylation of MSK1 at the ERK-dependent site, Thr581, which is critical for kinase activation (Brami-Cherrier et al., 2005; McCoy et al., 2005). Phosphorylation of histone H3 was studied at the MSK1 dependent site, Ser10 (Brami-Cherrier et al., 2005).

Immunofluorescence analysis revealed that LID was accompanied by increased phosphorylation of MSK1 and histone H3. Simple regression analysis showed a positive correlation between severity of dyskinesia and the number of phospho-MSK1- and phospho-histone H3-positive MSNs. It has been reported that, in hemiparkinsonian rats, acute administration of L-DOPA increases the phosphorylation of MSK1 at Ser376 (Westin et al., 2007), an autophosphorylation site important for the catalytic activity of MSK1 (McCoy et al., 2005). This observation supports the idea of enhanced MSK1 signaling and histone H3 phosphorylation in LID.

Acetylation of histone H3 is critical for transcriptional regulation and acts in synergism with phosphorylation (Nowak and Corces, 2004). We therefore investigated the levels of phospho-Ser10-acetyl-Lys14-histone H3 in the striata of dyskinetic mice. We found a positive correlation between the severity of dyskinesia and the increased level of phospho-acetylated histone H3.

Since activation of ERK signaling results in increased gene expression, we examined whether dyskinesia was accompanied by increased transcriptional activity. We found that the number of neurons immunoreactive for c-fos was significantly higher in dyskinetic mice as compared to mice showing low dyskinesia. In summary, these data indicate that, in the striatum of dyskinetic mice, activation of ERK promotes the phosphorylation of MSK1 and histone H3, leading to increased gene expression.

4.10 ACTIVATION OF MSK1 AND HISTONE H3 IS RESTRICTED TO STRIATONIGRAL MSNs AND REQUIRES ACTIVATION OF D1Rs (PAPER II)

In Paper II, we utilized *Drd1a*- and *Drd2*-EGFP mice to examine the localization of phospho-Thr581-MSK1 and phospho-Ser10-acetyl-Lys14-histone H3 in the MSNs of the direct and indirect pathway. Moreover, we investigated the role of D1Rs and D2Rs in the modification of the state of phosphorylation of these proteins. Our results indicate that, in the DA depleted striatum, L-DOPA induced phosphorylation of MSK1 and acetyl-Lys14-histone H3 selectively in the MSNs of the direct pathway. This pattern of distribution overlaps with that of activated ERK, further indicating that MSK1 and histone H3 represents the nuclear effectors targets of ERK.

Diskinetic mice showed the same pattern of phosphorylation of MSK1 and histone H3 observed after acute L-DOPA treatment. Importantly, in these mice phosphorylated MSK1 and histone H3, remained restricted to striatonigral MSNs. The changes in phospho-acetylated-histone H3 are more sustained in the dorsolateral striatum, which is considered to be specifically involved in the development of AIMs (Andersson et al., 1999). Recently, our results on the regulation of phospho-Ser10-acetyl-Lys14-histone H3 have been confirmed by another group (Darmopil et al., 2009).

The increase in phospho-Thr581-MSK1 and phospho-Ser10-acetyl-Lys14-histone H3 induced by L-DOPA was blocked by SCH2339 (0.125 mg/kg), but not by raclopride (0.25 mg/kg). Similar results, indicating the selective involvement of D1Rs in the action of L-DOPA, have been obtained for phospho-Ser376-MSK1 in hemiparkinsonian rats (Westin et al., 2007) and for phospho-Ser10-acetyl-Lys14-histone H3 in 6-OHDA lesioned D1R-null mice (Darmopil et al., 2009).

4.11 BLOCKADE OF ERK1/2 SIGNALING COUNTERACTS THE DEVELOPMENT OF LID (PAPER I)

In Paper I, we investigated the possible role played by increased ERK signaling in LID by using SL327, a MEK inhibitor which prevents phosphorylation of ERK. Administration of SL327 (75 mg/kg; i.p.) abolished the phosphorylation of ERK1/2, MSK1, histone H3 and acetyl histone H3 induced by L-DOPA (20 mg/kg), further demonstrating that MSK1 and histone H3 are regulated through ERK. Most

importantly, we found that, when combined with L-DOPA, SL327 reduced dyskinesia. Recently, these results were confirmed in dyskinetic rats by using lovastatin, a drug which also reduces ERK signaling (Schuster et al., 2008).

The data described above suggest that SL327 may counteract dyskinesia via inhibition of long-term modifications occurring in striatonigral MSNs and related to abnormal regulation of nuclear targets of ERK (e.g., MSK1 and histone H3) implicated in transcriptional control. Whereas the demonstration of the direct involvement of MSK1 in LID will require additional studies, it is interesting to note that ERK-dependent activation of MSK1 has been implicated in psychomotor sensitization induced by cocaine (Brami-Cherrier et al., 2005).

4.12 L-DOPA ACTIVATES MTORC1 SIGNALING IN THE DA DEPLETED STRIATUM: PHOSPHORYLATION OF S6K, S6 AND 4E-BP (PAPER III)

It has been reported that ERK participates in the activation of mTOR, which is an important regulator of protein synthesis [(Costa-Mattioli et al., 2009); cf. Introduction]. In Paper III we examined the effect produced by L-DOPA on multiple targets of mTORC1, which is specifically involved in the regulation of translational efficiency.

Administration of L-DOPA to 6-OHDA lesioned mice increased the state of phosphorylation of S6K at Thr389 and 4E-BP at Ser65, which are directly regulated by mTORC1 (Pullen and Thomas, 1997; Hay and Sonenberg, 2004). L-DOPA also increased the phosphorylation of S6 at Ser240/Ser244 (a dual site regulated by S6K) and Ser235/Ser236 (another dual site regulated by S6K and ERK) (Ruvinsky and Meyuhas, 2006). Phosphorylation of these sites is necessary to activate S6 (Ruvinsky and Meyuhas, 2006). The ability of L-DOPA to promote mTORC1 signaling in the striatum was dependent on DA depletion, because neither L-DOPA nor the 6-OHDA lesion alone affected the state of phosphorylation of S6K, S6 and 4E-BP.

These results indicate that, in a mouse model of PD, administration of L-DOPA activates mTORC1 signaling and for the first time link DAergic transmission and mTOR signaling in the striatum. Changes in mTOR signaling have been proposed to occur in neurodegenerative processes (Malagelada et al., 2006; Malagelada et al., 2008). For instance, it has been reported that RTP801, a protein inhibitor of mTOR, is highly expressed in the SNc of PD patient and MPTP-treated mice (Malagelada et al.,

2006; Malagelada et al., 2008). However, the studies described in this thesis, which are limited to the striatum, suggest that the upregulation of RTP801 is restricted to DAergic neurons of the nigrostriatal pathway.

4.13 ACTIVATION OF S6K, S6 AND 4E-BP OCCURS IN MSNs OF THE STRIATONIGRAL PATHWAY (PAPER III)

We next examined the role of D1Rs and D2Rs in the activation of mTORC1 signaling induced by L-DOPA (Paper III). Administration of the D1R antagonist (SCH23390; 0.125 mg/kg) abolished the ability of L-DOPA (20 mg/kg) to stimulate the phosphorylation of S6K, S6 and 4E-BP in DA depleted striatum. In contrast, a D2R antagonist (raclopride; 0.2 mg/kg) did not change the effects produced by L-DOPA. These results were confirmed by immunofluorescence analysis of phosphorylated S6.

We also studied the localization of S6 phosphorylation using *Drd1a*- and *Drd2*-EGFP mice. We found that L-DOPA-induced phosphorylation of S6 at Ser240/244 and Ser235/236 occurred selectively in striatonigral MSNs. Overall these data show that L-DOPA promotes mTORC1 signaling in the DA depleted striatum via activation of sensitized D1Rs and that this effect is restricted to the MSNs of the direct pathway.

4.14 L-DOPA INCREASES MNKS-DEPENDENT PHOSPHORYLATION OF EIF4E (PAPER III)

mTORC1-dependent phosphorylation of 4E-BP results in the dissociation of eIF4E, which leads to the formation of the eIF4F initiation complex (Pyronnet et al., 1999). eIF-4E is phosphorylated at Ser209 by Mnks, which, in turn, are activated by ERK1/2 (Waskiewicz et al., 1997; Pyronnet et al., 1999). In Paper III, we found that, in 6-OHDA lesioned mice, administration of L-DOPA increased phospho-Thr197/202-Mnks and phospho-Ser209-eIF4E and that this effect was blocked by SCH23390 (D1R antagonist) but not by raclopride (D2R antagonist).

These results suggest that activation of ERK promotes the phosphorylation of Mnks and eIF4E in the MSNs of the direct pathway. Phosphorylation of eIF4E is regarded as an index of eIF4F complex assembly (Pyronnet et al., 1999).

Hyperphosphorylation of eIF4E together with activation of mTORC1 signaling may lead to increase protein translation in 6-OHDA lesioned mice treated with L-DOPA.

4.15 ERK IS INVOLVED IN L-DOPA-MEDIATED ACTIVATION OF MTORC1 (PAPER III)

It has been reported that ERK promotes activation of mTORC1 through activation of p90RSK and phosphorylation/activation of Raptor (Carrière et al., 2008). ERK can also increase the phosphorylation of TSC2 (Roux et al., 2004; Ma et al., 2005), which results in activation of mTORC1, via stimulation of Rheb (Long et al., 2005). Therefore, we investigated the involvement of ERK in L-DOPA-induced activation of mTORC1 (Paper III).

Striatal slices obtained from 6-OHDA lesioned mice were incubated with a D1R agonist (SKF81297; 1μM) in the presence or absence of an inhibitor of MEK (U0126; 25μM). We found an increase in the state of phosphorylation of S6 and 4E-BP, confirming the critical role played by D1Rs in L-DOPA induced activation of mTORC1. This effect was paralleled by enhanced ERK phosphorylation, which was blocked by U0126. Most importantly, U0126 also abolished the phosphorylation of S6 and 4E-BP mediated by SKF81297 without affecting the level of phospho-Ser845-GluR1, which is a PKA target. These results indicate that ERK is involved in the D1R-dependent activation of mTORC1 signaling pathway.

4.16 ACTIVATION OF MTORC1 SIGNALING IS ASSOCIATED WITH LID (PAPER III)

Our findings demonstrated that, in a mouse model of PD, administration of L-DOPA results in activation of mTORC1 signaling and in the formation of the initiation of translation complex, eIF4F. Increased translational activity may be involved in long-term adaptive response to L-DOPA, including LID.

The involvement of abnormal mTORC1 signaling in dyskinesia was suggested by Western blotting experiments, in which we show a positive correlation between severity of LID and increased phosphorylation of S6K, S6, 4E-BP, eIF4E and Mnks (Paper III). These results were confirmed by immunofluorescence analyses performed in *Drd1a*- and *Drd2*-EGFP mice and showing increased levels of phosphorylated S6 in the striatonigral MSNs of dyskinetic mice.

4.17 BLOCKADE OF MTORC1 SIGNALING REDUCES LID (PAPER III)

The activation of the mTORC1 pathway in striatonigral MSNs persists during chronic L-DOPA administration and correlates with the severity of AIMs. We therefore examined the role of mTORC1 in the development of dyskinesia by using the specific mTORC1 inhibitor, rapamycin (Paper III).

We found that rapamycin reduced the phosphorylation of S6K, S6, 4E-BP and eIF4E produced by L-DOPA, without affecting the phosphorylation of ERK and GluR1. Importantly, downstream nuclear targets of ERK, such as histone H3, were not affected by rapamycin. The abolishment of L-DOPA-induced eIF4E phosphorylation by rapamycin supports the idea that Mnks-mediated phosphorylation of eIF4E occurs only after its dissociation from the repressor 4E-BP.

We then proceeded by examining the effect of rapamycin on LID. 6-OHDA lesioned mice were treated with L-DOPA (10 mg/kg) in the presence or absence of rapamycin (2 and 5 mg/kg; i.p.) for 9 days. On the 10^{th} day the mice received only L-DOPA and the AIMs were analyzed. A strong reduction of dyskinesia was found in the mice treated with a combination of L-DOPA and rapamycin. The ability of rapamycin to inhibit LID raised the possibility that this drug might also affect the antiparkinsonian, positive action of L-DOPA. We tested this possibility by examining the anti-akinetic effect of L-DOPA in the presence or absence of rapamycin, using the cylinder test. We found that rapamycin did not modify the ability of L-DOPA to improve forelimb akinesia induced by DA depletion.

5 CONCLUSIONS AND FUTURE DIRECTIONS

The major goal of this thesis was the elucidation of signal transduction processes occurring in the striatum and linked to PD and dyskinesia. Our studies have identified a series of abnormalities affecting cAMP, ERK and mTOR signaling and implicated in LID. We have also provided evidence demonstrating that these changes affect a specific group of striatal projection neurons, corresponding to the MSNs of the direct, striatonigral pathway.

One important question concerns the further characterization of mTOR signaling in dyskinesia. Activation of mTOR leads to the formation of two complexes: mTORC1, which includes mTOR and the interacting protein Raptor, and mTORC2, which includes mTOR and Rictor. mTORC1 is involved in the regulation of mRNA translation, whereas mTORC2 is implicated in actin organization and cell survival. Rapamycin inhibits preferentially mTORC1, but its action is often limited in time and insufficient to produce a complete inactivation of mTORC1 signaling. Moreover, rapamycin can act as a cell-type-specific inhibitor of mTORC2 (Guertin and Sabatini, 2009). It will be important to examine the relative contribution of mTORC1 and mTORC2 to striatal signaling and, particularly, to the development of dyskinesia.

The involvement of mTOR in dyskinesia suggests that some of the mRNAs whose translation is promoted via mTOR signaling (and suppressed by rapamycin) may be responsible for LID. One possible strategy to address this point is to use BAC transgenic mice that express EGFP-tagged ribosomal protein L10a in D1, or D2 receptor expressing MSNs (Doyle et al., 2008). The use of these mice will allow the purification of polysomal mRNAs from striatal MSNs of the direct and indirect pathway. It will then be possible to examine the effect of rapamycin on the translational profile of the MSNs of the direct and indirect pathway.

It will be also necessary to examine the effect of rapamycin and rapamycin analogs for their ability to reduce dyskinesia in a more advanced animal model of dyskinesia. In particular, mTORC1 inhibitors could be tested in MPTP-intoxicated monkeys subjected to chronic administration of L-DOPA. These studies will further strengthen the idea of the involvement of mTOR signaling in dyskinesia and will ultimately demonstrate the efficacy of drugs interfering with this signaling pathway for the treatment of this disorder.

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