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Leucine-rich repeat kinase 2, synaptic morphology and motor behavior

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ABSTRACT

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are known to cause late-onset, familial forms of Parkinson's Disease, with prevalence up to 40% in specific populations. These mutations are also linked to sporadic PD, highlighting the importance of the related protein in the development of the disease.

LRRK2 is a large, complex, multi-domain protein, with two main enzymatic activities: a GTPase domain (bearing three pathogenic mutations) and a kinase domain, in which the most common mutation (G2019S) is found.

Even though the precise cellular functions of LRRK2 are not known, LRRK2 has been proposed to take part in key signaling pathways, ultimately governing cellular functions such as synaptic transmission, synaptic vesicle dynamics, autophagy and membrane-to-Golgi trafficking.

Major efforts have been dedicated to development and characterization of animal models of LRRK2-induced parkinsonism. However, transgenic models reported so far failed in showing parkinsonian phenotype and neuropathology.

In this work, we used three different mouse lines (non-transgenic, BAC hLRRK2-G2019S and LRRK2 knockout) to explore spontaneous and physiologically-stimulated motor behavior as well motor responses to dopaminergic compounds. We found that the G2019S mutation leads to motor impairment which is responsive to dopamine agonists, while ablation of the protein causes hyperactivity and reduced anxious-like behavior. Moreover, mice expressing the G2019S mutation and knockout mice showed opposite responses to dopamine receptor stimulation.

In order to investigate the role of endogenous LRRK2 on synaptic morphology and connectivity, we co-cultured striatal medium spiny neurons (MSN) and cortical neurons and applied the LRRK2 kinase inhibitor LRRK2-IN1. We observed a tendency for an increased presynaptic drive towards MSNs and reduction of their postsynaptic receptiveness.

These results show that LRRK2 is profoundly involved in regulation of motor and non-motor behavior, with pathogenic mutation leading to the development of a parkinsonian phenotype. This

role of LRRK2 is intimately associated with dopamine machinery. We also propose LRRK2 to participate in the maintenance and regulation of cortico-striatal synapses *in vitro*.

ABSTRACT	3
INTRODUCTION	8
Parkinson's Disease: clinical aspects	9
Parkinson's Disease: therapeutic interventions	11
Evolution of the classical view of PD	14
Genetics of PD	15
Dominantly inherited mutations	16
<i>α-synuclein</i>	16
<i>Leucine-rich repeat kinase 2</i>	18
<i>Vacuolar protein sorting 35</i>	19
<i>Eukaryotic translation initiation factor 4-gamma</i>	20
Recessively inherited mutations	22
<i>Parkin</i>	22
<i>PTEN-induced kinase 1</i>	23
<i>DJ-1</i>	24
LRRK2: structure, properties and neurobiology	25
<i>Biochemistry and cellular biology of LRRK2</i>	25
<i>LRRK2 functions in animal models</i>	29
AIMS OF STUDIES	33
MATERIALS AND METHODS	35
IN VIVO EXPERIMENTS	36
Subjects	36
Behavioral experiments	36

<i>Physiologically-stimulated motor activity</i>	36
<i>Spontaneous motor activity</i>	38
<i>Injected drugs</i>	39
IN VITRO EXPERIMENTS	40
Primary neuronal culture and transfection	40
Treatment protocol	41
Immunocytochemistry	41
Microscopy and image analysis	42
DATA PRESENTATION AND STATISTICAL ANALYSIS	43
RESULTS	44
IN VIVO EXPERIMENTS	45
Characterization of motor phenotype in wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice	45
<i>Effect of aging</i>	45
<i>Physiologically-stimulated motor activity: motor characterization</i>	46
<i>Spontaneous motor activity</i>	48
<i>Pharmacological testing in 6 month-old mice</i>	49
L-DOPA	50
Pramipexole	50
Haloperidol	51
SCH23390	52
SKF38393	53
IN VITRO EXPERIMENTS	55
Co-cultured striatal and cortical neurons	55
<i>Effect of LRRK2-IN1 in MSNs</i>	57

<i>Effect of LRRK2-IN1 in CTX neurons</i>	61
DISCUSSION	65
LRRK2 plays an age-dependent role on motor abilities	66
LRRK2 and the DA system interact in the modulation of motor behavior	69
LRRK2 kinase activity partially modulates synaptic morphology	71
Concluding remarks	73
ORIGINAL PAPERS	74
REFERENCES	75

INTRODUCTION

Parkinson's Disease: clinical aspects

Parkinson's disease (PD) is a complex, multifactorial neurologic disorder. It is the second most common neurodegenerative disease after Alzheimer's disease and the most common motor disease.

The disease was first described in 1817 by James Parkinson in the publication "An Essay on the Shaking Palsy", where he defined the disorder as "Shaking palsy (Paralysis Agitans): Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured".

This definition largely influenced our current vision of PD, which is still identified by a triad of motor manifestations including rigidity, bradykinesia and resting tremor. This triad accurately follows the original definition by James Parkinson. However, PD is now defined as a chronic and progressive neurodegenerative disorder characterized by motor and non-motor symptoms (Jankovic, 2008). In fact, "senses and intellects" are heavily affected during PD.

The appearance of motor symptoms is related to the degeneration of dopamine (DA) neurons in the substantia nigra pars compacta (SNc), resulting in depletion of DA in the striatum and other projection areas. Some of the surviving neurons present eosinophilic intracytoplasmic inclusions termed Lewy bodies (LB), which are proteinaceous in nature (Weintraub et al., 2008) and appear to represent the aftermath of the underlying pathology. However, recent evidence suggests these LBs are not deleterious to cells but may be even cytoprotective (McNaught and Olanow, 2006).

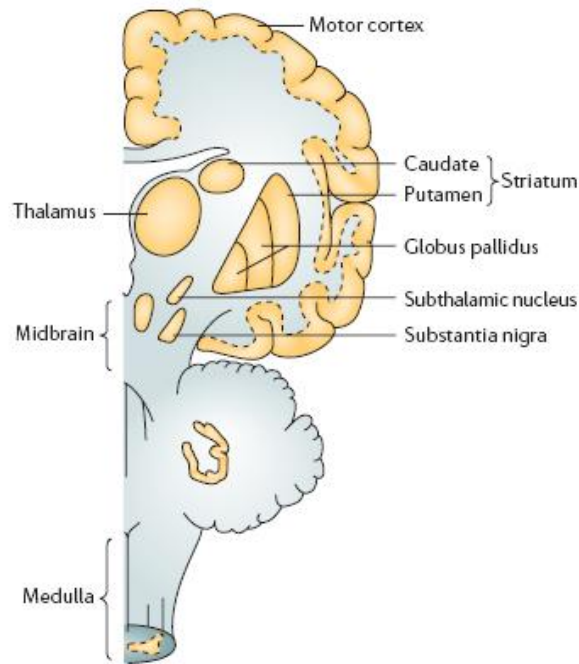


Fig. 1. Main brain areas affected in PD.

The standard method to assess motor symptoms and disability in PD is the Unified Parkinson's Disease Rating Scale (UPDRS; Ramaker et al., 2002), which can be applied both for patient management and clinical research. The cardinal motor symptoms of PD are rigidity, bradykinesia and resting tremor, complicated in later phases by postural instability, due to impairment of postural reflexes (Pallone 2007). Motor symptoms usually begin asymmetrically but gradually extend to the other side of the body (contralateral side). Asymmetric resting tremor is the common initial symptom (70%-90% of patients; Pallone 2007) and even though it is the most evident, it is rarely a major cause of disability.

On the other hand, the most disabling motor symptom is bradykinesia (slowness of movement), with akinesia (inability to initiate a movement) as its extreme. Bradykinesia also presents as very slow movement, hypophonia (weak voice or whispering as a result of uncoordination of muscles of vocalization), reduced dexterity, a masked face and drooling, decreased blink rate and a slow, shuffling gait.

The clinical manifestation of PD is not limited to motor deficit but also includes a variety of non-motor symptoms that significantly affect patients' quality of life (Chaudhuri et al., 2006). In this view, the UPDRS is undergoing revisions with the aim to improve sensitivity and integrate non-motor features of the disease (Goetz et al., 2007). Indeed, these signs and symptoms could even precede motor deficits by years and be underestimated in the clinic. It is now accepted that motor features, defined as parkinsonism, represent only one trait of a complex and multifaceted disorder as PD, comprising REM sleep disorders, anosmia, depression, dementia and autonomic failure (Langston 2006; Chaudhuri and Schapira, 2009). These features contribute to motor disability along the course of the disease, dominating the clinical picture as PD progresses, and eventually may contribute to shortened life expectancy (Chaudhuri et al., 2006).

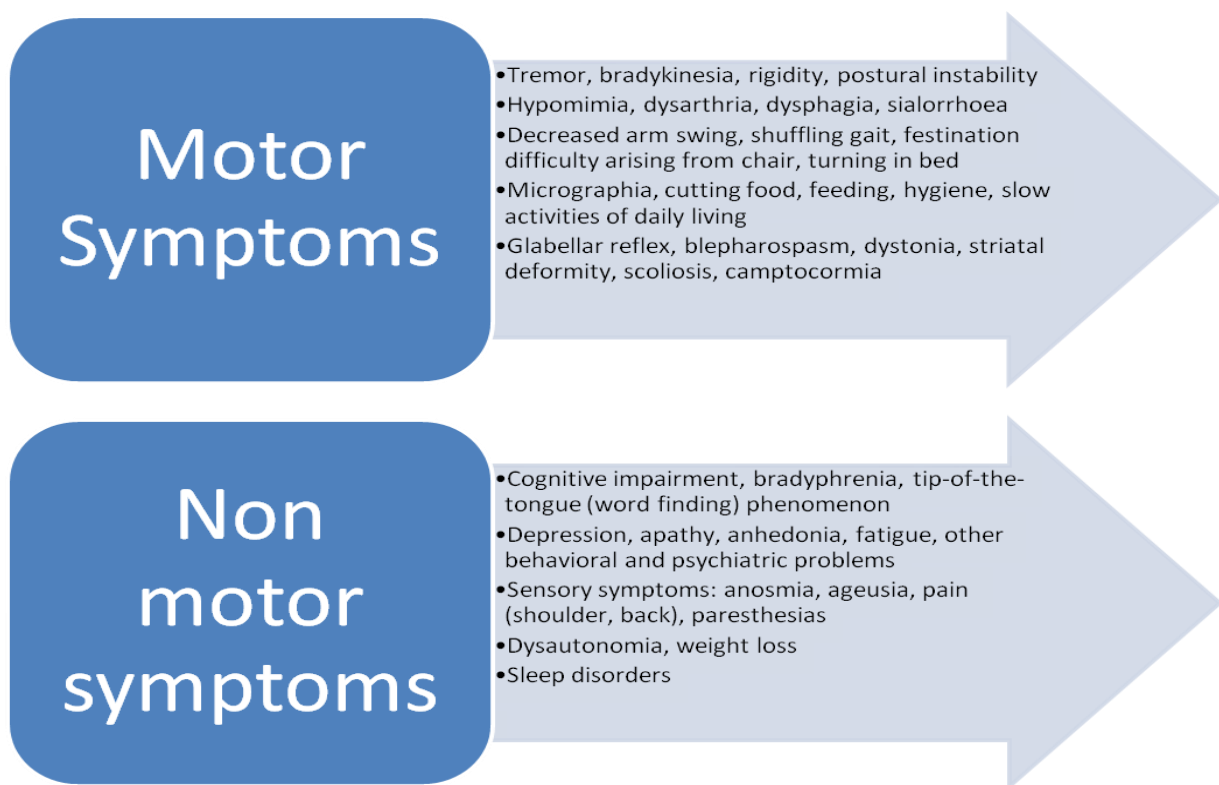


Fig. 2. Table summarizing PD symptomatology.

Parkinson’s Disease: therapeutic interventions

Since its introduction, L-DOPA remains the most effective symptomatic treatment of motor deficits, although it has less impact on non-motor symptoms (Chaudhuri and Schapira, 2009). Currently, no

treatment has proved effective in halting or slowing the progression of the neurodegenerative process (Meissner et al., 2011), leaving neuroprotection as an unmet clinical need.

Thus, the most important goals of clinical management are to preserve functional independence and health-related quality of life (Weintraub et al., 2008). In this view, treatment is directed towards symptomatic relief for both motor and non-motor features (Rezak 2007).

As mentioned above, L-DOPA is the cornerstone of the therapy of PD. As a prodrug, levodopa crosses the blood-brain barrier (BBB) and is decarboxylated to DA in the residual nigrostriatal neurons (Pallone 2007) and, as DA loss progresses, also in striatal serotonin (5-HT) terminals. The drug is always given in combination with a peripheral dopa-decarboxylase inhibitor, in order to improve central bioavailability and minimize adverse effects, such as nausea and hypotension, caused by peripheral action of DA (Rao et al 2006). L-DOPA is highly effective in the first phases of therapy, usually up to 5 years ('honeymoon period'). As treatment continues and PD progresses, motor complications, namely wearing-off (shortening of effect duration), dyskinesia (abnormal, involuntary movement) and motor fluctuations appear in virtually all patients.

Since the aim of L-DOPA therapy is to rescue the impairment in DA transmission, other therapeutic strategies involve the use of direct DA receptor agonists. These are used as monotherapy in early PD or in combination with L-DOPA in more advanced phases of the disease. There is an increasing use of these agents as initial therapy to help delay the need for L-DOPA and reduce overall L-DOPA dosage. However, effects are less robust and patients will eventually require L-DOPA.

Other pharmacological agents are currently used in the treatment of PD, and most of them maximize the beneficial effects of L-DOPA by augmenting its efficacy and reducing its adverse effects, or cover symptoms not adequately managed by L-DOPA alone. These agents include: MAO-B (monoamino oxidase-B) inhibitors, that prevent DA metabolism in the brain; COMT (Catechol O-Methyltransferase) inhibitors, that inhibit peripheral metabolism of L-DOPA and/or central metabolism of DA; anticholinergic agents that antagonize muscarinic receptors and mainly relieve resting tremor.

Apart from pharmacological treatments, surgery options are also considered in PD therapy. Deep brain stimulation (DBS) of the subthalamic nucleus (STN) is an effective surgical procedure in selected candidates for treating medically resistant motor symptoms of PD (Okun et al., 2007).

Gene therapy is a novel therapeutic strategy applied to PD treatment, but with little results obtained so far (Meissner et al., 2011). For example, different clinical studies investigated the effects of the injections of viral vectors that drive the expression of neurturin/glia-derived neurotrophic factor (GDNF) in neurons (Gill et al., 2003; Lang et al., 2006; Marks et al., 2010; Bartus et al., 2011), failing to lead to significant improvements when a large placebo-controlled design was employed (Lang et al., 2006).

Recently, there has been great interest in human stem cells, which have been shown to survive, innervate to some extent and reverse motor dysfunction in rodent and nonhuman primate models of PD (Bjorklund et al., 2002; Cai et al., 2009; Redmond et al., 2007). Human embryonic stem cells are easy to manipulate, but they can form teratomas (Bjorklund et al., 2002). Immunological reactions could be avoided using induced pluripotent stem cells (iPS cells; Kiskinis et al., 2010; Soldner et al., 2009). Unfortunately, they did not show particular efficacy. More experimental data are needed to validate this strategy (see Obeso et al., 2010).

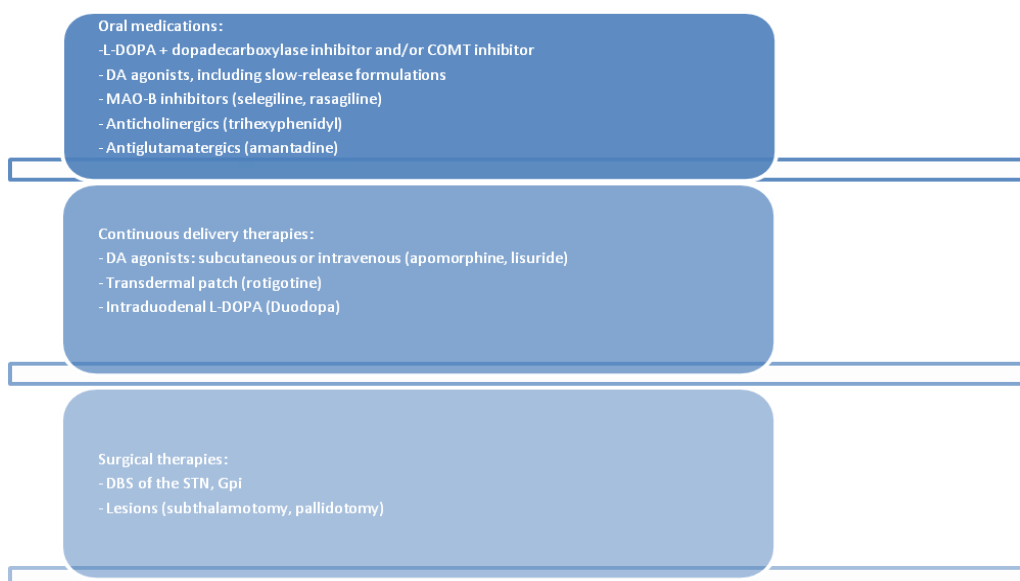


Fig. 3. Therapeutic treatments currently used in PD.

Evolution of the classical view of PD

The neuropathological origin of motor symptoms in PD is surprisingly still questionable. Neuronal cell loss has been reported in pre-supplementary motor cortical area in PD patients, and it has been suggested that this damage could occur early in the disease (MacDonald and Halliday 2002). In fact, changes in cortical activation patterns have been reported in concurrence with onset of motor symptoms (Fukuda et al., 2001; Sabatini et al., 2000; Thobois et al., 2000; Cunnington et al., 2001), questioning about the primary cause of motor dysfunction.

The neuronal damage associated with PD causes alterations in neurotransmitter release, not limited to a decrease of DA release in striatum and prefrontal cortex, as could be deduced by the classical histopathological view. The reported functional antagonism of DA and acetylcholine (ACh) transmission at the striatal level leads to increased ACh release (Pisani et al., 2007) although the opposite is true at the cortical level (Francis and Perry, 2007). Indeed, as mentioned earlier, anticholinergic drugs were among the first treatments clinically used to control PD motor symptoms. The 5-HT system is also affected: neuronal loss in the 5-HT-producing raphe nuclei has been reported in PD (Paulus and Jellinger, 1991; Gai et al., 1995). Consistently, reduced 5-HT levels appeared lower in the caudate nucleus and putamen (see Huot et al., 2011) and neocortex (Francis and Perry, 2007). However, animal models of parkinsonism gave conflicting results regarding the 5-HT system. In the MPTP-lesioned mouse a reduction of striatal 5-HT levels accompanied by 5-HT hyperinnervation was reported (Rozas et al., 1998). In other PD animal models the situation appears more variable (for a review, see Huot et al., 2011).

A major advancement in the field has been provided by Braak and colleagues (2003) who analysed and staged the LBs accumulation and pattern in PD patients of different ages, leading to the conclusion that PD spreads from lower brainstem to higher cortical areas. Central to the hypothesis of PD as a prionopathy, was also the evidence that α -synuclein acts and spreads like the prion protein, capable of neuron-to-neuron transmission (Olanow and Prusiner 2009; Desplats et al.,

2009), and that Lewy body-like pathology spreads from host tissue to embryonic nigral transplants (Kordower et al., 2008).

Among non-motor symptoms, dementia is one of the most common (Hely et al., 2008) and difficult to treat (Meissner et al., 2011). PD dementia (PDD) is close in phenotype to Dementia with Lewy Bodies (DLB), which is characterized by α -synuclein-positive inclusions and parkinsonism (Langston 2006), suggesting PD, PDD and DLB might be the same disease but with different points of origin.

It appears clear that the current view of PD is mostly an end-stage one, with attention focused on motor symptoms. The burst on the scene of SNCA (α -synuclein) has paved the way to disclose earlier events in PD pathogenesis.

Genetics of PD

Heritability in PD has long been debated, until Polymeropoulos and colleagues (1997) firstly described pathogenic missense mutations in the SNCA gene (coding for α -synuclein), followed by the discovery of additional genes.

At the moment, gene mutations shown to be causative of PD are grouped in 2 categories: dominantly inherited and recessively inherited mutations.

PARK loci	Gene	Chromosome	Form of PD	Mutations	Origin
PARK1	<i>SNCA</i>	4q21	AD	A30P, E46K, A53T	Greece and Italy
PARK2	<i>Parkin</i>	6q25.2–q27	AR J	Various mutations, exonic deletions, duplications and triplication	Japan
PARK3	Unknown	2p13	AD	–	Europe
PARK4	<i>SNCA</i>	4q21	AD	Duplication and triplication	Iowa
PARK5	<i>UCHL1</i>	4p14	AD and idiopathic	I93M and S18Y	Germany
PARK6	<i>PINK1</i>	1p35–p36	AR	G309D, exonic deletions	Italy
PARK7	<i>DJ-1</i>	1p36	AR and EO	Homozygous exon, deletion L166P	Europe
PARK8	<i>LRRK2</i>	12q12	AD and idiopathic	R1441C/G/H, Y1699C G2019S I2020T G2385R	Japan
PARK9	<i>ATP13A2</i>	1p36	Kufor-Rakeb syndrome and EO PD	Loss-of-function mutations	Jordan, Italy and Brazil
PARK10	Unknown	1p32	Idiopathic	–	Iceland
PARK11	Unknown	2q36–q37	AD and idiopathic	–	North America
PARK12	Unknown	X	Familial	–	North America
PARK13	<i>HTRA2</i>	2p13	Idiopathic	A141S G399S	Germany

Fig. 4. The 13 PARK loci described to date. VPS35 and eIF4G1 are excluded from this table since they do not map on the original loci. AD, autosomal dominant; AR, autosomal recessive; J, juvenile; EO, early onset.

Dominantly inherited mutations

α -Synuclein

As said, genetics studies in PD were boosted by the discovery of pathogenic, mutant forms of the SNCA gene. The related protein, α -synuclein, is expressed throughout the brain and has potential roles in learning, synaptic plasticity, vesicle dynamics and DA synthesis (Lotharius and Brundin 2002; Sidhu et al., 2004).

Missense mutations and gene multiplication increase the propensity of α -synuclein to aggregate, and indeed aggregates of beta-sheet-rich fibrillar forms of α -synuclein are the main constituents of LBs in PD and other LB diseases (Spillantini et al., 1998). The function of α -synuclein is still unclear but localization within nerve terminal and interaction with membranes suggest a role in regulation of vesicle dynamics, trafficking at the presynaptic terminal, brain lipid metabolism (Willingham et al., 2003; Darios et al., 2010) and neurotransmitters release at the presynaptic level

through promotion of assembly of the SNARE complex (Burre et al., 2010). Thus, it appears that membrane and lipid regulation pathways could be crucial in developing α -synuclein-related PD.

In addition to single-point mutations, duplication and triplication of the SNCA locus elevate α -synuclein expression and cause familial PD (Singleton et al 2003; Farrer et al 2004). However, it remains unclear whether α -synuclein expression is elevated in the brains of sporadic PD patients.

The implication of α -synuclein in PD pathology appears to be related to its structural properties. Given its presence in LBs as fibrillar forms, it has been thought that insoluble aggregates are cytotoxic. However, α -synuclein monomers interact to form pre-fibrillar aggregates or protofibrils, which in turn can form insoluble fibrils (Gosavi et al., 2002; Conway et al., 2000; Conway et al., 2001). A recent hypothesis proposes that protofibrils are the cytotoxic species, whereas the fibrillar aggregates of the protein could represent a cytoprotective mechanism in PD (Dev et al., 2003; Caughey and Lansbury, 2003). Supporting this hypothesis, α -synuclein protofibrils are increased in the brains of PD and DLB patients (Sharon et al., 2003), and have been associated with neurotoxicity in α -synuclein-overexpressing cells and mouse models (Masliah et al., 2000; Gosavi et al., 2002).

A number of animal models have been developed to study α -synuclein role in PD pathology. Overexpression of the protein in transgenic animals has been the most common method. In this regard, the promoter used to drive the overexpression appears to play an important role: the tyrosine hydroxylase (TH) promoter was used in several mouse lines, leading to interesting results on the induction of neurodegeneration (Thiruchelvam et al., 2004). However, this does not represent an ideal strategy because expression is restricted to catecholaminergic neurons, and models should mimic the broad but regionally selective α -synuclein pathology observed in patients (Halliday et al., 2006). In fact, other promoters like PDGF β and Thy1 have been used. Both confer broad expression of α -synuclein in neurons but with different patterns (Rockenstein et al., 2002) and functional outcomes. In particular, one line with the PDGF β promoter develops DAergic deficits in the striatum (Masliah et al., 2000, 2005). The Thy1 lines have a wide range of phenotypes, depending

on mutations, background strain, level of expression and/or the insertion site. Mice overexpressing wild-type α -synuclein under the control of Thy1 promoter have been extensively characterized. In these mice the protein is widespread at high levels in cortical and subcortical regions (including SNc; Rockenstein et al., 2002) with proteinase K-resistant inclusions of α -synuclein in key regions such as olfactory bulb, SN and locus coeruleus (Fernagut et al., 2007). This pattern represents a hallmark of α -synuclein pathology in PD brains (Neumann et al., 2004; Halliday et al., 2006).

In addition to transgenic methods, α -synuclein has been overexpressed using viral vectors. This approach leads to a rapid degeneration of nigrostriatal neurons, which is not yet reproduced by genetic mutations in mice and rats (Kirik and Bjorklund, 2003), even with the wild-type protein (Kirik et al., 2002).

Even though genetic models often lack nigrostriatal DA cell loss, considered a “hallmark of PD”, they strikingly reproduce early characteristics of the disease (Halliday and McCann, 2008; Braak et al., 2004) making them particularly useful for assessing pathogenic mechanisms and early therapeutic strategies.

Leucine-rich repeat kinase 2

Mutations in leucine-rich repeat kinase 2 (LRRK2) gene have recently been demonstrated to cause autosomal-dominant, late-onset PD (Zimprich et al., 2004; Paisan-Ruiz et al., 2004). The gene is located in the locus PARK8, which was originally mapped as an autosomal dominant trait in a Japanese family with asymmetrical, L-DOPA-responsive, late-onset PD (Funayama et al., 2002). Glycine to serine substitution in position 2019 is the most common LRRK2 mutation, and in specific populations it might account for 18-30% of PD cases (Ozelius et al., 2006; Lesage et al., 2006). Of note, disease penetrance in G2019S carriers is age dependent, representing a critical consideration in genetic counseling (Kachergus et al., 2005). Interestingly, the G2019S mutation is associated with a common haplotype throughout the world. This haplotype is smallest in Arabic

patients and indicates that this mutation might have originated in the Middle East (Kachergus et al., 2005; Ozelius et al., 2006; Lesage et al., 2006).

Since the LRRK2 protein is the main subject of this thesis, its structure, properties and biological significance will be introduced in a dedicated chapter.

Vacuolar protein sorting 35

Very recently, pathogenic mutations, and in particular the D620N substitution, were identified within vacuolar protein sorting 35 (VPS35) as a novel genetic determinant of autosomal dominant late-onset parkinsonism (Vilarino-Guell et al., 2011; Zimprich et al., 2011).

VPS35 is a component of the retromer cargo-recognition complex, critical for membrane-protein recycling and endosome-trans-Golgi network (TGN) trafficking. The TGN is the last sorting station of the secretory pathway from which soluble or membrane proteins and lipids are sorted for subsequent transport to different destinations: the cell surface, the endosomal system and synaptic domains in neurons (Anitei et al., 2010). Within the TGN, transmembrane proteins are sorted for delivery to the plasma membrane and the endosomal/lysosome systems. This includes transmembrane proteins cycling between the TGN and endosomes, such as mannose-6-phosphate receptors (MPR), which deliver bound lysosomal enzymes to endosomes in a mannose-6-phosphate (M6P) dependent manner (Munier-Lehmann et al., 1996; Ghosh et al., 2003). After unloading their bound ligands in endosomes, they return either to cell surface or TGN; membrane coats containing clathrin, GGA and AP-1 mediate the exit of hydrolase-MPR from TGN, whereas the retromer complex mediates the retrieval of unoccupied MPR from endosomes (Anitei et al., 2010; Rojas et al., 2008; Bonifacino and Hurley, 2008).

In addition to MPR recycling, retromer function is necessary for normal endocytosis, as in the case of progranulin, linked to fronto-temporal dementia (Anitei et al., 2010; Hu et al., 2010), and for processing of the amyloid precursor protein (APP) linked to Alzheimer's Disease (Nielsen et al., 2007; Vieira et al., 2010). Moreover, the retromer complex has important roles in actin cytoskeletal

organization, synapse formation (Korolchuk et al., 2007), Wnt signaling (Franch-Marro et al., 2008), mitochondrial peroxisome trafficking (Braschi et al., 2010) and nuclear export (Mingot et al., 2004). The role of retromer might be particularly relevant in neurons, given they are excitable cells that support ~10.000 times more plasma membrane surface than other cell types. Indeed, protein trafficking, recycling and degradation are essential for the creation and maintenance of neuronal architecture, synaptic plasticity and connectivity (Lasiacka and Winckler, 2011; Sann et al., 2009). VPS35 has not been extensively studied in the context of neurological disorders, but these recent genetic findings will prompt the investigation of its role in neurobiology.

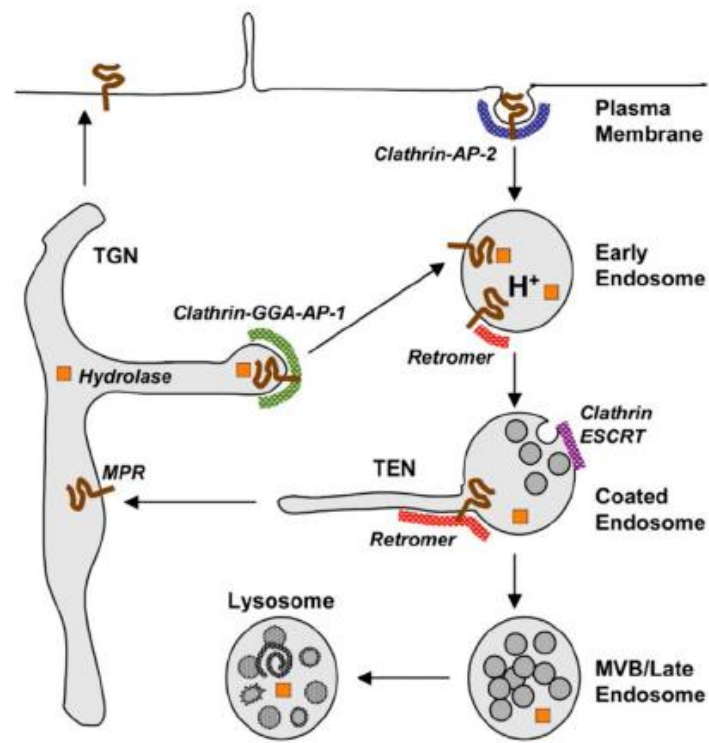


Fig. 5. An example of retromer function: sorting of acid hydrolases by transmembrana MPR cycling between the TGN and endosomes. From Bonifacino and Hurley (2008).

Eukaryotic translation initiation factor 4-gamma

Very recently, genome-wide analysis of families with autosomal-dominant parkinsonism revealed a missense mutation (R1205H) in eukaryotic translation initiation factor 4-gamma (eIF4G1; Chartier-Harlin et al., 2011). The observed clinical phenotype is consistent with late-onset PD. Symptoms

start insidiously with asymmetric resting tremor or akinetic rigidity, and become progressively mixed. In the same study, eIF4G1-R1205H was found to segregate with idiopathic PD. Moreover, the shared haplotype observed suggests that the mutation originates from an ancestral founder and segregates with disease in seemingly unrelated families.

eIF4G1 is the core scaffold of a multisubunit complex that regulates translation initiation of mRNAs encoding mitochondrial, cell survival and growth genes in response to different stresses (Ramirez-Valle et al., 2008; Silvera et al., 2009). This complex is composed, other than eIF4G1, by cap-binding protein eIF4E and ATP-dependent RNA helicase eIF4A. Notably, the R1205H mutation in eIF4G1 perturbs binding with eIF4E while the wild-type protein does not; moreover, oxidative stress results in loss of mitochondrial membrane potential selectively in cells overexpressing the mutant protein (Chartier-Harlin et al., 2011), an effect also observed after eIF4G1 silencing (Ramirez-Valle et al., 2008). This data indicate that the mutation causes a loss of function of the protein.

The mutation in eIF4G1 affects mRNA translation initiation in parkinsonism and might point to a convergent pathway for different forms of disease (Hawkes et al., 2009; Zhou et al., 2008). In this regard, it is important to note that availability of eIF4E is generally the rate-limiting step of translation initiation and is regulated by phosphorylation of eIF4E-binding proteins (4E-BP) through the mammalian target of rapamycin (mTOR) pathway (Ramirez-Valle et al., 2008; Ma and Blenis, 2009). The 4E-BP protein has been shown to be a substrate of human LRRK2, with pathogenic mutants of this protein causing hyperphosphorylation of 4E-BP and reduced resistance to oxidative stress (Gehrke et al., 2010). Finally, activation of mTOR signaling and 4E-BP phosphorylation are associated with L-DOPA-induced dyskinesia (Santini et al., 2009), further stressing the involvement of translation initiation in the context of parkinsonism.

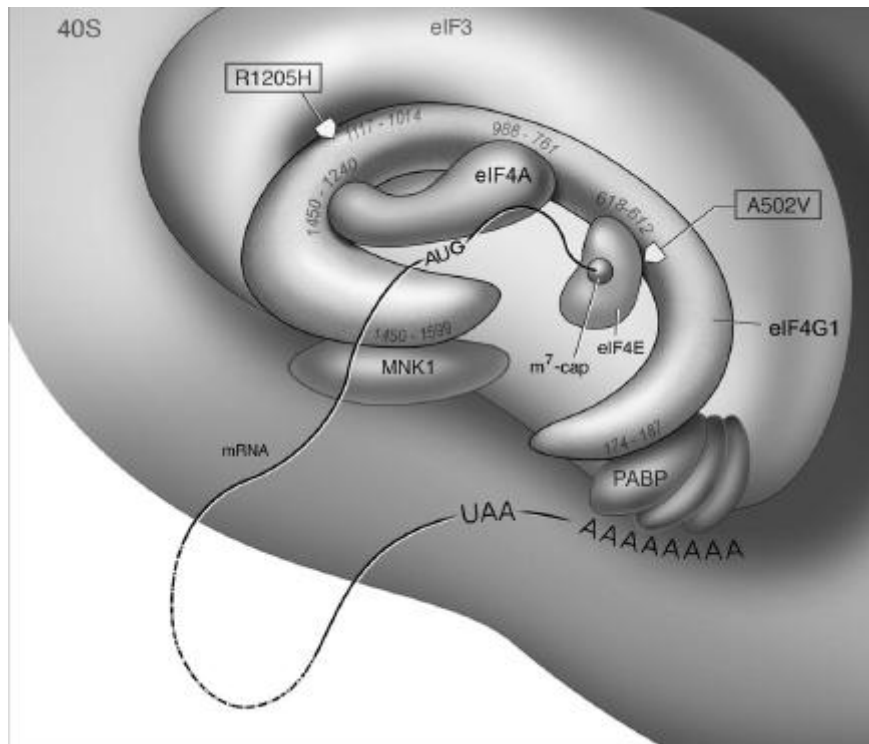


Fig. 6. Scheme of eIF4G1 protein interactions. Approximate positions of A502V and R1205H mutations are highlighted.

Recessively inherited mutations

Recessively inherited deletions and missense mutations have been identified in family-based linkage studies, but they are rare overall, causing <1% of early-onset parkinsonism (Lockhart et al., 2004).

Parkin

Mutations in the parkin gene were originally identified in Japanese families with autosomal recessive, juvenile parkinsonism (Kitada et al., 1998). The protein contains an N-terminal ubiquitin-like domain and two RING-finger domains. It is thought to function as an E3-ligase, conjugating ubiquitin to proteins targeted to proteasome degradation (Shimura et al., 2000). Point mutations are the most common genetic lesions in parkin, although exonic rearrangements, deletions and duplications are also common (Mata et al., 2004). Other than that, patients with homozygous exonic deletions leading to complete loss of parkin expression show selective loss of DA neurons in SN

and locus coeruleus without LB or neurofibrillary tangle pathology, in contrast to that described in patients with heterozygous mutations (Pramstaller et al., 2005). These different outcomes might be mutation-specific (difference in alteration of parkin E3-ligase activity and substrate specificity; Sriram et al., 2005), but, as shown for LRRK2 mutations, different end-stage pathologies might share the same primary cause (Farrer 2006).

Animal models modeling parkin loss-of-function have been described; in parkin-null mice mitochondrial dysfunction and oxidative stress have been implicated in disease pathogenesis. However, none of the parkin knockout mice have any substantial DAergic or behavioral abnormalities (Goldberg et al., 2005; Itier et al., 2003; Perez and Palmiter, 2005; Von Coelln et al., 2004). Interestingly, parkin overexpression appears to be neuroprotective, indicating its upregulation as one route to therapy (Lo Bianco et al., 2004; Greene et al., 2005).

PTEN-induced kinase 1

Homozygous mutations in PTEN-induced kinase 1 (PINK1) were originally found to co-segregate with early-onset parkinsonism in a family-based linkage study (Valente et al., 2001). Mutations were then identified in 1-2% of cases of early-onset disease (Hatano et al., 2004). PINK1 protein comprises a mitochondrial targeting motif and a highly conserved kinase domain, which is shared with the Ca²⁺/calmodulin family of serine-threonine kinases (Valente et al., 2001).

Protein mutations has different impacts on protein stability, localization and kinase activity (Petit et al., 2005; Beilina et al., 2005). The wild-type protein seems to protect from stress-induced mitochondrial dysfunction and apoptosis (Deng et al., 2005). Also in this case, transgenic mouse models have been developed. Similar to parkin knockout mice, PINK1 knockout mice did not show any major abnormalities (Gautier et al., 2008; Gispert et al., 2009), particularly in DA neurons (Kitada et al., 2007). Again, similar to parkin knockout mice, ablation of PINK1 resulted in mitochondrial defects (Gautier et al., 2008; Palacino et al., 2004). Of note, *Drosophila* flies lacking PINK1 exhibited behavioral deficits along with mitochondrial pathology. Transgenic expression of

parkin in these flies ameliorated the PINK1 loss-of-function phenotype, whereas transgenic expression of PINK1 had no effect on parkin loss-of-function phenotypes (Clark et al., 2006; Park et al., 2006). This led to the accepted hypothesis that parkin and PINK1 are engaged in a common signaling pathway, with PINK1 working upstream of parkin.

DJ-1

The DJ-1 protein is a member of the ThiJ/PfpI family of molecular chaperones (Gasser, 2009; Moore et al., 2006). These chaperones are induced during oxidative stress and the protein primarily exists as a dimer localized to mitochondria (Tao et al., 2003; Zhang et al., 2005). PD-associated mutations produce DJ-1 loss of function by causing defective dimer formation or lack of expression (Macedo et al., 2003; Moore et al., 2003). The DJ-1 protein is a redox-sensitive molecular chaperone regulating, among other, redox-dependent kinase signaling pathways, and acting as a regulator of antioxidant gene expression (Kahle et al., 2009). Indeed, primary cultures from DJ-1-null mice showed increased sensitivity to oxidative stress, whereas overexpression provided a protective effect (Goldberg et al., 2005; Kim et al., 2005).

DJ-1 knockout mice have been developed as well, without any major detectable abnormalities. However, in some DJ-1 knockout animals changes in the nigrostriatal transmission and mitochondrial dysfunction were reported (Andres-Mateos et al., 2007; Goldberg et al., 2005; Kim et al., 2005).

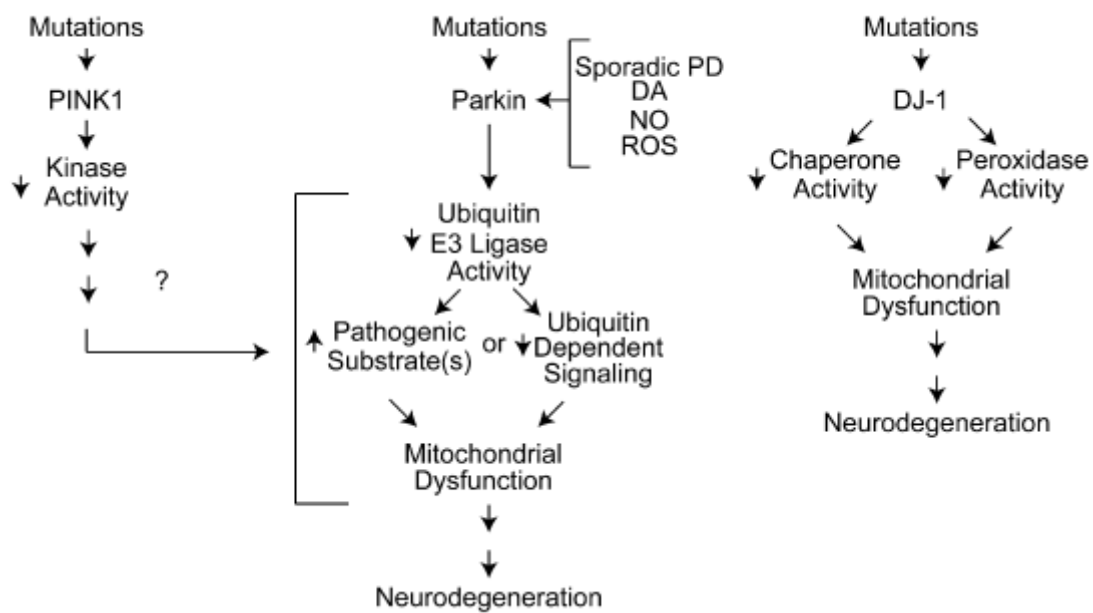


Fig. 7. Possible molecular mechanisms in autosomal recessive PD (Dawson et al., 2010).

LRRK2: structure, properties and neurobiology

Biochemistry and cellular biology of LRRK2

The LRRK2 protein, also named dardarin, derives its name for the presence of leucine-rich repeats (LRR) and a kinase domain in its structure. Between these two regions, a GTPase sequence called ROC (Ras of complex proteins; Bosgraaf and Van Haastert, 2003) and an adjacent COR (C-Terminal of ROC) domain can be found. These domains are characteristic of the ROCO superfamily of proteins (Marin et al., 2008), which all contain ROC-COR tandem domains, but not all have kinase domains. LRRK2 kinase domain has been hypothesized to derive evolutionarily from different sources and to be quite divergent in sequence from the origin (Marin 2006, 2008; Marin et al., 2008). It was initially proposed that the kinase domain was related to the MLKs (mixed lineage kinases), but analysis of all kinase domains throughout the human genome suggests that LRRK2 is part of a small offshoot group of the RIPK (receptor-interacting protein kinase) family of kinases, which are partially similar to the IRAK (interleukin 1 receptor-activated kinase) family and more distant from the MLKs (Manning et al., 2002).

The N-terminal region of LRRK2 is composed of different repeat sequences (LRR, heat, Ankyrin). These are very likely to be protein-protein interaction motifs, indicating that LRRK2 can act as a scaffold for several other proteins. This view is confirmed by the presence of a WD40 domain at the C-end of the protein, which can also interact with lipids (McArdle and Hofmann, 2008). Indeed LRRK2 has been reported to interact with lipid rafts in synaptic terminals and to associate with membrane structures (Hatano et al., 2007). The complexity of LRRK2 function is increased by its ability to self-interact (Gloeckner et al., 2006) and to form a dimer (Greggio et al., 2008).

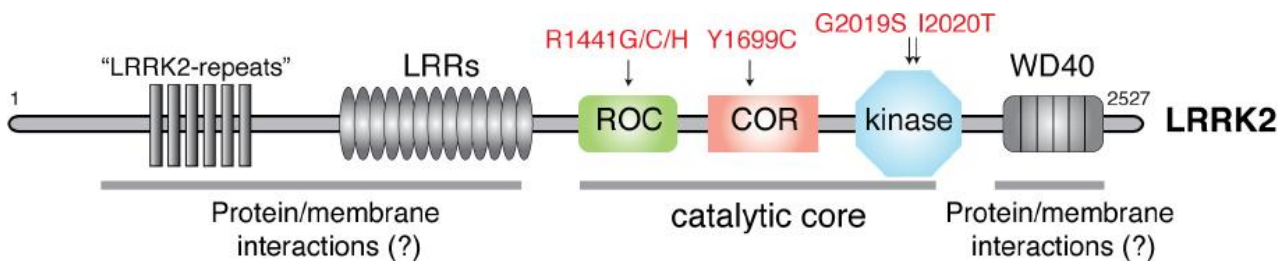


Fig. 8. Schematic representation of LRRK2 protein structure (in red, PD-causing mutations).

Mutations in LRRK2 proven to be pathogenic affect different domains of the protein: R1441C/G/H substitutions are located in the enzymatic ROC domain, Y1699C is found in the COR domain, G2019S and I2020T mutations are in the kinase domain (Paisà-Ruiz et al., 2008). The most common mutation, G2019S, is proposed to exert its pathogenicity through an increase of kinase activity (Greggio et al., 2006; Jaleel et al., 2007; Nichols et al., 2010; West et al., 2005). On the other hand, point mutations located in the ROC and COR domains have been shown to decrease GTPase activity (Guo et al., 2007; Lewis et al., 2007). Finally, the I2020T substitution has been reported to stimulate phosphorylation of 4E-BP [eIF4E (eukaryotic initiation factor 4E)-binding protein] in some models (Imai et al., 2008) but has no effect on kinase activity in other assays (Nichols et al., 2010). Thus, there might be mechanistic differences between different mutations located in the same domain, with the caveat that these observations could also be due to methodological differences in the various assays (Greggio and Cookson, 2009).

The role of LRRK2 in the cellular context has not yet been defined, mostly due to the uncertainty about molecular substrates and/or partners of LRRK2. The first indications came from studies showing cytotoxic effects of mutant LRRK2. Iaccarino and colleagues (2007) reported R1441C-LRRK2 to cause apoptosis in cell lines, through release of cytochrome c and caspase-3 activation. Moreover, they showed that LRRK2-mediated cell death is dependent upon mitochondrial dysfunction and apoptosome formation. Interestingly, LRRK2 constructs devoid of LRR or WD40 domains did not induce apoptosis, highlighting the importance of non-enzymatic sequences in the protein function. Later, LRRK2 has been reported to interact with the adaptor FADD (Fas-associated protein with death domain), with PD-mutants inducing apoptosis through caspase-8 activation (Ho et al., 2009). This study proposed the apoptotic extrinsic pathway (mediated by cell surface death receptors) as opposed to the intrinsic (mitochondria-mediated) pathway, as a mechanism for LRRK2 cytotoxicity.

However, signaling pathways and mechanisms underlying LRRK2-mediated effects are still under debate. Great efforts have been spent on the identification of molecules interacting with LRRK2 in cells. As previously mentioned, 4E-BP has been shown to undergo phosphorylation in cell cultures overexpressing LRRK2. This leads to inactivation of 4E-BP and, possibly, dysregulation of protein translation (Imai et al., 2008). The Ezrin-Radixin-Moesin (ERM) family of proteins may also be a LRRK2 target (Parisiadou et al., 2009). These proteins are involved in cell maintenance, regulating cellular shape, growth and motility (Mangeat et al., 1999; Bretscher et al., 2002). Far from being the only interactions reported, LRRK2 has been shown to activate the ERK (extracellular signal regulated kinase) pathway in a kinase-dependent manner: PD mutations delay the phosphorylation of MEK2, the upstream activator of ERK, resulting in delayed activation of ERK itself (Carballo-Carbajal et al., 2010). Consistently, oxidative stress stimulated phosphoERK to a lower extent and induced greater cell death in Y1699C-LRRK2 overexpressing cells compared to cells expressing the wild-type protein (Liou et al., 2008). In addition, inactivation of LRRK2 led to decreased amount of phosphorylated 4E-BP, thus probably acting along the Akt/TOR signaling pathway (in

which ERK is also involved; Tain et al., 2009). The involvement of LRRK2 in MAPK (mitogen-activated protein kinases) pathway is further suggested by its ability to phosphorylate MAPKK members MKK3/6 and MKK4/7. Moreover, PD mutations located in the LRRK2 kinase domain showed increased phosphorylation of MKK6 (Gloeckner et al., 2009). Along their signaling pathways, MKK3/6 and MKK4/7 activate, respectively, the generically pro-apoptotic p38 and JNK (c-Jun N-terminal kinase; Berwick and Harvey, 2011). Disappointingly, different studies reported no effect of LRRK2 on p38 phosphorylation and JNK activity (West et al., 2007; Carballo-Carbajal et al., 2010), when overexpressed in HEK cell lines. Only West and colleagues (2007) reported activated JNK signaling in SH-SY5Y cell lines overexpressing LRRK2.

Finally, LRRK2 has also been linked to the Wnt signaling pathway through the interaction with central components such as disheveled (DVL) proteins 1-3 (Sancho et al., 2009) and glycogen synthase kinase-3 β (GSK-3 β ; Lin et al., 2010). In the same studies, pathogenic LRRK2 mutations modified these interactions.

Along with cell lines, LRRK2 functions have been extensively studied in primary neuronal cultures, in order to disclose the role of the protein on neuronal cell physiology. Transgenic expression of human G2019S-LRRK2 in neurons derived from *Drosophila* induced dendrite arborization defects in DA but not 5-HT cells. In this context, mutant LRRK2 toxicity is mediated by phosphorylation of tau through GSK3 β (Lin et al., 2010). Consistently, evidence of reduced neurite outgrowth and branching (Dachsel et al., 2010), reduced dendritic arborization and length together with reduced neurogenesis in dentate gyrus (DG) and subventricular zone (SVZ; Winner et al., 2011) have been obtained in primary cultures from LRRK2 transgenic mice. Interestingly, neuronal cultures from LRRK2 knockout mice showed increased process length and branching (Dachsel et al., 2010). Moreover, LRRK2-G2019S reduced the density of dendritic spines, with a particular reduction in mushroom spines (Winner et al., 2011), which are usually more abundant in mature neurons (Nimchinsky et al., 2002; Sala, 2002). Thus, it has been hypothesized that mutant LRRK2 might

cause neurotoxic effects through inhibition of neurite outgrowth and interference with maturation of filopodia into mature spines.

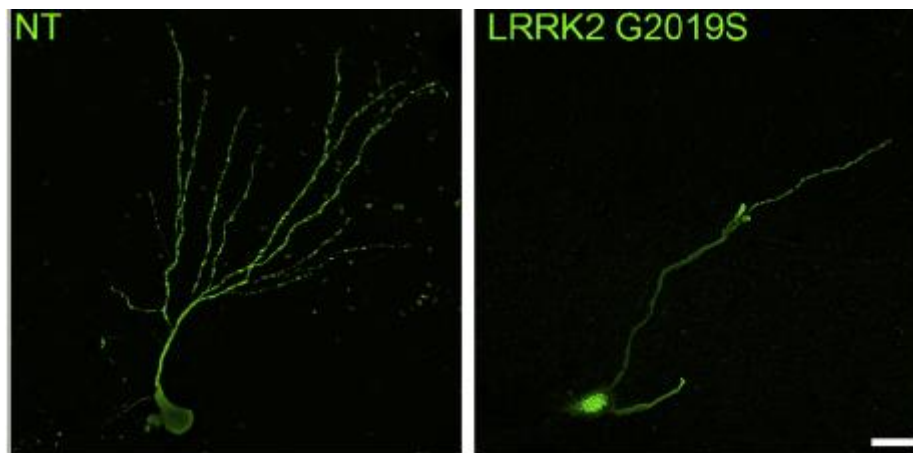


Fig. 9. Defects in neurite outgrowth in neurons from LRRK2-G2019S mice (Winner et al., 2011).

LRRK2 functions in animal models

The LRRK2 protein has been extensively studied in a variety of animal models, including *Drosophila*, *C. Elegans* and rodents. A thorough analysis of non-mammalian models is beyond the scope of this thesis, thus only rodents studies will be reviewed.

In the mouse brain, LRRK2 expression was detected at high levels in the hippocampus, striatum, cortex and cerebellum, with no prominent glial staining. Notably, intense labeling was found in striatal medium spiny neurons. At the midbrain level, LRRK2 staining was higher in SNc than SNr (Melrose et al., 2007). Consistently, the majority of DA neurons in rat SN express LRRK2. Of note, LRRK2 is preferentially expressed in SN compared to the adjacent VTA (ventral tegmental area; Han et al., 2008).

Mice overexpressing human LRRK2 through bacterial artificial chromosome (BAC) transgenesis were firstly reported. This model provided a genetic tool for studying gene function in vivo. BAC carries an intact genomic regulatory sequence and confers precise temporal and spatial expression pattern of the transgene under the control of the endogenous promoter (Heintz, 2001).

In one of the initial studies, R1441G-LRRK2 BAC mice showed an age-dependent, L-DOPA responsive motor deficit. Moreover, these mice showed slightly decreased striatal DA release and axonal pathology with hyperphosphorylated tau. However, no nigral neuronal degeneration was reported (Li et al., 2009). At variance, BAC G2019S-LRRK2 mice failed to reproduce a parkinsonian behavioral phenotype (Li et al., 2010; Melrose et al., 2010), although a reduction of striatal DA content as well as spontaneous and stimulus-evoked extracellular DA release was found (Li et al., 2010; Melrose et al., 2010). Histopathological signs, e.g. accumulation of hyperphosphorylated tau, were reported in older mice (Melrose et al., 2010), although these were not accompanied by DA cell death in SN.

At the same time, different groups induced a targeted insertion of a PD pathogenic mutation in the LRRK2 gene (knock-in models, KI). This technique generates a transgenic mouse with physiological levels of expression of the gene of interest.

KI mice bearing the R1441C mutation (Tong et al., 2009) are behaviorally normal even at old ages. However, pharmacological manipulation of the DA system mice showed reduced locomotor response to amphetamine and quinpirole, with respect to controls. While the number of DA neurons in SNc and striatal DA content were unchanged, spontaneous firing activity in mesencephalic brain slices was less responsive to DA drugs, consistent with behavioral data. Thus, dysregulation of the nigrostriatal DA system is suggested by the different models so far presented (Tong et al., 2009). Lately, KI G2019S mice have also been generated (Herzig et al., 2011). However, these mice did not show abnormalities in DA transmission and responses to DA agonists. Recently, mice expressing full-length human LRRK2 under the control of a cytomegalovirus-enhanced (CMVE) human platelet-derived growth factor β -chain (CMVE-PDGFB) promoter (Ramonet et al., 2011) have been generated. In these mice, a different histopathological situation has been reported at 19-21 months of age: G2019S-LRRK2 mice displayed reduction in the number of TH⁺ SNc neurons but not striatal TH⁺ fiber density. This might be due to compensatory re-sprouting of surviving neuronal processes. However, motor behavior was normal at all ages tested. Consistent with

previous studies, primary mesencephalic cell cultures obtained from these animals showed reduced neurite length and branching.

Overall, these results suggest that LRRK2 transgenic animals hardly show neurodegeneration in SNc (considered a hallmark of PD), but do have alterations in the activity and morphology of DA neurons. Moreover, mutant LRRK2 impairs maturation of dendrites and spines, thus negatively affecting synaptic communication.

Mutation	Genetic approach (strain)	Neuropathology	Abnormal motor behavior	Dopamine system dysfunction
R1441G	BAC transgenics (FVB)	Tau pathology and axonal dystrophy	Progressive and L-dopa-responsive abnormal motor activity	Impaired dopamine release by microdialysis
G2019S	BAC transgenics (C57/BL6)	Enhanced striatal phospho-tau staining compared with only LRRK2-Wt overexpression line	Not up to 18–20 months (but abnormal > 20 months with low penetrance)	Decrease in striatal DA content; reduced striatal DA release and uptake by voltametry analysis
G2019S	BAC transgenics (FVB and JOR)	Phospho-tau accumulation in brain of aged mice	N/D	Reduction in extracellular dopamine levels at striatum
G2019S	Tet inducible transgenics (C57/BL6)	No changes	Increased ambulatory activities at 12 months of age	N/D
α -Syn-A53T/LRRK2-G2019S	Tet-inducible transgenics (C57/BL6)	Microglial activation; accelerated neurodegeneration and enhanced α -syn accumulation compared with A53T single transgenic mice	N/D	N/D
R1441C	Knock-in (129 and C57/BL6)	No changes	Reduced response to AMPH in locomotor activity	Reduced catecholamine release in cultured mutant chromaffin cells
LRRK2 ^{-/-}	Knockout (129 and C57/BL6)	No changes	N/D	No changes
LRRK2 ^{-/-}	Knockout (C57/BL6)	No changes	No major progressive behavioral deficits	No changes
LRRK2 ^{-/-}	Knockout	Moderate increase of activated microglia	No changes	N/D
G2019S	HSV delivery in mice (C57/BL6)	Loss of TH-positive neurons	N/D	N/D
G2019S	Adenovirus vector in rat (Wistar)	Progressive loss of TH- and VMAT2-positive dopaminergic neurons; abnormal hyperphosphorylation of tau in dystrophic nigral neuritic processes (WT and G2019S LRRK2 overexpression)	N/D	N/D

Fig. 10. Summary of LRRK2 transgenic models reported to date (Yue and Lachenmayer, 2011).

Different studies independently reported generation of animal lines with targeted deletion of the endogenous LRRK2 gene (knock-out, KO). Data presented so far indicate that the LRRK2 gene is not essential for mouse survival and does not play a role in early neural development, differentiation or viability. In addition, all KO mice are viable and fertile and exhibit no detectable motor function

abnormality (Tong et al., 2010; Lin et al., 2009; Andres-Mateos et al., 2009). Interestingly, deletion of the LRRK2 gene induced renal abnormality at old age, with cell type-dependent impairment of protein degradation pathways, accumulation of α -synuclein and enhanced apoptotic cell death (Tong et al., 2010). However, these observations have not been mechanistically linked to pathological changes in the brain, which actually appears unaffected in these animals.

Along with genetically modified models, viral delivery of LRRK2 was also reported (Lee et al., 2010; Dusanchet et al., 2011). In striking contrast with the previously reported models, intrastriatal delivery of LRRK2 mutants through HSV (Lee et al., 2010) or AAV (Dusanchet et al., 2011) carriers induced neuronal cell death in SNc. This difference may be due to activation of glial inflammatory pathways elicited by the viral construct and the specific spatial and temporal expression of the protein (Yue and Lachenmayer, 2011).

AIMS OF STUDIES

The main aim of this study was to investigate the role played by LRRK2 in cell and animal neurophysiology, with particular attention to motor behavior and DA transmission *in vivo*, and synaptic morphology *in vitro*. The study has been divided in two independent parts, corresponding to the work performed at the University of Ferrara (part #1) and the University of British Columbia (part #2).

Part 1. *In vivo* phenotypic characterization of LRRK2 effects on motor activity and motor responses to DA receptors ligands. The first part of the study involved the use of three different mouse lines: wild-type mice as controls; transgenic BAC mice carrying the G2019S mutation in the human LRRK2 gene; knock-out (LRRK2-KO) mice bearing deletion of the endogenous LRRK2 gene. First, we performed a motor characterization of these mice through tests specific for spontaneous and exercise-induced motor activity. Mice were divided in age-matched groups (3, 6 and 12 month-old), in order to investigate whether LRRK2 affects the worsening of motor function associated with aging. Then, the motor responses of these mice to DA receptor agonists and antagonists were assessed.

Part 2. *In vitro* investigation of LRRK2 effects on synaptic morphology in cortico-striatal co-cultures. This study was designed to fulfill two aims at the same time. First, we planned to test the novel LRRK2 selective kinase inhibitor LRRK2-IN1 (Deng et al., 2011) in rat primary neuronal cultures. Second, we investigated the involvement of LRRK2 in the development and maintenance of dendritic spines and synaptic contacts. In this view, we employed a novel approach using co-cultures of two different neuronal populations, namely cortical cells and striatal medium spiny neurons (MSNs).

MATERIALS AND METHODS

IN VIVO EXPERIMENTS

Subjects

Non-transgenic wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice, backcrossed on a C57BL/6J strain, were obtained from Mayo Clinic (Jacksonville, FL, USA) through a collaboration with Prof. Matthew Farrer and Dr. Heather Melrose. Colonies were grown in the *vivarium* of the Section of Pharmacology, at the Department of Experimental and Clinical Medicine (University of Ferrara). Animals were kept under regular lighting conditions (12 h light/dark cycle) and given food and water *ad libitum*. The experimental protocols performed in the present study were approved by the Italian Ministry of Health and the Ethical Committee of the University of Ferrara, and adequate measures were taken to minimize animal pain and discomfort.

Behavioral experiments

Physiologically-stimulated motor activity

Three behavioral tests were used to evaluate different motor functions as previously described: the bar, drag and rotarod tests (Marti et al., 2005; Viaro et al., 2008). The bar test (Kuschinski and Hornykiewicz, 1972; for a review see Sanberg et al., 1988) measures the animal ability to respond to an externally imposed static posture, and can be taken as a measure of akinesia (time to initiate a movement). The drag test (modification of the “wheelbarrow test”; Schallert et al., 1979) measures animal ability to balance body posture using the forelimbs in response to an externally applied dynamic stimulus (i.e. backward dragging), and can be taken as an index of akinesia and bradykinesia (slowness of movements). The fixed-speed rotarod test (Rozas et al., 1997) measures overall motor performance as an integration of coordination, gait, balance, muscle tone and motivation to run. The three tests were performed in a fixed sequence (bar, drag and rotarod). During behavioral phenotyping the sequence was performed daily during the light cycle (09.00 – 11.00), for four consecutive days (D1 to D4). For pharmacological testing, the sequence was

repeated within the same experimental session, before (control) and after (at 10 and 90 min) drug injection. When L-DOPA was tested, post injection time analysis was set at 30 min.

- Bar test: mice were placed on a table and each forepaw was placed alternatively on blocks of increasing heights (1.5, 3 and 6 cm). Total time (in s) spent by each paw on the blocks was recorded (cut-off time 20 s). Performance was expressed as total time spent on bar. Since performance at the left and right forepaw did not differ, data were pooled together.

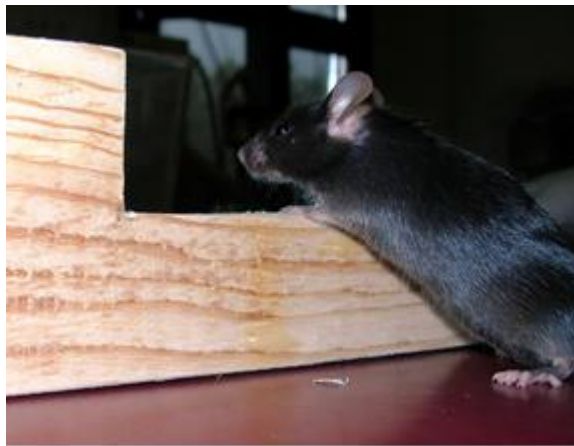


Fig. 12 . Mouse undergoing the bar test.

- Drag test: mice were lifted from the tail (allowing forepaws to rest on the table) and dragged backwards at a constant speed (~20 cm/s) for a fixed distance (100 cm). The number of steps made by each forepaw was counted by two separate observers. Performance was expressed as total number of steps, pooling together the left and right forepaw.



Fig. 13. Mouse undergoing the drag test.

- Rotarod test: the fixed-speed rotarod test was employed using an established protocol (Marti et al., 2005; Viaro et al., 2008). Briefly, mice were tested on a rotating rod (diameter of the cylinder 8 cm), whose speed was stepwise increased (every 180 s) from 5 to 55 rpm. The time spent on the rod was recorded.



Fig. 14. Mouse performing the rotarod test.

For behavioral phenotyping, untrained mice were tested at three different ages: 3, 6 and 12 months old. Pharmacological testing was then performed on 6 month-old mice.

Spontaneous motor activity

Experiments were performed during the light cycle (between 09.00 and 13.00) according to Guerrini et al. (2009). Six-months old wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice were monitored for 30 min. For these experiments the ANY-maze video tracking system was used (Ugo Basile, application version 4.52c Beta). Mice were positioned in a square plastic cage (40 cm \times 40 cm), one mouse per cage. Four mice were monitored in parallel. The central zone of the open field was defined as the central 20 cm \times 20 cm square. Mouse horizontal activity was monitored by a camera while vertical activity was measured by an infrared beam array. The parameters measured were: cumulative distance traveled (total distance in meter that the animal traveled during the test), immobility time (the animal is considered immobile when 90% of it remains in the same place for a

minimum of 2.5 s), number of rearings (the number of beam breaks due to vertical movements), number of entries in the central zone, and time spent by the animal in the central area of the field. An entry in the central zone was marked when the entire area of the animal was in the central square, and the time in the central zone is defined as the amount of time in seconds that the animal spent in the central square.

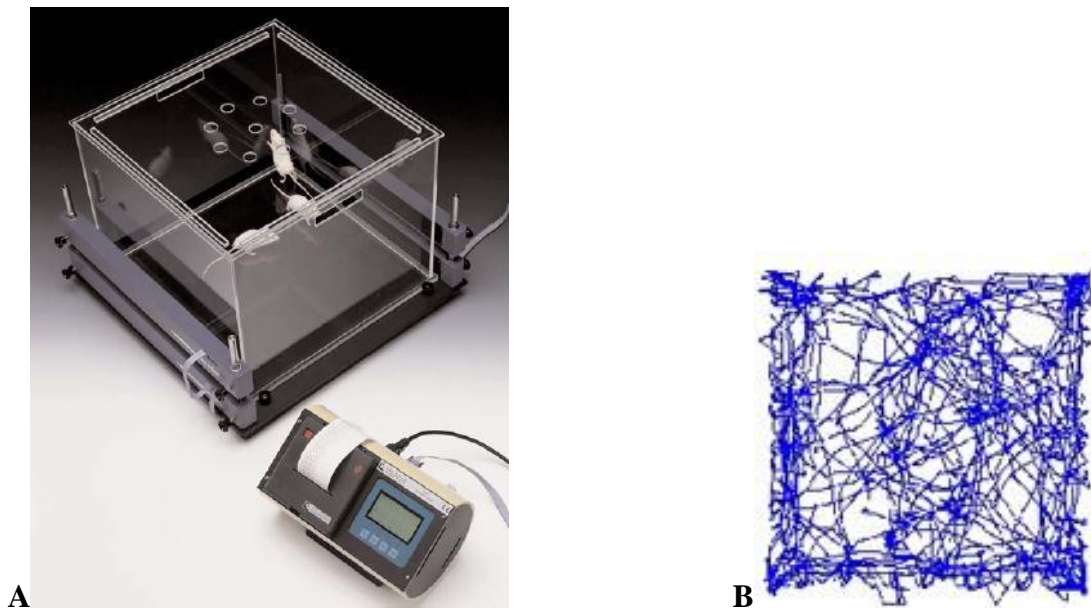


Fig. 15. **A.** Activity cage for open field test, equipped with infrared laser beams for assessment of vertical activity. **B.** Typical tracking of mouse horizontal locomotion in the cage.

Injected drugs

L-DOPA and benserazide were purchased from Sigma Chemical Company (St. Louis, MO, USA). Haloperidol, SCH23390 and SKF38393 were purchased from Tocris Bioscience (Bristol, UK). Pramipexole was purchased from McTony Bio and Chem (Vancouver, BC, Canada). All drugs were freshly dissolved in saline just prior to use and the volume injected was 10 μ l/g body weight.

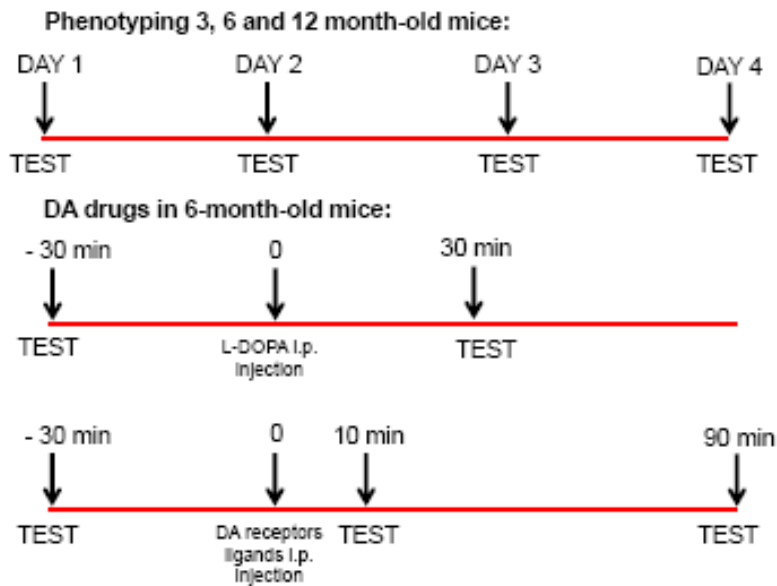


Fig 16. Experimental design adopted in physiologically-stimulated motor behavior and pharmacological experiments.

IN VITRO EXPERIMENTS

Primary neuronal culture and transfection

Cultures were prepared as in Fan et al (2010). Briefly, pregnant Wistar rats were anesthetized with halothane and decapitated at embryonic day 17-18. Embryos were extracted and the brains removed and placed on ice in Hank's Balanced Salt Solution (HBSS, GIBCO). For transfections, 3-5 millions cells were suspended in 100 μ l of electroporation buffer (Mirus Bio) with 1-5 μ g of endonuclease-free DNA, placed in a cuvette (0.2 μ m, Biorad) and electroporated (AMAXA nucleofector I: program 03 for cortex and 05 striatum). Solution was removed from the cuvette and resuspended in D minimum essential medium (GIBCO) plus 10% fetal bovine serum (FBS; DMEM+), and plated as described above. After 2-4h DMEM+ was replaced with 500 μ l plating medium (PM, 2% B27, Invitrogen; penicillin/streptomycin; 2 mM α -glutamine; neurobasal medium, GIBCO). At day in vitro (DIV) 4, 500 μ l was added, with subsequent half media changes every 3-5 days. DNA plasmid was a YFP on a CAG promoter (a gift from S. Kaech and G. Banker, Oregon Health Sciences University, Portland, with permission of J. Miyazaki for the CAG promoter; Kaech and Banker, 2006; Niwa et al., 1991). In cortico-striatal co-cultures, either cortical

(CTX) or striatal MSNs were YFP-nucleofected to differentiate between cell types and then plated with non-transfected cells, either MSNs or CTX, at a ratio of 1:1.

Treatment protocol

Cell cultures were divided in three treatment groups, namely DMSO (vehicle), LRRK2-IN1 1 μ M and LRRK2-IN1 10 μ M. Each treatment was applied for 3, 24 or 72 h before fixation. However, all cells were fixed at 21 DIV, to obtain comparable growth trends. LRRK2-IN1 was obtained by the laboratory of Prof. Matthew Farrer (Centre for Applied Neurogenetics, University of British Columbia, Vancouver, BC, Canada) from the Michael J. Fox Foundation LRRK2 Consortium, through Drs. Dario Alessi and Nathaniel Gray (University of Dundee, UK).

Immunocytochemistry

Coverslips were fixed in 4% paraformaldehyde (PFA) + 4% sucrose for 10 min and rinsed 3 times with phosphate buffered saline (PBS). Next, coverslips were washed (5 min, PBS plus 0.03% triton X-100; Sigma, PBST) and blocked (30 min, 10% normal goat serum, NGS, in PBS). Primary antibodies were incubated overnight with shaking at 4°C in PBST plus 2% NGS, subsequently incubated at RT for 1h, washed 3 times with PBST, and incubated for 1.5h at RT with secondary antibodies (Alexa-488 and Alexa-568 conjugated α -chicken and α -mouse, Molecular probes; α -guinea pig AMCA, Jackson Laboratories). For live staining, cells were incubated for 10 min at 37°C with α -GFP (cross reactive with YFP) in conditioned media, rinsed 2 times with media, fixed in PFA-sucrose for 10 min, rinsed 3 times with PBS, and incubated with secondary antibody for 1.5h. For labeling with VGlut1 and PSD95, cells were subsequently permeabilized with methanol for 5 min at -20°C, rinsed 3 times with PBS, washed for 5 min with PBST, then treated with primary antibodies overnight, washed 3 times with PBST, and incubated with secondary antibodies as above. Coverslips were slide mounted with fluoromount (Southern Biotech).

Antibodies included: α -green fluorescent protein (GFP, chicken, AbCam ab13970, 1:1000), α -post synaptic density protein 95 (PSD-95, mouse, Thermo Scientific, MA 1-045, 1:1000), α -vesicular glutamate transporter 1 (VGluT1, guinea pig, Chemicon, AB5905, 1:4000).

Microscopy and image analysis

Images were acquired using an Olympus Fluoview-1000 confocal laser scanning microscope; acquisition was performed using a 63x objective (1.4 Oil Plan-Apochromat; 4-8 stacks of 0.5 μ m) and extended focus projections were created from 4-5 images containing all of the visible dendritic surface staining and the glass-attached somatic membrane. For cluster detection and localization images were manually thresholded by a blinded experimenter, and analysis of co-localization was conducted in small regions of interest around three secondary dendrites. Cluster analysis was performed in ImageJ; co-localization was calculated using an ImageJ colocalization plugin (<http://rsb.info.nih.gov/ij/plugins/colocalization.html>) as in Tapia et al. (2011). Points of colocalisation were defined as regions > 1 pixel in size where the intensity ratio of the two channels was > 50 .

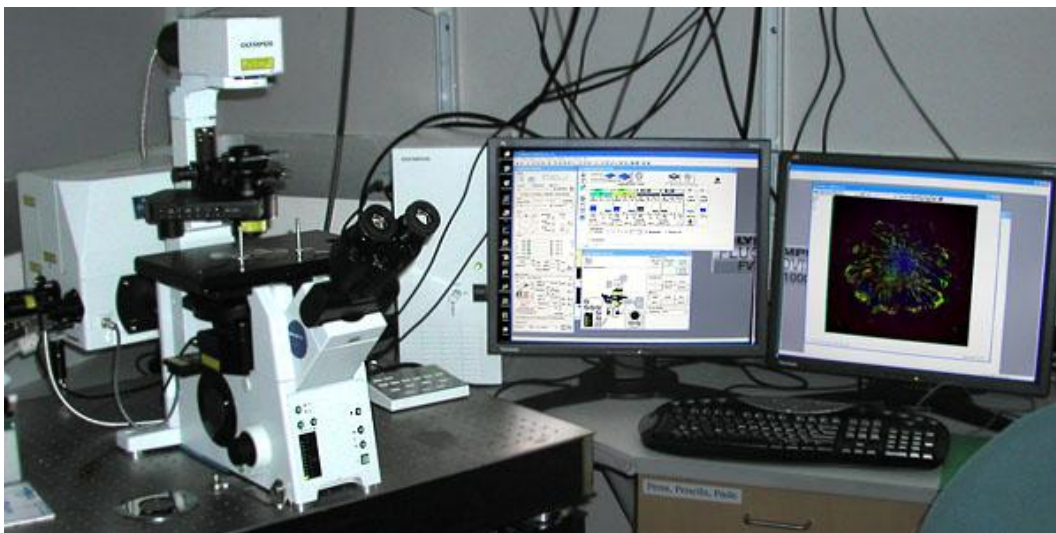


Fig. 11. The Olympus Fluoview-1000 confocal laser scanning microscope system.

DATA PRESENTATION AND STATISTICAL ANALYSIS

For in vivo experiments, data are presented as absolute values with mean \pm SEM. Statistical analysis was performed by one-way repeated measure (RM) ANOVA, implemented on a excel spreadsheet, followed by Bonferroni post-hoc test. In both cases, limit of significance was set at $p < 0.05$. For in vitro experiments, data are presented as mean \pm SEM obtained from 3 different cultures. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Turkey's Multiple Comparison post-hoc test, using GraphPad Prism software (San Diego, CA, USA).

RESULTS

IN VIVO EXPERIMENTS

Characterization of motor phenotype in wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice

Effect of aging

The fixed sequence of bar, drag and rotarod tests was performed on untrained mice of 3, 6 and 12 months. The comparison of the different ages within each genotype yielded to a differential aging profile. In the bar test, (Fig. 17A) wild-type mice showed an age-dependent increase of the time spent on the blocks, with 12 month-old mice being more akinetic than younger ones (~411%). In the same conditions, BAC hLRRK2-G2019S were significantly akinetic yet at 6 months (~141%), reaching the highest level of akinesia at 12 months (~365%). The responses of LRRK2-KO mice did not worsen over time.

In the drag test (Fig. 17B), wild-type and BAC hLRRK2-G2019S mice showed reduction of the number of steps, which was evident at 6 months (~23% and ~24%, respectively) and maintained at 12 months (~22% and ~28%, respectively). Conversely, LRRK2-KO mice did not show worsening of stepping activity over time and displayed the same stepping activity at 3, 6 and 12 months of age. Finally, in the rotarod test (Fig. 17C), no differences were observed in any of the three genotypes at all ages under investigation.

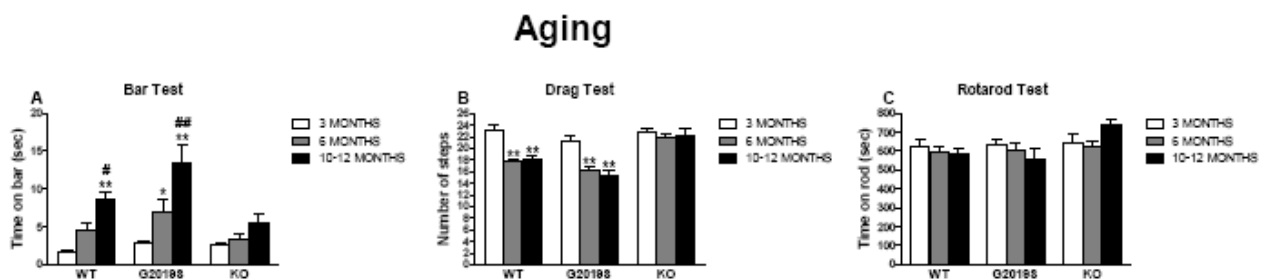


Fig. 17. Motor profile of mice during aging (3, 6 and 12 months of age) as observed in the bar (A), drag (B) and rotarod (C) tests. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 14-16 determinations per group. * $p < 0.05$, ** $p < 0.01$, significantly different from 3 months of age; # $p < 0.05$, ## $p < 0.01$, significantly different from 6 months of age

Physiologically-stimulated motor activity: motor characterization

Previous studies in the same types of mice (Melrose et al., 2010) did not report major differences in motor behavior, even at old ages. Thus, we designed a behavioral protocol in which mice were tested for 4 consecutive days, in order to disclose motor deficits as a consequence of repetitive behavioral stimulation and motor learning.

- 3 month-old mice. In the bar test (Fig. 18A), no major differences in motor profiles among genotypes were observed, with BAC hLRRK2-G2019S mice showing occasional (D1 and D3) mild increases in immobility time. Likewise, the only difference detected in the drag test (Fig. 18B) was a greater activity of LRRK2-KO mice at D2. Finally, in the rotarod test (Fig. 18C) no difference was observed among genotypes over the whole testing period.

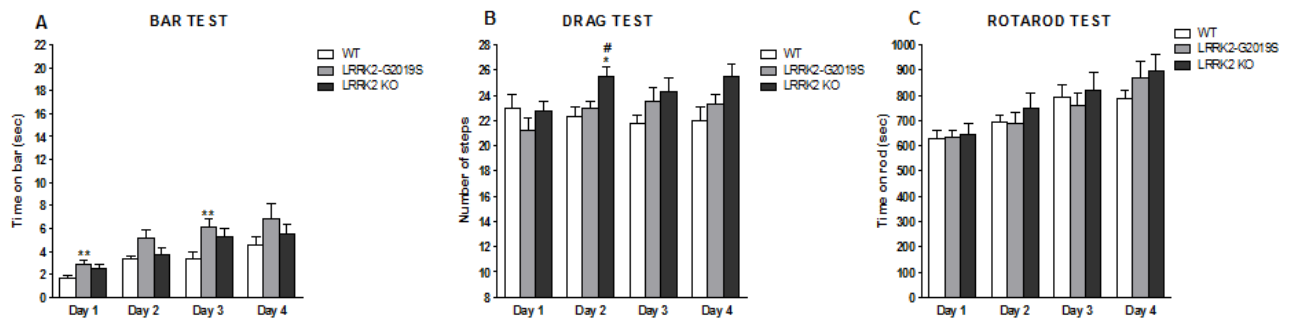


Fig. 18. Effect of a 4-day training protocol in the bar (A), drag (B) and rotarod (C) tests in 3-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 14-16 determinations per group. ** $p < 0.01$, significantly different from wild-type; # $p < 0.05$, significantly different from BAC hLRRK2-G2019S.

- 6 month-old mice. In the bar test (Fig. 19A), BAC hLRRK2-G2019S mice showed a trend towards higher immobility time, reaching significance at D4 (~112%). Oppositely, LRRK2-KO mice showed significantly less akinesia than wild-type mice at D3 (~56%) and D4 (~52%). Likewise, BAC hLRRK2-G2019S mice developed a deficit in stepping activity at

D3 (~15%) and D4 (~19%) in the drag test (Fig. 19B) whereas LRRK2-KO mice showed greater stepping activity at D1 through D4 (~20%). No significant difference among genotypes was found in the rotarod test (Fig. 19C).

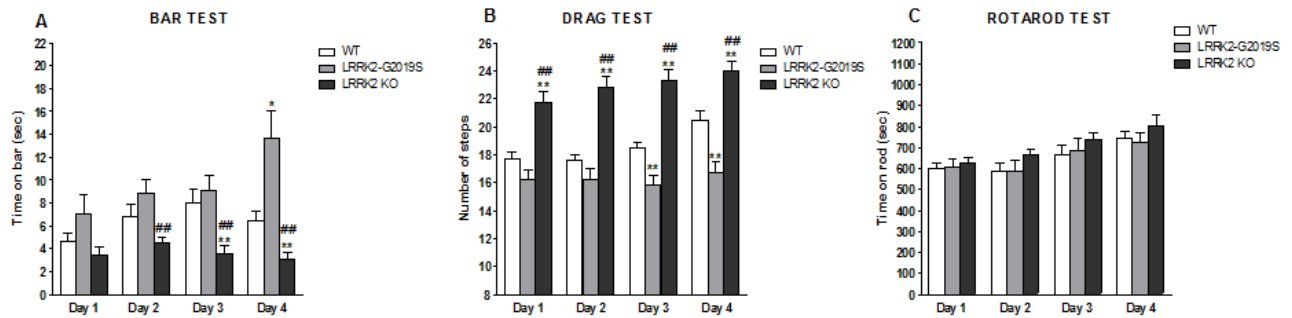


Fig. 19. Effect of a 4-day training protocol in the bar (A), drag (B) and rotarod (C) tests in 6-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 14-16 determinations per group. * p <0.05, ** p <0.01, significantly different from wild-type; ### p <0.01, significantly different from BAC hLRRK2-G2019S.

- 12 month-old mice. In the bar test (Fig. 20A), higher levels of akinesia in BAC hLRRK2-G2019S were observed at D3 (~74%) and D4 (~84%) whereas LRRK2-KO animals showed a reduction of immobility time throughout the 4 testing sessions, although this did not reach the level of significance. In the drag test (Fig. 20B), behavioral outcomes were similar to 6 month-old mice, with BAC hLRRK2-G2019S mice showing motor impairment at an earlier time point (i.e. D2; ~18%). LRRK2-KO animals were consistently hyperactive, from D1 (~22%) through D4 (~21%). In the rotarod test (Fig. 20C), the motor performance of wild-type and BAC hLRRK2-G2019S mice was not different whereas that of LRRK2-KO animals was significantly higher at D1 (~25% increase), D3 (~21%) and D4 (~28%).

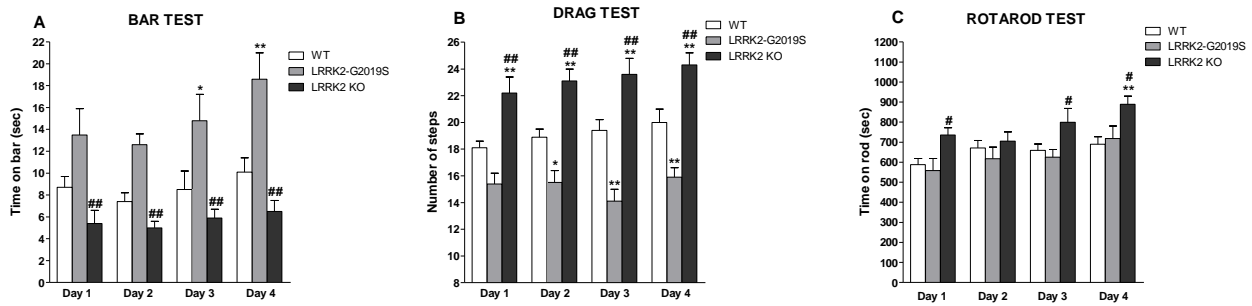


Fig. 20. Effect of a 4-day training protocol in the bar (A), drag (B) and rotarod (C) tests in 12-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 14-16 determinations per group. * $p < 0.05$, ** $p < 0.01$, significantly different from wild-type; # $p < 0.05$, ## $p < 0.01$, significantly different from BAC hLRRK2-G2019S.

Spontaneous motor activity

In order to assess the spontaneous locomotion activity and exploratory behavior, we challenged the three mouse lines in the open field test (Fig. 21). The total distance traveled and the immobility time during the 30-min session did not differ among wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice (Fig. 21A-B). Conversely, vertical motor activity was higher in LRRK2-KO mice (~63%) with a greater number of rearings recorded by the system (Fig. 21C). Exploratory behavior showed similar trends, with a non significant increase in the number of entries in the central zone of the arena (Fig. 21D), but a significantly greater amount of time spent in the same zone for LRRK2-KO mice (~78%), with respect to both wild-type and BAC hLRRK2-G2019S mice (Fig. 21E).

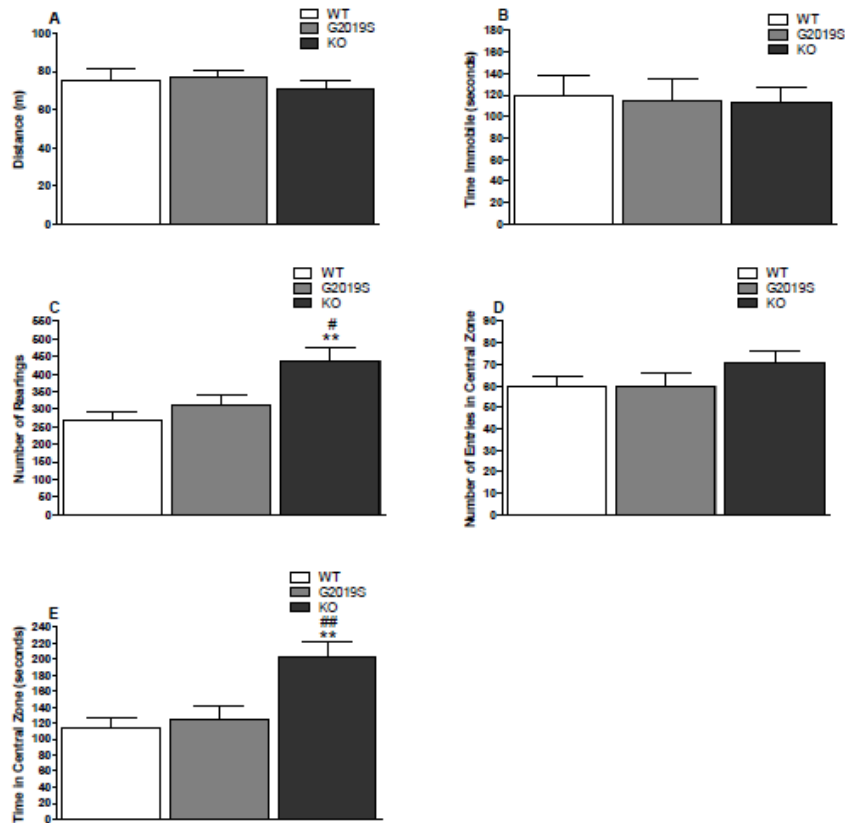


Fig. 21. Spontaneous locomotor activity and anxious-like behavior in 6-month old mice. Data are expressed as distance traveled (A), immobility time (B), number of rearings (C), number of entries in the central zone (D) and time spent in the central zone (E) and are means \pm SEM of 18-20 determinations per group. ** $p < 0.01$, significantly different from wild-type; # $p < 0.05$, ## $p < 0.01$, significantly different from BAC hLRRK2-G2019S.

Pharmacological testing in 6 month-old mice

The identification of an age-dependent motor deficit in BAC hLRRK2-G2019S mice prompted us to investigate the nature of this impairment. In particular, we set to test whether this motor deficit could be reversed by DA agonists, and thus be reminiscent of a parkinsonian phenotype. Thus, we performed the fixed sequence of bar, drag and rotarod tests in 6 month-old mice before and after systemic administration of L-DOPA, DA receptors agonists and DA receptors antagonists.

L-DOPA

The antiparkinsonian drug of reference, L-DOPA, was systemically injected (i.p.) at a dose (10 mg/Kg) previously found effective in reversing the impairment of stepping activity in the MPTP-treated mouse, a well-established model of parkinsonism (Viaro et al., 2010). L-DOPA was given in combination with peripheral dopa-decarboxylase inhibitor benserazide (25 mg/Kg, i.p.).

L-DOPA failed to reduce the immobility time of wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice (Fig. 22A). Conversely, in the drag test (Fig. 22B) L-DOPA rescued stepping activity in BAC hLRRK2-G2019S mice (~40%), being ineffective in the other genotypes. Finally, L-DOPA did not affect the rotarod performance of the 3 genotypes (Fig. 22C). Overall, these data suggest that the motor impairment induced by G2019S mutation is DA-dependent.

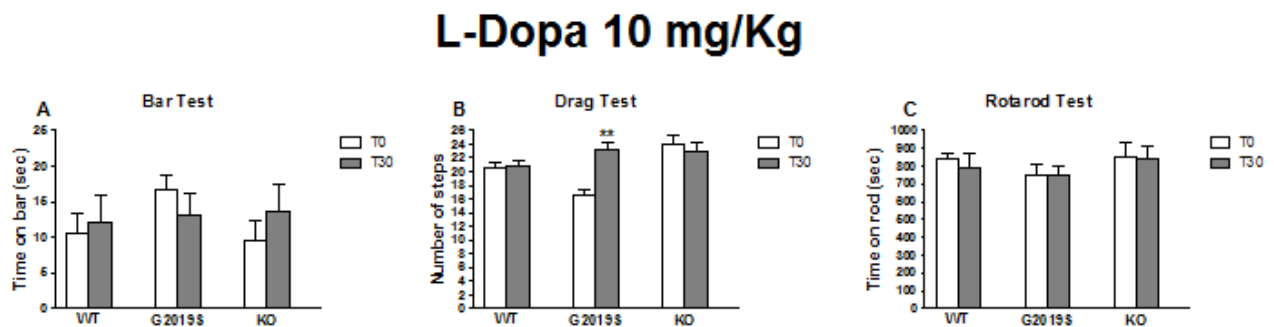


Fig. 22. Effect of systemic injection of L-DOPA (10 mg/Kg plus benserazide 25 mg/Kg, i.p.) in the bar (A), drag (B) and rotarod (C) tests in 6-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 8-10 determinations per group. ** $p < 0.01$, significantly different from T0.

Pramipexole

To further confirm the parkinsonian nature of the phenotype observed in BAC hLRRK2-G2019S mice, we tested the D2/D3 receptor agonist pramipexole (PPX), which is widely employed in PD therapy. We selected a dose of 0.001 mg/Kg (i.p.) which has been demonstrated to positively modulate motor behavior in MPTP-treated mice (Viaro et al., 2010). In the bar test (Fig. 23A), no motor response was detected in wild-type and BAC hLRRK2-G2019S mice whereas an increase of immobility time was recorded in LRRK2-KO mice 90 min after PPX injection (~259%). In the drag

test (Fig. 23B), PPX did not change stepping ability in wild-type mice, while BAC hLRRK2-G2019S mice showed a delayed improvement at the 90-min time-point (~26%). Consistent with the bar test, PPX inhibited motor activity in LRRK2-KO animals (~18%) at 90 min after administration. Finally, no effects on rotarod performance were induced by PPX in any genotypes (Fig. 23C).

PPX 0.001 mg/Kg

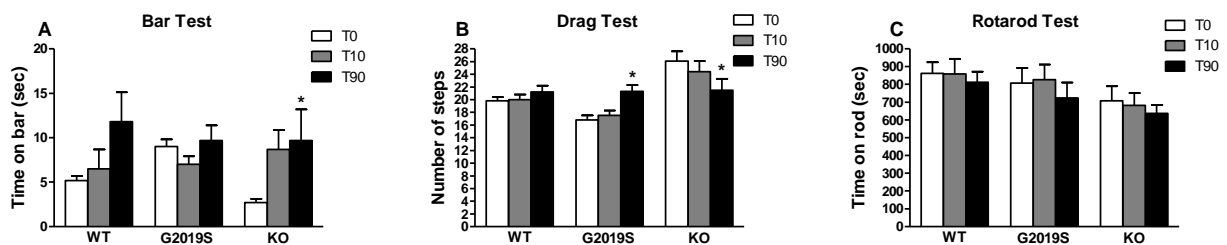


Fig. 23. Effect of systemic injection of PPX (0.001 mg/Kg, i.p.) in the bar (A), drag (B) and rotarod (C) tests in 6-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 8-10 determinations per group. * $p < 0.05$, ** $p < 0.01$, significantly different from T0.

Haloperidol

Since we observed different responses to D2/D3 agonists in LRRK2 mutants and LRRK2-KO mice, we tested the motor responses to the D2/D3 antagonist haloperidol. From previous studies, we selected the effective dose of 0.8 mg/Kg (i.p.; Mabrouk et al., 2010).

In the bar test (Fig. 24A), haloperidol induced high levels of akinesia in wild-type, BAC hLRRK2-G2019S and LRRK2-KO mice, both at 10 (463%, 182%, 364%, respectively) and 90 min (636%, 350% and 578%, respectively) after drug injection. In the drag test (Fig. 24B), haloperidol induced a marked reduction of stepping activity in wild-type (~35%) and KO mice (~60%). Strikingly, BAC hLRRK2-G2019S were unresponsive to the motor inhibition induced by haloperidol. In the rotarod

test (Fig. 24C), haloperidol caused marked inhibition of overall motor performance comparably in wild-type (~78%), BAC hLRRK2-G2019S (~84%) and KO mice (~85%).

Haloperidol 0.8 mg/Kg

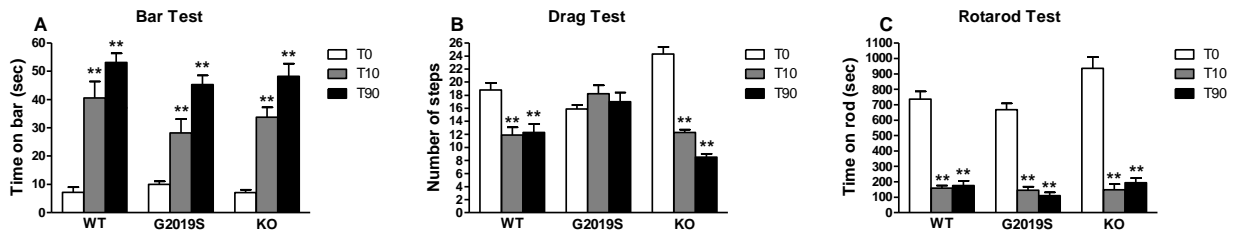


Fig. 24. Effect of systemic injection of haloperidol (0.8 mg/Kg, i.p.) in the bar (A), drag (B) and rotarod (C) tests in 6-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 8-10 determinations per group. ** $p < 0.01$, significantly different from T0.

SCH23390

In order to obtain a comprehensive picture of the cross-talk between LRRK2 and DA receptors in the modulation of motor behavior, we planned to pharmacologically manipulate also the D1 receptors, challenging mice with the selective D1/D5 receptor antagonist SCH23390 and the D1/D5 receptor agonist SKF38393.

As for SCH23390, we selected a threshold dose (0.01 mg/Kg, i.p.) according to a dose-response curve performed in C57Bl/6J mice (Viaro and Morari, personal communication). SCH23390 increased (~77%) the immobility time in the bar test at 90 min after treatment (Fig. 25A). Conversely, neither BAC hLRRK2-G2019S nor KO mice responded to D1 receptors blockade. In the drag test (Fig. 25B), SCH23390 was ineffective in modulating stepping activity of wild-type and KO mice but it greatly ameliorated stepping activity in BAC hLRRK2-G2019S mice at both time-points (10 and 90 min after injection, 30% and 46%, respectively). Finally, SCH23390 did not affect the rotarod performance of any genotypes (Fig. 25C)

SCH23390 0.01 mg/Kg

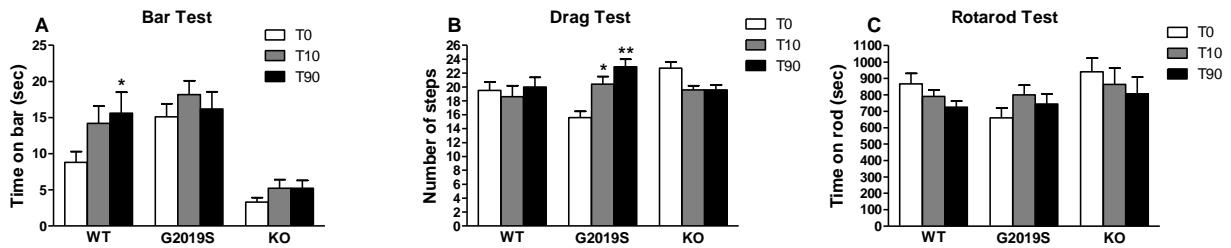


Fig. 25. Effect of systemic injection of SCH23390 (0.01 mg/Kg, i.p.) in the bar (A), drag (B) and rotarod (C) tests in 6-month old mice. Data are expressed as time on bar (A), number of steps (B) and time on the rod (C) and are means \pm SEM of 8-10 determinations per group. * $p < 0.05$, ** $p < 0.01$, significantly different from T0.

SKF38393

Then, we administered the D1/D5 receptor agonist SKF38393, at the threshold dose of 0.1 mg/Kg, reported to be ineffective in C57Bl/6J mice (Viaro and Morari, personal communication). For technical reasons, no rotarod test was performed in this set of experiments.

In the bar test (Fig. 26A), both wild-type and KO animals were unresponsive to D1 receptors stimulation. Oppositely, BAC hLRRK2-G2019S mice showed a marked reduction in the immobility time, both 10 and 90 min after administration (57% and 58%, respectively). Consistently, SKF38393 did not affect stepping activity of wild-type mice in the drag test (Fig. 26B). However, it improved stepping activity in BAC hLRRK2-G2019S mice (~38%), causing a delayed impairment in KO mice (~21%).

SKF38393 0.1 mg/Kg

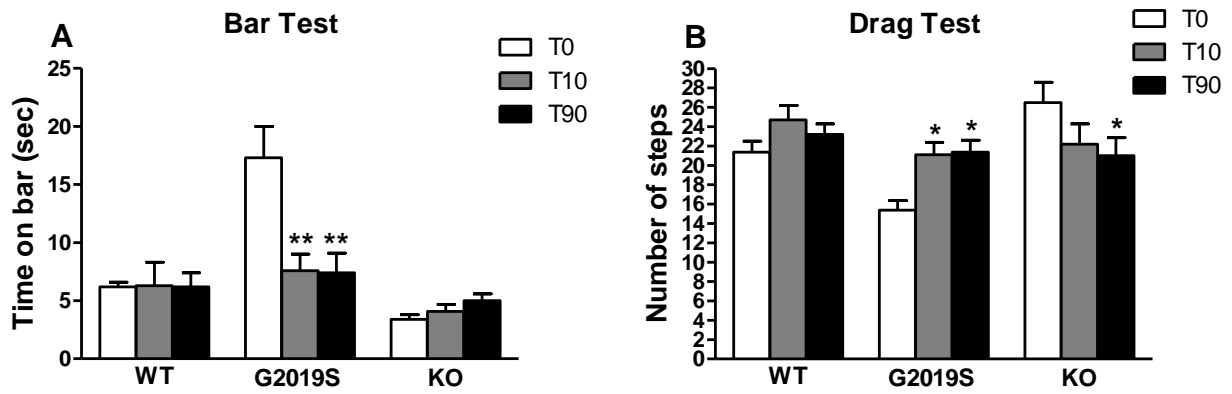


Fig. 26. Effect of systemic injection of SKF38393 (0.1 mg/Kg, i.p.) in the bar (A) and drag (B) tests in 6-month old mice. Data are expressed as time on bar (A) and number of steps (B) and are means \pm SEM of 8-10 determinations per group. * $p < 0.05$, ** $p < 0.01$, significantly different from T0.

IN VITRO EXPERIMENTS

Co-cultured striatal and cortical neurons

The characterization of CTX-MSN rat primary co-cultures has been reported previously (Kaufman et al., 2012). Here we confirm the ability to identify, using immunofluorescence and confocal laser scanning microscopy, the 2 distinct neuronal subpopulations and the localization of synaptic markers (i.e. VGluT1 and PSD95) in dendritic spines of cortical (Fig. 27) and striatal neurons (Fig. 28). Software-assisted analysis of acquired images allows evaluation of different parameters. In order to investigate the effects on synaptic markers and dendritic spines we evaluated: i) VGluT1 and PSD95 clusters size; ii) percentage colocalisation of VGluT1 and PSD95; iii) density of dendritic protrusions (i.e. filopodia); iv) percentage of protrusions labeled with PSD95 (i.e. postsynaptic sites); v) density of VGluT1 and PSD95 clusters (per 10 μm length); vi) density of colocalised points (per 10 μm length). To independently analyze the 2 different neuronal subtypes, in one given co-culture only MSNs or CTX neurons were labeled with YFP.

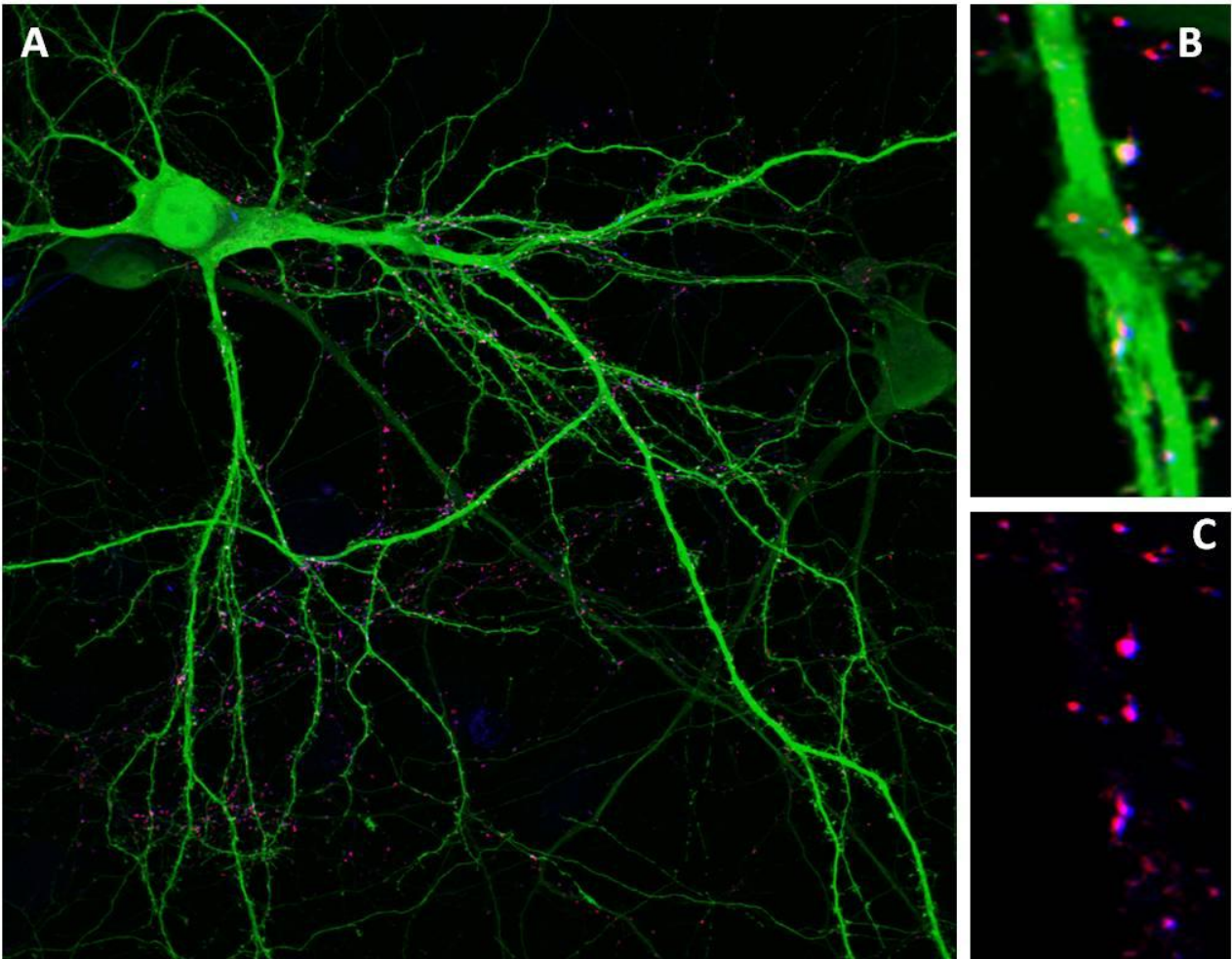


Fig. 27. A. Cortical neuron with triple immunolabeling (green=GFP, BLUE=VGlut1 and red=PSD95). B. Magnification of a secondary dendrite. C. VGlut1 and PSD95 colocalisation (purple) in the same tract.

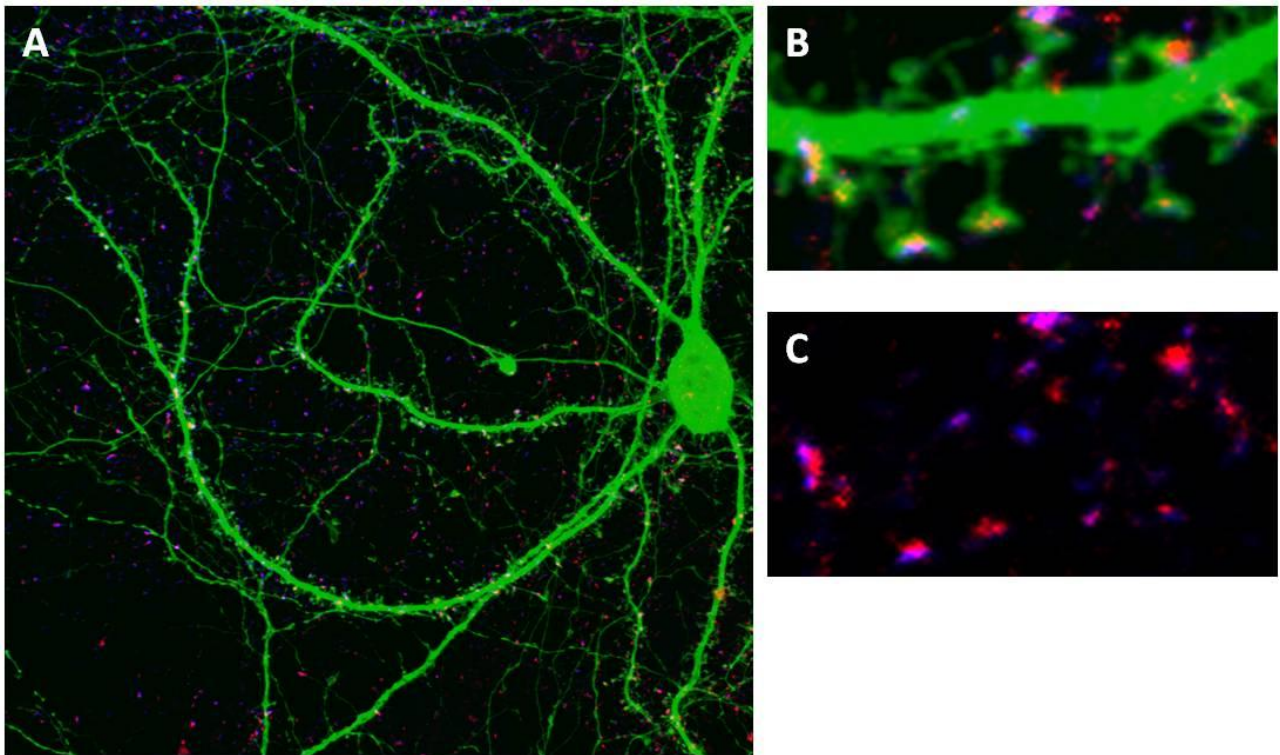


Fig. 28. **A.** Striatal MSN with triple immunolabeling (green=GFP, BLUE=VGlut1 and red=PSD95). **B.** Magnification of a secondary dendrite. **C.** VGlut1 and PSD95 colocalisation (purple) in the same tract.

Effect of LRRK2-IN1 in MSNs

Inhibition of LRRK2 kinase activity led to rearrangement in the size of synaptic markers. Specifically (Fig. 29A), VGlut1 clusters size increased with respect to vehicle by ~117% and ~105% following 72h application of 1 and 10 μ M LRRK2-IN1, respectively. No effect was detected at earlier time-points. Qualitatively similar results were observed in PSD95 clusters size, but the treatment did not significantly affect clusters size when compared to vehicle (Fig. 29B). However, a significant increase was observed at 72h compared to 24h and 3h application of 1 μ M LRRK2-IN1.

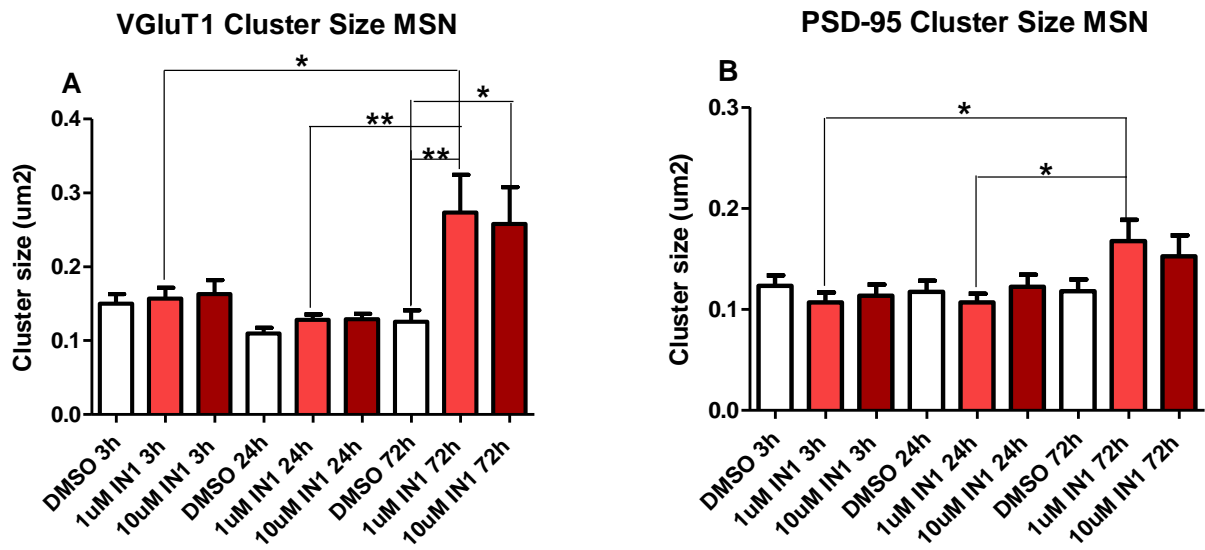


Fig. 29. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on the size of synaptic clusters of VGlut1 (A) and PSD-95 (B) in cultured MSNs. Data are expressed as area (µm²) of puncta and are mean ± SEM of 22-24 determinations per group. *p<0.05, **p<0.01, significantly different comparison.

The colocalisation percentages of VGlut1 with PSD95 and PSD95 with VGlut1 were not modified by LRRK2-IN1 (Fig. 30).

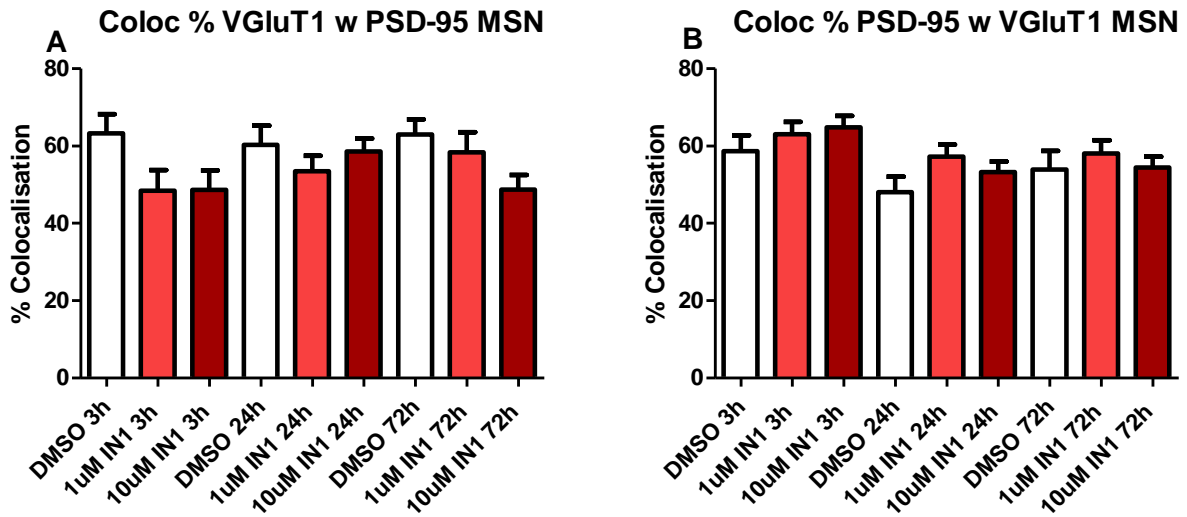


Fig. 30. Effect of LRRK2-IN1 (1 and 10 μ M, in the media) on colocalisation of the synaptic markers VGlut1 (A) and PSD95 (B) in MSNs. Data are expressed as percentage colocalisation of VGlut1 with PSD-95 (A) and PSD-95 with VGlut1 (B) and are mean \pm SEM of 22-24 determinations per group.

In MSNs, the density of protrusions in secondary dendrites was not modified by the treatment at any time points (Fig. 31A). Consistently, the percentage of protrusions labeled with anti-PSD95 antibody did not change (Fig. 31B).

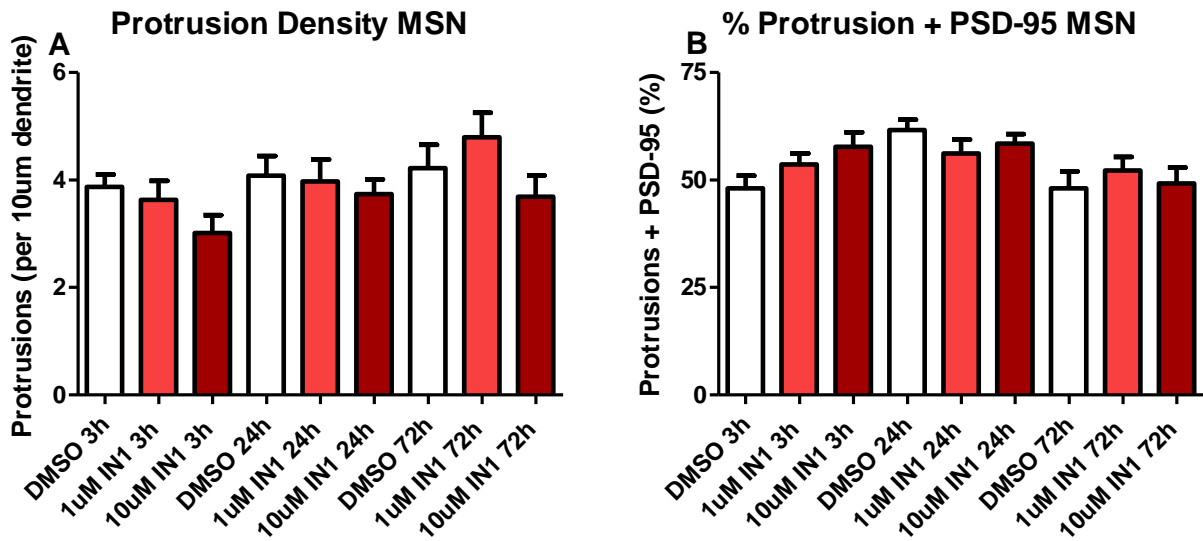


Fig. 31. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on protrusion density (A) and percentage of PSD95-positive protrusions (B) in MSNs. Data are expressed as number of protrusion per 10 µm dendrite (A) and percentage of PSD-95-positive protrusions (B) and are mean ± SEM of 22-24 determinations per group.

The density of VGluT1 and PSD95 clusters in MSN secondary dendrites did not yield significant changes with LRRK2-IN1 (Fig. 32).

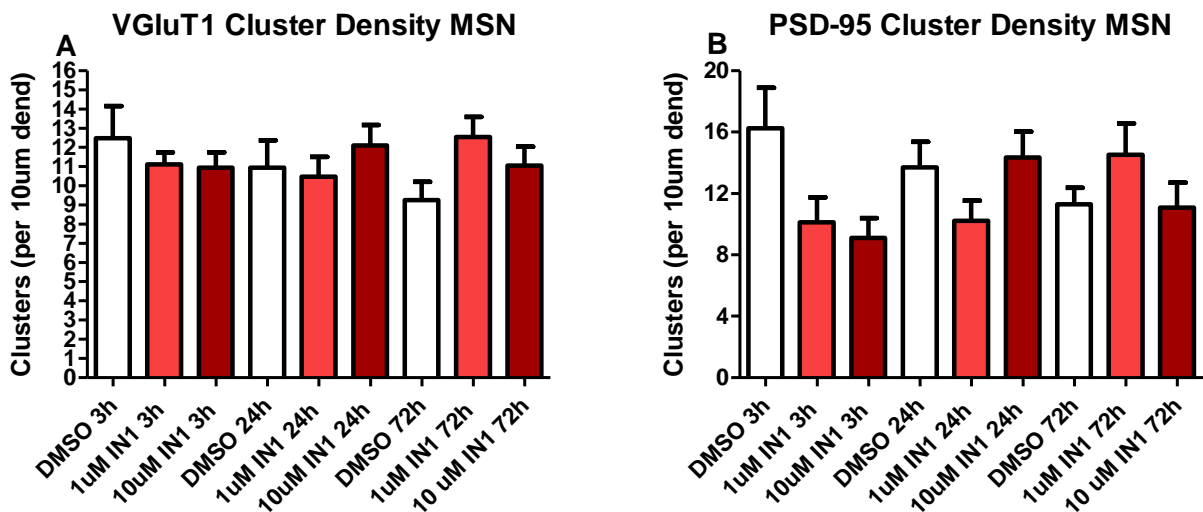


Fig. 32. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on clusters densities of the synaptic markers VGluT1 (A) and PSD-95 (B) in MSNs. Data are expressed as number of VGluT1 clusters (A) and PSD-95 clusters (B) per 10 µm dendrite and are mean ± SEM of 22-24 determinations per group.

Finally, analysis of the density of co-localised clusters did not yield to significant effects of treatment (Fig. 33).

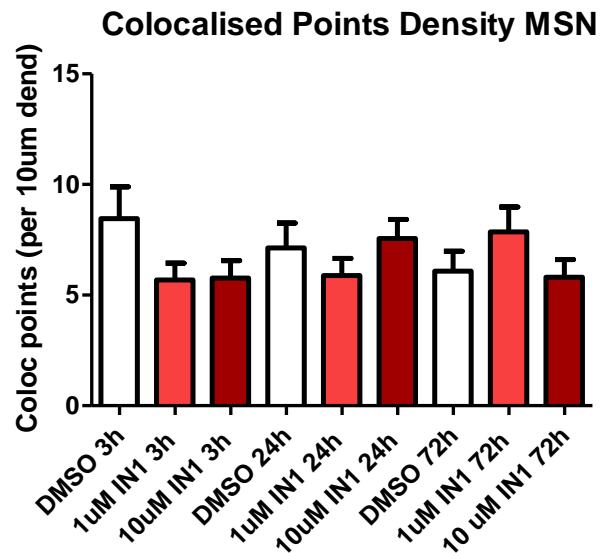


Fig. 33. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on VGluT1-PSD95 co-localised clusters in MSNs. Data are expressed as number of co-clusters per 10 µm dendrite and are mean ± SEM of 22-24 determinations per group.

Effect of LRRK2-IN1 in CTX neurons

Differently from MSNs, pharmacological inhibition of LRRK2 kinase activity did not induce significant changes in the size of synaptic markers in CTX neurons (Fig. 34). The area of VGluT1 clusters (presynaptic; Fig 34A) and PSD95 (postsynaptic marker; Fig. 34B) was not modified by treatment.

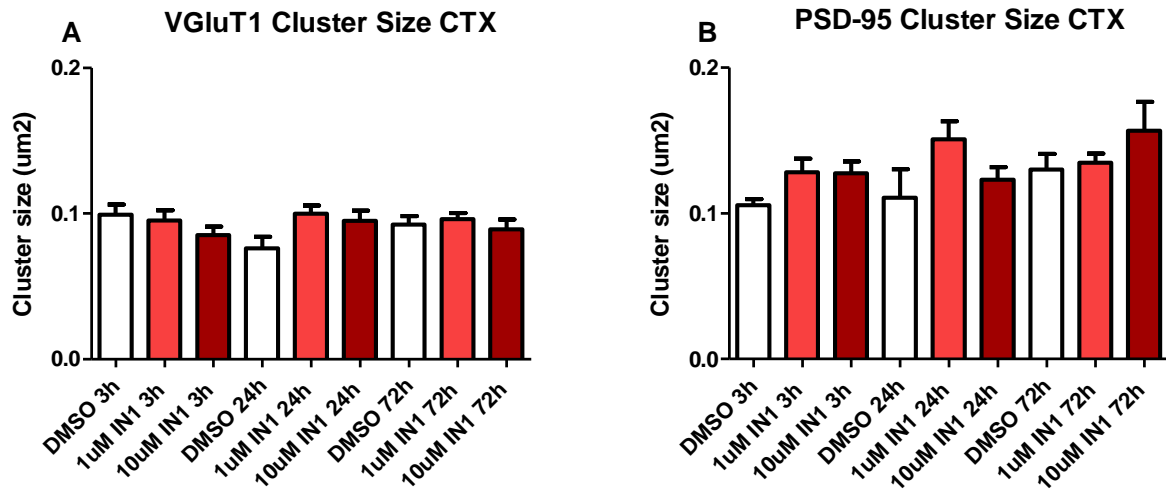


Fig. 34. Effect of LRRK2-IN1 (1 and 10 μM, in the media) on the size of VGLuT1 (A) and PSD-95 (B) synaptic clusters in cultured CTX neurons. Data are expressed as area (μm²) of puncta and are mean ± SEM of 22-24 determinations per group.

In line with the observations in MSNs, the percentages of colocalisation of the synaptic proteins were not significantly affected by LRRK2-IN1 (Fig. 35).

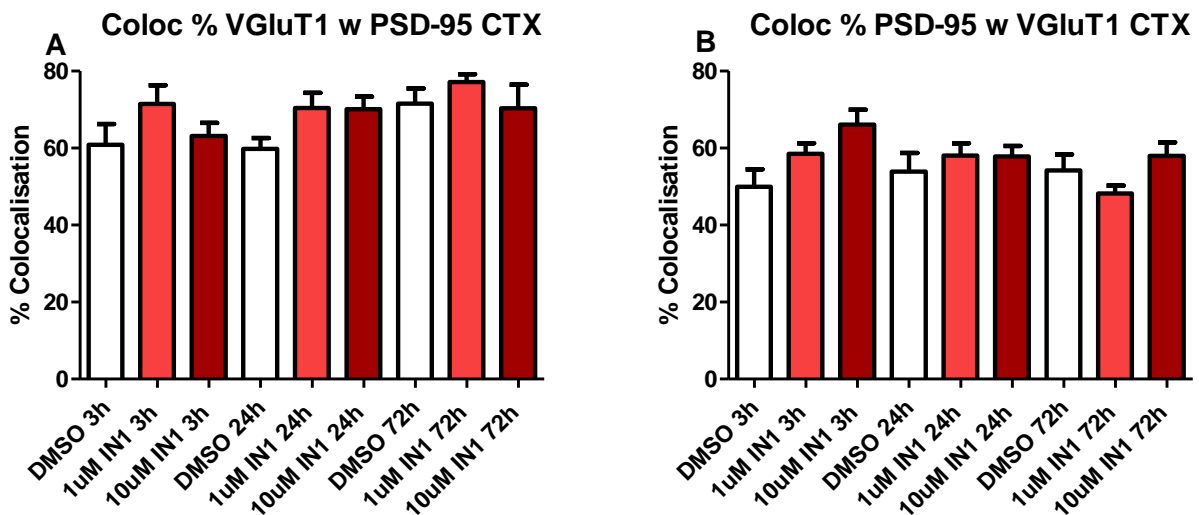


Fig. 35. Effect of LRRK2-IN1 (1 and 10 μM, in the media) on colocalisation of the synaptic markers VGLuT1 (A) and PSD95 (B) in cTX neurons. Data are expressed as percentage colocalisation of VGLuT1 with PSD-95 (A) and PSD-95 with VGLuT1 (B) and are mean ± SEM of 22-24 determinations per group.

Consistent with data from MSNs, LRRK2-IN1 did not affect the density of filopodia in cortical secondary dendrites (Fig. 36A). Notably, even though drug application in neuronal cultures did not modify the percentage of PSD95-positive protrusions with respect to vehicle (i.e. DMSO), the same concentration (1 μ M) had different outcomes at different time points, namely the percentage of filopodia labeled with PSD95 was higher after 3h when compared to 24h and 72h (~21% and ~28%, respectively; Fig. 36B).

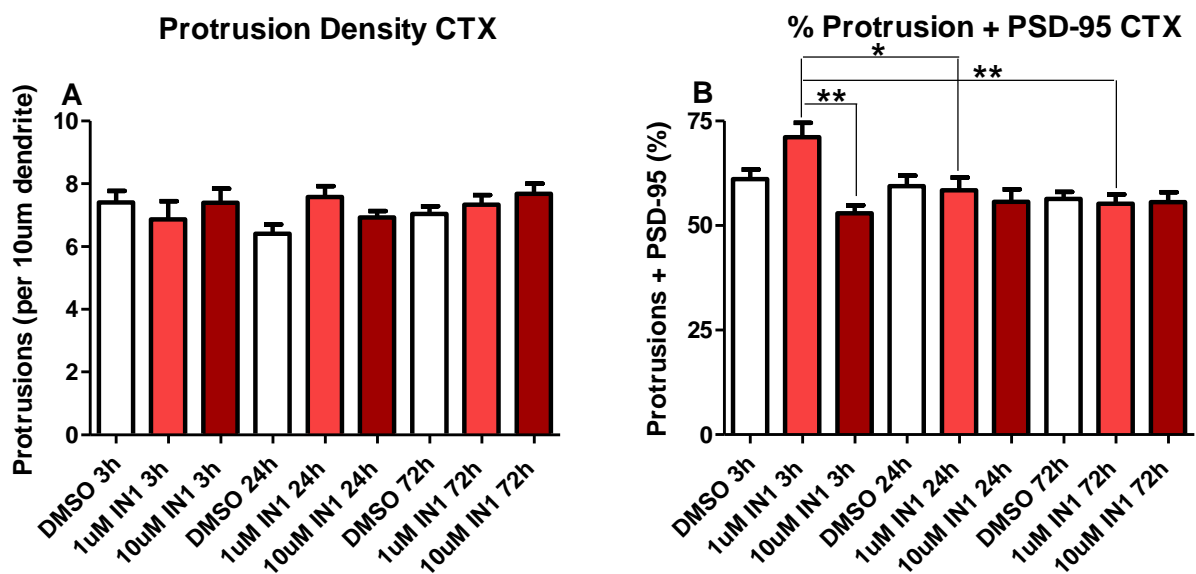


Fig. 36. Effect of LRRK2-IN1 (1 and 10 μ M, in the media) on protrusion density (A) and percentage of PSD95-positive protrusions (B) in CTX neurons. Data are expressed as number of protrusion per 10 μ m dendrite (A) and percentage of PSD-95-positive protrusions (B) and are mean \pm SEM of 22-24 determinations per group. * p <0.05,

** p <0.01, significantly different comparison.

Analysis of VGluT1 and PSD95 clusters densities in CTX secondary dendrites did not lead to significant results, probably due to variability in the data sets (Fig. 37).

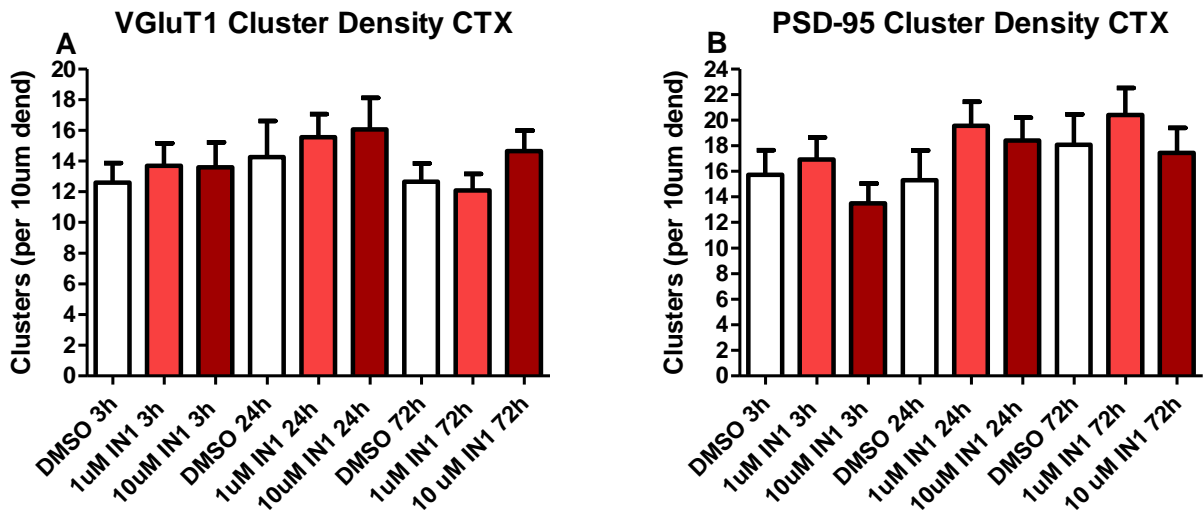


Fig. 37. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on clusters densities of the synaptic markers VGLuT1 (A) and PSD-95 (B) in CTX neurons. Data are expressed as number of VGLuT1 clusters (A) and PSD-95 clusters (B) per 10 µm dendrite and are mean ± SEM of 22-24 determinations per group.

Finally, the density of VGLuT1-PSD95 colocalised clusters was analyzed also in CTX cells (Fig. 38), but no significant effects of LRRK2-IN1 were observed.

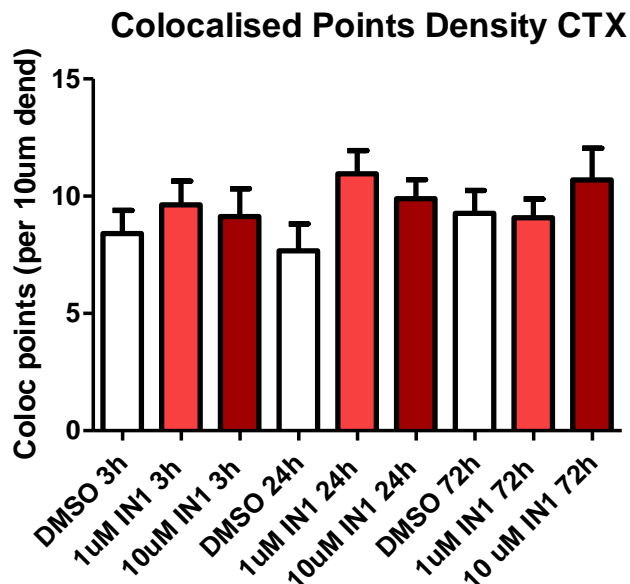


Fig. 38. Effect of LRRK2-IN1 (1 and 10 µM, in the media) on VGLuT1-PSD95 co-localised clusters in CTX neurons. Data are expressed as number of co-clusters per 10 µm dendrite and are mean ± SEM of 22-24 determinations per group.

DISCUSSION

The discovery that familial PD is associated with highly penetrant mutations in LRRK2 has raised great enthusiasm towards a novel molecular pathway involved in the pathology. At the same time, however, the scientific community had to deal with a considerable amount of discordances due to inconsistent characterization of animal (in particular, rodent) models and lack of selective pharmacological tools. Moreover, the protein is still far from being well defined in its cellular role. Synaptic rearrangements and defects have been reported in a number of neurological diseases (Wishart et al., 2006). Consistently, mutations in LRRK2 have been shown to cause neurotransmission deficits (Tong et al., 2009; Lee et al., 2010), suggesting that synaptic dysfunction might be a primary effect of PD-causing mutants.

Along with influence on synapses, LRRK2 has also been implicated in mitochondrial homeostasis, neurite outgrowth and dendrite degeneration in different cellular and animal models (MacLeod et al., 2006; Plowey et al., 2008; Dachsel et al., 2010; Lin et al., 2010; Chan et al., 2011; Winner et al., 2011). However, the role of LRRK2 in motor function as well as the behavioral consequences of LRRK2 mutations are still under debate, and a well-established behavioral phenotype in transgenic animals has not been described yet.

LRRK2 plays an age-dependent role on motor abilities

PD-related LRRK2 mutants have been shown to impair motor abilities in older animals (Li et al., 2009) and to affect motor responses to DA drugs (Tong et al., 2009). On the other hand, no study has to date reported motor abnormalities caused by G2019S substitution in transgenic mice (Melrose et al., 2010; Li et al., 2010). As a matter of fact, to the best of our knowledge, the role played by the endogenous protein itself on motor behavior has not yet been fully addressed.

In this study, we report a surprising role of LRRK2 in motor worsening associated with aging. Indeed, LRRK2-KO mice did not show impairment of their motor performance up to 12 months of age, in contrast to that observed in wild-type and mutant animals. Conversely, the pathogenic G2019S substitution (leading to increased kinase activity) appeared to accelerate the onset of motor

worsening during aging. Behavioral deficits have been shown in C57BL/6J mice along their lifespan, both in motor and non motor parameters (Fahlström et al., 2011). These observations might be related to the hypothetical mitochondrial role of LRRK2. Indeed, LRRK2 has been shown to associate with the outer membrane of mitochondria (West et al., 2005). Moreover, mitochondrial impairment has been reported in biopsies from LRRK2-G2019S PD patients (Mortiboys et al., 2010). Consistently, pathogenic LRRK2 mutants induced apoptosis through the mitochondrial pathway and release of cytochrome c (Iaccarino et al., 2007), a pathway known to respond to reactive oxygen species (ROS) and MPP⁺ toxicity (Perier et al., 2007). Interestingly, the p66Shc gene encodes for an adaptor protein involved in mitochondria ROS production (Orsini et al., 2004) and its ablation leads to prolonged lifespan (Migliaccio et al., 1999). Thus, a role for these pathways in the aging process appears likely.

Despite the differences across genotypes observed over aging, we did not observe any difference between age-matched wild-type and BAC hLRRK2-G2019S mice on the first day of testing. Thus, we designed a more thorough behavioral protocol with the aim to disclose differences in motor learning and/or motor function during repetitive (4 consecutive days) motor stimulation of age-matched mice.

First of all, these experiments confirmed the age-dependence of motor worsening associated with LRRK2 mutation, since major differences among genotypes were observed starting from 6 months but not earlier time (3 months). Indeed, at 6 months of age, BAC hLRRK2-G2019S mice exhibited a behavioral deficit after 3 days of testing. This pattern was also observed in older animals (12 month-old), when stepping impairment became evident even at an earlier time-point (Day 2). This is the first evidence for the G2019S mutation causing motor deficits in transgenic compared to non transgenic animals. A similar result has been reported for the R1441G substitution in BAC hLRRK2 transgenic mice (Li et al., 2009). In our study, we also report hyperactivity in LRRK2-KO animals from 6 months onwards. In this case, the behavioral phenotype was evident already at the first day of testing, remaining stably above the level of performance of their littermates. Thus, the

hyperactivity caused by genetic deletion of LRRK2 is dependent on age but not on repeated motor testing. The finding that wild-type mice do not modify their performance when challenged over a 4-day testing protocol is consistent with a previous study (Volta et al., 2010) and indicates that the phenotypes observed are tightly influenced by LRRK2. Moreover, the deficits observed are specifically dependent on the reiterated testing. Indeed, no changes in spontaneous locomotor activity have been observed, both in term of distance traveled and immobility time within the activity cage. This contrasts with a previous study reporting that BAC hLRRK2-G2019S mice traveled for longer distances (Melrose et al., 2010). The discrepancy may relate to the fact that Melrose and coworkers (2010) tested transgenic mice bred on a FVB strain, whereas we worked with C57BL/6J backcrossed mice. Interestingly, in the same experimental setting we showed a reduced anxious-like behavior in LRRK2-KO mice in the absence of spontaneous hyperactivity. Our results also differ from other studies reporting no behavioral changes in spontaneous activity of G2019S knockin mice (Tong et al., 2009; Herzig et al., 2011). This might be due to the low levels of transgene expression as well as to the different protocol of motor testing. Indeed, in the same study, also LRRK2-KO mice had a normal motor activity.

The protocol we employed, thus, appears critical in the development of behavioral deficits in BAC hLRRK2-G2019S mice. Although we do not have an easy explanation for this phenomenon, Li and colleagues (2010) reported an impaired sustainability of striatal DA release in BAC hLRRK2-G2019S mice. Indeed, repetitive stimulation of DAergic axon terminals leads to a decline in extracellular concentration of evoked DA release, as measured by fast-scan cyclic voltammetry (FSCV). In striata from BAC hLRRK2-G2019S mice, this decline appeared amplified, indicating a reduced capability to cope with the excitotoxic release demand. Thus, repeated motor testing may challenge the nigrostriatal DA system, causing a “hypodopaminergic” mutant mice to fail in coping with the higher demand of striatal DA release. These events might lead to a delayed behavioral impairment, with respect to normal animals.

LRRK2 and the DA system interact in the modulation of motor behavior

The discovery of motor deficit in BAC hLRRK2-G2019S mice prompted us to assess the response to classical antiparkinsonian drugs, which is mandatory for model validation.

We observed a selective response to L-DOPA only in BAC hLRRK2-G2019S mice, as reported for mice carrying the R1441G mutation (Li et al., 2009). Pathogenic substitutions in LRRK2 are thought to induce axonal and nerve terminal pathology in the nigrostriatal DA system (Li et al., 2009; Melrose et al., 2010; Li et al., 2010). Therefore, motor impairment might be due to decreased activity along the nigrostriatal DA system, even in the absence of overt cell death. L-DOPA may reverse the hypokinetic phenotype through its classical action of DA levels supply (Lindgren et al., 2010). We might also hypothesize a more specific action on D2/D3 DA receptors, based on the analogous response elicited by the D2/D3 agonist PPX in BAC hLRRK2-G2019S mice. The effects of L-DOPA and PPX in BAC hLRRK2-G2019S mice are surprisingly similar to those elicited in MPTP-treated mice (Viario et al., 2010) indicating that motor deficit has a dopaminergic nature. Indeed, impairment of DA transmission or DA loss are known to cause compensatory up-regulation of postsynaptic DA D2 receptors allowing the antiparkinsonian action of dopaminomimetics. Consistent with this view, PPX was ineffective in wild-type mice and even worsened motor activity in LRRK2-KO animals. We therefore asked if enhanced LRRK2 activity (caused by G2019S mutation; West et al., 2005; Gloeckner et al., 2006) or its genetic deletion oppositely modulate signaling at D2/D3 receptors. Of note, reduced responses to D2 DA receptor agonists have been reported in LRRK2-R1441C knockin mice, both in behavioral and electrophysiological paradigms (Tong et al., 2009), although no motor responses to DA agonists and antagonists have been shown in LRRK2-G2019S knockin mice (Herzig et al., 2011). We therefore investigated the behavioral outcome of pharmacological blockade of D2 receptors by haloperidol. Surprisingly, despite similar levels of catalepsy were elicited in all the three genotypes (as observed in the bar test), BAC hLRRK2-G2019S mice were insensitive to the inhibitory effect of haloperidol on stepping activity. Therefore, D2/D3 DA receptors appear functional but less saturated by endogenous DA in these

LRRK2 mutants, again pointing to a deficient endogenous transmission. Indeed, haloperidol-induced catalepsy is mediated by blockade of striatal post-synaptic D2 receptors (Bateup et al., 2010) and impairment of the nigrostriatal DA system has been observed in LRRK2 BAC mutant mice (Li et al., 2009; Melrose et al., 2010; Li et al., 2010).

Although the response to haloperidol was not different in absolute values between wild-type and LRRK2-KO animals, a greater impairment in percentage values was observed in LRRK2-KO mice. This finding adds to the paradoxical inhibitory response of LRRK2-KO mice to PPX, pointing to the existence of a hyperdopaminergic phenotype in these animals. In fact, motor impairment induced by DA agonists is mediated by pre-synaptic D2/D3 (auto)receptors (Mierau and Schingnitz 1992; Maj et al., 1997; Siuciak and Fujiawara, 2004), and one might expect that in face of a greater DA release D2 autoreceptor function is up-regulated to maintain physiological transmission. On the contrary, hypodopaminergic conditions lead to supersensitivity of post-synaptic D2-like receptors allowing DA receptor agonists to promote motor function (Seeman, 2007), which accounts for the positive effects observed in BAC hLRRK2-G2019S mice treated with PPX.

In light of these data, we finally investigated the role played by D1-like DA receptors in transgenic LRRK2 mice. Activation of D1 DA receptors located on MSNs projecting to the SNr are thought to promote motor activity through the striatonigral (direct) pathway (Deniau and Chevalier 1985). Consistently, wild-type mice showed increased akinesia when treated with the D1 antagonist SCH23390. However, the facilitatory effect of D1 agonists only emerges when DA levels drop, as in parkinsonian conditions. Consistently, the D1 agonist SKF38393 attenuated akinesia (bar test) and bradykinesia (drag test) in BAC hLRRK2-G2019S mice being ineffective in wild-type and LRRK2-KO animals (actually a delayed reduction of stepping was observed in this genotype).

Paradoxically, however, also D1 receptor blockade increased stepping activity in BAC hLRRK2-G2019S mice, indicating a greater tonic inhibitory influence of endogenous DA via D1 receptors in this genotype. These results are hard to explain. One might hypothesize that SKF38393 and SCH23390 interact with different populations of D1 receptors. For instance, D1 receptors are also

present on striatopallidal MSNs (Surmeier et al., 1996) and their activation would lead to motor impairment. Thus, blockade of these receptors would promote motor activity. Alternatively, D1 receptors are also expressed on striatopallidal nerve terminals in GP (Barone et al., 1987) where they stimulate pallidal GABA release (Mabrouk et al., 2011). Activation of this pathway would also lead to movement inhibition, which will be reversed by D1 receptor blockade.

LRRK2 kinase activity partially modulates synaptic morphology

Co-culture of MSNs and CTX neurons allowed to reproduce, on a smaller scale, a neuronal pathway essential for physiological functions. Indeed, in the absence of glutamate transmission, MSNs do not develop their characteristic spiny morphology (Segal et al., 2003). In our conditions, striatal MSNs have been shown to develop more complex dendritic architecture and dendritic spine-like protrusions (Kaufman et al., 2012).

The application of the LRRK2 kinase inhibitor LRRK2-IN1 produced an increase in the size of VGluT1 clusters, as analysed in imaged MSNs. In this regard, when considering VGluT1, it is important to note that the expression of this marker occurs in the pre-synaptic compartment, thus in the neuronal subtype not labeled by YFP.

Specifically, the size of cortical VGluT1 clusters increased after 72h treatment with LRRK2-IN1. This might indicate that LRRK2, through its phosphorylation activity, acts as a constraint of the glutamatergic cortical inputs onto MSNs. Notably, the responsiveness of striatal MSNs to this increased cortical drive is limited, as indicated by the absence of a clear effect of LRRK2-IN1 on the amount of PSD95-positive dendritic protrusions. It could be hypothesized that inhibition of LRRK2 increases the signaling ability of CTX neurons on MSNs. This effect appears to be correlated to a specific action on cortico-striatal synapses, since no effect has been observed on striatal VGluT1 cluster size, analyzed in imaged CTX neurons. The enhancement in the CTX-to-MSN signaling appears not to be related to an increase in the number of synaptic contacts, since no changes in the density of co-localised clusters have been observed following LRRK2-IN1

treatment, both in imaged MSNs and CTX neurons. Consistently with our observations, silencing LRRK2 expression in cortical neurons induced a redistribution of vesicles in the presynaptic site and increased vesicle kinetics leading to enhanced postsynaptic current in glutamate-receptive neurons (Piccoli et al., 2011). This is in accordance with an increased cortical drive, induced by enhancement of vesicles activity without an increase in their number at the synaptic level. A possible mechanism might be represented by alterations in microtubule and cytoskeleton dynamics. Indeed, LRRK2 has been reported to have a role in these processes (Lee et al., 2010; Parisiadou et al., 2009). Thus, LRRK2-IN1 might act by increasing glutamate vesicles kinetics through alterations in the cytoskeletal structure.

In imaged CTX cells, a short application of LRRK2-IN1 produces higher percentages of PSD95-positive protrusions. This molecular adaptation might be a consequence of an increased signaling from the non labeled MSNs present in the culture or a direct LRRK2 regulation of the scaffolding machinery within the dendritic spines.

The pharmacological profile of the LRRK2 inhibitor used in this study (LRRK2-IN1), should also be considered in discussing the results. The compound has recently been developed and reported to be a potent and selective ATP-competitive inhibitor of LRRK2 kinase activity (Deng et al., 2011). Other LRRK2 kinase inhibitors have been employed but no one is selective (Covy et al., 2009; Anand et al., 2009; Nichols et al., 2009; Lee et al., 2010). Disappointingly, also the selectivity of LRRK2-IN1 has been recently questioned, since the inhibitor produced similar effects in LRRK2^{+/+} and LRRK2^{-/-} neuronal cultures (Loos et al., 2011). Thus, some selectivity concerns in LRRK2-IN1 effects might be raised. For this reason, we are designing and will perform a supplementary experiment in which the same analysis will be made after knock down of LRRK2 expression.

Concluding remarks

In this study we performed the first investigation of the involvement of the LRRK2 protein in synaptic morphology, in a recently developed strategy to model the cortico-striatal connection in vitro. Our results point towards a regulatory role of this neuronal pathway played by LRRK2, even though we faced uncertainties due to data variability and pharmacological selectivity issues about the kinase inhibitor employed. Nonetheless, our results are consistent with previous data and indicate that reduction of LRRK2 kinase activity leads to increased signaling from cortical neurons. This study also provides for the first time an age-dependent motor deficits in BAC hLRRK2-G2019S mice under a protocol of behavioral stimulation and motor learning. Importantly, these deficits are reversed by DA receptor agonists and reminiscent of a parkinsonian condition. At the same time, ablation of the *Lrrk2* gene prevents the decrease of motor abilities associated with aging and causes a constitutive hyperactive condition. Overall, it therefore appears that LRRK2 is a negative modulator of motor function. Finally, we show the complex impact of LRRK2 on D1 and D2 receptor-mediated control of motor behavior. Although the different motor responses to DA agonists might be interpreted on different activity of striatal DA terminals (with BAC hLRRK2-G2019S mice being hypodopaminergic and LRRK2-KO mice being hyperdopaminergic), further neurochemical and electrophysiological investigation are necessary to investigate whether LRRK2 directly modulates D1 and D2 receptor machinery. Nonetheless, this study offers a phenotypic model of genetic parkinsonism useful for screening of symptomatic drugs and investigating early pathogenic events in this form of PD (PARK8) .

ORIGINAL PAPERS

Mabrouk OS, **Volta M**, Marti M, Morari M. Stimulation of delta opioid receptors located in substantia nigra reticulata but not globus pallidus or striatum restores motor activity in 6-hydroxydopamine lesioned rats: new insights into the role of delta receptors in parkinsonism. *J Neurochem.* 2008; 107: 1647-1659.

Volta M, Marti M, McDonald J, Molinari S, Camarda V et al. Pharmacological profile and antiparkinsonian properties of the novel nociceptin/orphanin FQ receptor antagonist 1-[1-cyclooctylmethyl-5-(1-hydroxy-1-methyl-ethyl)-1,2,3,6-tetrahydro-pyridin-4-yl]-3-ethyl-1,3-dihydro-benzoimidazol-2-one (GF-4). *Peptides.* 2010; 31: 1194-1204.

Volta M, Mabrouk OS, Bido S, Marti M, Morari M. Further evidence for an involvement of nociceptin/orphanin FQ in the pathophysiology of Parkinson's disease: a behavioral and neurochemical study in reserpinized mice. *J Neurochem.* 2010; 115: 1543-1555.

Volta M, Viaro R, Trapella C, Marti M, Morari M. Dopamine-nociceptin/orphanin FQ interactions in the substantia nigra reticulata of hemiparkinsonian rats: involvement of D2/D3 receptors and impact on nigro-thalamic neurons and motor activity. *Exp Neurol.* 2011; 228: 126-137.

Esposito E, Mariani P, Ravani L, Contado C, **Volta M** et al. Nanoparticulate lipid dispersions for bromocriptine delivery: Characterization and in vivo study. *Eur J Pharm Biopharm.* 2012; 80: 306-314.

REFERENCES

Anand VS, Reichling LJ, Lipinski K, Stochaj W, Duan W et al. FEBS J 2009; 276: 466-78.

Andres-Mateos E, Perier C, Zhang L, Blanchard-Fillion B, Greco TM et al. DJ-1 gene deletion reveals that DJ-1 is an atypical peroxiredoxin-like peroxidase. Proc Natl Acad Sci USA 2007; 104: 14807-14812.

Andres-Mateos E, Mejias R, Sasaki M, Li X, Lin BM et al. Unexpected lack of hypersensitivity in LRRK2 knock-out mice to MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine). J Neurosci 2009; 29: 15846-15850.

Anitei M, Wassmer T, Stange C, Hoflack B. Bidirectional transport between the trans-Golgi network and the endosomal system. Mol Membr Biol 2010; 27: 443-456.

Barone P, Tucci I, Parashos SA, Chase TN. D-1 dopamine receptor changes after striatal quinolinic acid lesion. Eur J Pharmacol 1987; 138: 141-145.

Bartus RT, Herzog CD, Chu Y, Wilson A, Brown L et al. Bioactivity of AAV2-Neurturin gene therapy (CERE-120): differences between Parkinson's disease and nonhuman primate brains. Mov Disord 2011; 26: 27-36.

Bateup HS, Santini E, Shen W, Birnbaum S, Valjent E et al. Distinct subclasses of medium spiny neurons differentially regulate striatal motor behaviors. Proc Natl Acad Sci U S A 2010; 107: 14845-14850.

Beilina A, Van Der Brug M, Ahmad R, Kesavapany S, Miller DW et al. Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. Proc Natl Acad Sci USA 2005; 102: 5703-5708.

Berwick DC, Harvey K. LRRK2 signaling pathways: the key to unlocking neurodegeneration? Trends Cell Biol 2011; 21: 257-265.

Bjorklund LM, Sánchez-Pernaute R, Chung S, Anderson T, Chen IY et al. Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. Proc Natl Acad Sci USA 2002; 99: 2344-2349.

Bonifacino JS, Hurley JH. Retromer. Curr Opin Cell Biol 2008; 20: 427-436.

Bosgraaf L, Van Haastert PJ. Roc, a Ras/GTPase domain in complex proteins. Biochim Biophys Acta 2003; 1643: 5-10.

Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN et al. Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 2003; 24: 197-211.

Braak H, Ghebremedhin E, Rüb U, Bratzke H, Del Tredici K. Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 2004; 318: 121-134.

Braschi E, Goyon V, Zunino R, Mohanty A, Xu L et al. Vps35 mediates vesicle transport between the mitochondria and peroxisomes. Curr Biol 2010; 20: 1310-1315.

Bretscher A, Edwards K, Fehon RG. ERM proteins and merlin: integrators at the cell cortex. Nat Rev Mol Cell Biol 2002; 3: 586-599.

Burre J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, et al. Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. *Science* 2010; 329: 1663-1667.

Cai J, Yang M, Poremsky E, Kidd S, Schneider JS et al. Dopaminergic neurons derived from human induced pluripotent stem cells survive and integrate into 6-OHDA lesioned rats. *Stem Cells Dev* 2010; 19: 1017-1023.

Carballo-Carbajal I, Weber-Endress S, Rovelli G, Chan D, Wolozin B et al. Leucine-rich repeat kinase 2 induces alpha-synuclein expression via the extracellular signal-regulated kinase pathway. *J Biol Chem* 2010; 285: 821-827.

Caughey B, Lansbury PT. Protofibrils, pores, fibrils and neurodegeneration: separating the responsible protein aggregates from the innocent bystanders. *Annu Rev Neurosci* 2003; 26: 267-298.

Chan D, Citro A, Cordy JM, Shen GC, Wolozin B. Rac1 protein rescues neurite retraction caused by G2019S leucine-rich repeat kinase 2 (LRRK2). *J Biol Chem* 2011; 286: 16140-16149.

Chartier-Harlin MC, Dachsel JC, Vilarinho-Güell C, Lincoln SJ, Leprêtre F et al. Translation initiator EIF4G1 mutations in familial Parkinson disease. *Am J Hum Genet* 2011; 89: 398-406.

Chaudhuri KR, Healy DG, Schapira AHV; National Institute for Clinical Excellence. Non-motor symptoms of Parkinson's Disease: diagnosis and management. *Lancet Neurol* 2006; 5: 235-245.

Chaudhuri KR, Schapira AHV. Non-motor symptoms of Parkinson's Disease: dopaminergic pathophysiology and treatment. *Lancet Neurol* 2009; 8: 464-474.

Clark IE, Dodson MW, Jiang C, Cao JH, Huh JR et al. Drosophila pink1 is required for mitochondrial function and interacts genetically with parkin. *Nature* 2006; 441: 1162-1166.

Conway KA, Lee SJ, Rochet JC, Ding TT, Williamson RE et al. Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy. *Proc Natl Acad Sci USA* 2000; 97: 571-576.

Conway KA, Rochet JC, Bieganski RM, Lansbury PT Jr. Kinetic stabilization of the alpha-synuclein protofibril by a dopamine-alpha-synuclein adduct. *Science* 2001; 294: 1346-1349.

Covy JP, Giasson BI. Identification of compounds that inhibit the kinase activity of leucine-rich repeat kinase 2. *Biochem Biophys Res Commun* 2009; 378: 473-477.

Cunnington R, Egan G, O'Sullivan J, Hughes A, Bradshaw J et al. Motor imagery in Parkinson's disease: a PET study. *Mov Disord* 2001; 16: 849-857.

Dächsel JC, Behrouz B, Yue M, Beevers JE, Melrose HL et al. A comparative study of Lrrk2 function in primary neuronal cultures. *Parkinsonism Relat Disord* 2010; 16: 650-655.

Darios F, Ruiperez V, Lopez I, Villanueva J, Gutierrez LM et al. Alpha-synuclein sequesters arachidonic acid to modulate SNARE-mediated exocytosis. *EMBO Rep* 2010; 11: 528-533.

- Deng H, Jankovic J, Guo Y, Xie W, Le W. Small interfering RNA targeting the PINK1 induces apoptosis in dopaminergic cells SH-SY5Y. *Biochem Biophys Res Commun* 2005; 337: 1133–1138.
- Deng X, Dzamko N, Prescott A, Davies P, Liu Q et al. Characterization of a selective inhibitor of the Parkinson's disease kinase LRRK2. *Nat Chem Biol* 2011; 7: 203-205.
- Deniau JM, Chevalier G. Disinhibition as a basic process in the expression of striatal functions. II. The striato-nigral influence on thalamocortical cells of the ventromedial thalamic nucleus. *Brain Res* 1985; 334: 227–233.
- Desplats P, Lee HJ, Bae EJ, Patrick C, Rockenstein E et al. Inclusion formation and neuronal cell death through neuron-to-neuron transmission of α -synuclein. *Proc Natl Acad Sci USA* 2009; 106: 13010-13015.
- Dev KK, Hofele K, Barbieri S, Buchman VL, van der Putten H. Part II: alpha-synuclein and its molecular pathophysiological role in neurodegenerative disease. *Neuropharmacology* 2003; 45: 14-44.
- Dusonchet J, Kochubey O, Stafa K, Young SM Jr, Zufferey R et al. A rat model of progressive nigral neurodegeneration induced by the Parkinson's disease-associated G2019S mutation in LRRK2. *J Neurosci* 2011; 31: 907-912.
- Fahlström A, Zeberg H, Ulfhake B. Changes in behaviors of male C57BL/6J mice across adult life span and effects of dietary restriction. *Age (Dordr)* 2011 doi:10.1007/s11357-011-9320-7
- Fan J, Vasuta OC, Zhang LY, Wang L, George A et al. N-methyl-D-aspartate receptor subunit- and neuronal-type dependence of excitotoxic signaling through post-synaptic density 95. *J Neurochem* 2010; 115: 1045-1056.
- Farrer M, Kachergus J, Forno L, Lincoln S, Wang DS et al. Comparison of kindreds with parkinsonism and α -synuclein genomic multiplications. *Ann Neurol* 2004; 55: 174-179.
- Farrer MJ. Genetics of Parkinson's disease: paradigm shifts and future prospects. *Nat Rev Genet* 2006; 7: 306-318.
- Fernagut PO, Hutson CB, Fleming SM, Tetreault NA, Salcedo J et al. Behavioral and histopathological consequences of paraquat intoxication in mice: effects of alpha-synuclein over-expression. *Synapse* 2007; 61: 991-1001.
- Franch-Marro X, Wendler F, Guidato S, Griffith J, Baena-Lopez A et al. Wingless secretion requires endosome-to-Golgi retrieval of Wntless/Evi/Sprinter by the retromer complex. *Nat Cell Biol* 2008; 10: 170-177.
- Francis PT, Perry EK. Cholinergic and other neurotransmitter mechanisms in Parkinson's disease, Parkinson's disease dementia and dementia with Lewy bodies. *Mov Disord* 2007; 22: 351-357.
- Fukuda M, Mentis M, Ma Y, Dhawan V, Antonini A et al. Networks mediating the clinical effects of pallidal brain stimulation for Parkinson's disease: a PET study of resting-state glucose metabolism. *Brain* 2001; 124: 1601-1609.
- Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S et al. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. *Ann Neurol* 2002; 51: 296-301.

Gai WP, Blessing WW, Blumbergs PC. Ubiquitin-positive degenerating neuritis in the brainstem in Parkinson's disease. *Brain* 1995; 118: 1447-1459.

Gasser T. Molecular pathogenesis of Parkinson disease: insights from genetic studies. *Expert Rev Mol Med* 2009; 11: e22.

Gautier CA, Kitada T, Shen J. Loss of PINK1 causes mitochondrial functional defects and increased sensitivity to oxidative stress. *Proc Natl Acad Sci USA* 2008; 105: 11364–11369.

Gehrke S, Imai Y, Sokol N, Lu BW. Pathogenic LRRK2 negatively regulates microRNA-mediated translational repression. *Nature* 2010; 466: 637-641.

Ghosh P, Griffith J, Geuze HJ, Kornfeld S. Mammalian GGAs act together to sort mannose 6-phosphate receptors. *J Cell Biol* 2003; 163: 755-766.

Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R et al. Direct brain infusion of glial cell line-derived neurotrophic factor in Parkinson's disease. *Nature Med* 2003; 9: 589-595.

Gispert S, Ricciardi F, Kurz A, Azizov M, Hoepken HH et al. Parkinson phenotype in aged PINK1-deficient mice is accompanied by progressive mitochondrial dysfunction in absence of neurodegeneration. *PLoS ONE* 2009; 4: e5777.

Gloeckner CJ, Kinkl N, Schumacher A, Braun RJ, O'Neill E et al. The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet* 2006; 15: 223–232.

Gloeckner CJ, Schumacher A, Boldt K, Ueffing M. The Parkinson disease-associated protein kinase LRRK2 exhibits MAPKKK activity and phosphorylates MKK3/6 and MKK4/7, in vitro. *J Neurochem* 2009; 109: 959-968.

Goetz CG, Fahn S, Martinez-Martin P, Poewe W, Sampaio C et al. Movement Disorder society-sponsored revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): process, format, and clinimetric testing plan. *Mov Disord* 2007; 22: 41-47.

Goldberg MS, Pisani A, Haburcak M, Vortherms TA, Kitada T et al. Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familial Parkinsonism-linked gene DJ-1. *Neuron* 2005; 45: 489–496.

Gosavi N, Lee HJ, Lee JS, Patel S, Lee SJ. Golgi fragmentation occurs in the cells with prefibrillar alpha-synuclein aggregates and precedes the formation of fibrillar inclusion. *J Biol Chem* 2002; 277: 48984-48992.

Greene JC, Whitworth AJ, Andrews LA, Parker TJ, Pallanck LJ. Genetic and genomic studies of *Drosophila* parkin mutants implicate oxidative stress and innate immune responses in pathogenesis. *Hum Mol Genet* 2005; 14: 799–811.

Greggio E, Jain S, Kingsbury A, Bandopadhyay R, Lewis P et al. Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol Dis* 2006; 23: 329–341.

Greggio E, Zambrano I, Kaganovich A, Beilina A, Taymans JM et al. The Parkinson disease-associated leucine-rich repeat kinase 2 (LRRK2) is a dimer that undergoes intramolecular autophosphorylation. *J Biol Chem* 2008; 283: 16906–16914.

Greggio E, Cookson MR. Leucine-rich repeat kinase 2 mutations and Parkinson's disease: three questions. *ASN Neuro* 2009; 1: pii: e00002.

Guerrini R, Camarda V, Trapella C, Caló G, Rizzi A et al. Further studies at neuropeptide s position 5: discovery of novel neuropeptide S receptor antagonists. *J Med Chem* 2009; 52: 4068-4071.

Guo L, Gandhi PN, Wang W, Petersen RB, Wilson-Delfosse AL et al. The Parkinson's disease-associated protein, leucine-rich repeat kinase 2 (LRRK2), is an authentic GTPase that stimulates kinase activity. *Exp Cell Res* 2007; 313: 3658–3670.

Hallyday GM, Del Tredici K, Braak H. Critical appraisal of brain pathology staging related to presymptomatic and symptomatic cases of sporadic Parkinson's disease. *J Neural Transm Suppl* 2006; 70: 99-103.

Hallyday GM, McCann H. Human-based studies on alpha-synuclein deposition and relationship to Parkinson's disease symptoms. *Exp Neurol* 2008; 209: 12-21.

Han BS, Iacovitti L, Katano T, Hattori N, Seol W et al. Expression of the LRRK2 gene in the midbrain dopaminergic neurons of the substantia nigra. *Neurosci Lett* 2008; 442: 190-194.

Hatano Y, Li Y, Sato K, Asakawa S, Yamamura Y et al. Novel PINK1 mutations in early-onset parkinsonism. *Ann Neurol* 2004; 56: 424-427.

Hatano T, Kubo S, Imai S, Maeda M, Ishikawa K et al. Leucine-rich repeat kinase 2 associates with lipid rafts. *Hum Mol Genet* 2007; 16: 678–690.

Hawkes CH, Del Tredici K, Braak H. Parkinson's disease: the dual hit theory revisited. *Ann N Y Acad Sci* 2009; 1170: 615-22.

Heintz N. BAC to the future: the use of bac transgenic mice for neuroscience research. *Nat Rev Neurosci* 2001; 2: 861-870.

Hely MA, Reid WG, Adena MA, Halliday GM, Morris JG. The Sidney multicenter study of Parkinson's disease: the inevitability of dementia at 20 years. *Mov Disord* 2008; 23: 837-844.

Herzig MC, Kolly C, Persohn E, Theil D, Schweizer T et al. LRRK2 protein levels are determined by kinase function and are crucial for kidney and lung homeostasis in mice. *Hum Mol Genet* 2011; 20: 4209-4223.

Ho CC, Rideout HJ, Ribe E, Troy CM, Dauer WT. The Parkinson disease protein leucine-rich repeat kinase 2 transduces death signals via Fas-associated protein with death domain and caspase-8 in a cellular model of neurodegeneration. *J Neurosci* 2009; 29: 1011-1016.

Hu F, Padukkavidana T, Vægter CB, Brady OA, Zheng Y et al. Sortilin-mediated endocytosis determines levels of the frontotemporal dementia protein, progranulin. *Neuron* 2010; 68: 654-667.

Huot P, Fox SH, Brotchie JM. The serotonergic system in Parkinson's disease. *Prog Neurobiol* 2011; 95: 163-212.

Iaccarino C, Crosio C, Vitale C, Sanna G, Carrì MT et al. Apoptotic mechanisms in mutant LRRK2-mediated cell death. *Hum Mol Genet* 2007; 16: 1319-1326.

Imai Y, Gehrke S, Wang HQ, Takahashi R, Hasegawa K et al. Phosphorylation of 4E-BP by LRRK2 affects the maintenance of dopaminergic neurons in *Drosophila*. *EMBO J.* 2008; 27: 2432-2443.

Itier JM, Ibanez P, Mena MA, Abbas N, Cohen-Salmon C et al. Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. *Hum Mol Genet* 2003; 12: 2277-2291.

Jaleel M, Nichols RJ, Deak M, Campbell DG, Gillardon F et al. LRRK2 phosphorylates moesin at threonine-558: characterization of how Parkinson's disease mutants affect kinase activity. *Biochem J* 2007; 405: 307-317.

Jankovic J. Parkinson's Disease: clinical features and diagnosis. *J Neurol Neurosurg Psychiatry* 2008; 79: 368-376.

Kachergus J, Mata IF, Hulihan M, Taylor JP, Lincoln S et al. Identification of a novel LRRK2 mutation linked to autosomal dominant parkinsonism: evidence of a common founder across European populations. *Am J Hum Genet* 2005; 76: 672-680.

Kaech S, Banker G. Culturing hippocampal neurons. *Nat Protoc* 2006; 1: 2406-2415.

Kahle PJ, Waak J, Gasser T. DJ-1 and prevention of oxidative stress in Parkinson's disease and other age-related disorders. *Free Radic Biol Med* 2009; 47: 1354-1361.

Kaufman AM, Milnerwood AJ, Sepers MD, Coquinco A, She K et al. Opposing roles of synaptic and extrasynaptic NMDA receptor signaling in co-cultured striatal and cortical neurons. *J Neurosci* 2012; *in press*.

Kim RH, Smith PD, Aleyasin H, Hayley S, Mount MP et al. Hypersensitivity of DJ-1-deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and oxidative stress. *Proc Natl Acad Sci USA* 2005; 102: 5215-5220.

Kirik D, Rosenblad C, Burger C, Lundberg C, Johansen TE et al. Parkinson-like neurodegeneration induced by targeted overexpression of alpha-synuclein in the nigrostriatal system. *J Neurosci* 2002; 22: 2780-2791.

Kirik D, Björklund A. Modeling CNS neurodegeneration by overexpression of disease-causing proteins using viral vectors. *Trends Neurosci* 2003; 26: 386-392.

Kiskinis E, Eggen K. Progress towards the clinical application of patient-specific pluripotent stem cells. *J Clin Invest* 2010; 120: 51-59.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 1998; 392: 605-608.

Kitada T, Pisani A, Porter DR, Yamaguchi H, Tscherter A et al. Impaired dopamine release and synaptic plasticity in the striatum of PINK1-deficient mice. *Proc Natl Acad Sci USA* 2007; 104: 11441–11446.

Kordower JH, Chu Y, Hauser RA, Freeman TB, Olanow CW. Parkinson's disease pathology in long-term embryonic nigral transplants in Parkinson's disease. *Nat Med* 2008; 14: 504-506.

Korolchuk VI, Schütz MM, Gómez-Llorente C, Rocha J, Lansu NR et al. *Drosophila* Vps35 function is necessary for normal endocytic trafficking and actin cytoskeleton organisation. *J Cell Sci* 2007; 120: 4367-4376.

Kuschinsky K, Hornykiewicz O. Morphine catalepsy in the rat: relation to striatal dopamine metabolism. *Eur J Pharmacol* 1972; 19: 119–122.

Lang AE, Gill S, Patel NK, Lozano A, Nutt JG et al. Randomized controlled trial of intraputamenal glial cell line-derived neurotrophic factor infusion in Parkinson's disease. *Ann Neurol* 2006; 59: 459-466.

Langston JW. The Parkinson's complex: parkinsonism is just the tip of the iceberg. *Ann Neurol* 2006; 59: 591-596.

Lasiecka ZM, Winckler B. Mechanisms of polarized membrane trafficking in neurons – Focusing in on endosomes. *Mol Cell Neurosci* 2011; 48: 278-287.

Lee BD, Shin JH, VanKampen J, Petrucelli L, West AB et al. Inhibitors of leucine-rich repeat kinase-2 protect against models of Parkinson's disease. *Nat Med* 2010; 16: 998-1000.

Lee S, Liu HP, Lin WY, Guo H, Lu B. LRRK2 kinase regulates synaptic morphology through distinct substrates at the presynaptic and postsynaptic compartments of the *Drosophila* neuromuscular junction. *J Neurosci.* 2010; 30: 16959-16969.

Lesage S, Dürr A, Tazir M, Lohmann E, Leutenegger AL et al. LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. *N Engl J Med* 2006; 354: 422-423.

Lewis PA, Greggio E, Beilina A, Jain S, Baker A et al. The R1441C mutation of LRRK2 disrupts GTP hydrolysis. *Biochem Biophys Res Commun* 2007; 357: 668–671.

Li Y, Liu W, Oo TF, Wang L, Tang Y et al. Mutant LRRK2(R1441G) BAC transgenic mice recapitulate cardinal features of Parkinson's disease. *Nat Neurosci* 2009; 12: 826-828.

Li X, Patel JC, Wang J, Avshalumov MV, Nicholson C et al. Enhanced striatal dopamine transmission and motor performance with LRRK2 overexpression in mice is eliminated by familial Parkinson's disease mutation G2019S. *J Neurosci.* 2010; 30: 1788–1797.

Lin X, Parisiadou L, Gu XL, Wang L, Shim H et al. Leucine-rich repeat kinase 2 regulates the progression of neuropathology induced by Parkinson's-disease-related mutant alpha-synuclein. *Neuron* 2009; 64: 807-827.

Lin CH, Tsai PI, Wu RM, Chien CT. LRRK2 G2019S mutation induces dendrite degeneration through mislocalization and phosphorylation of tau by recruiting autoactivated GSK3beta. *J Neurosci* 2010; 30: 13138-13149.

- Lindgren HS, Andersson DR, Lagerkvist S, Nissbrandt H, Cenci MA. L-DOPA-induced dopamine efflux in the striatum and the substantia nigra in a rat model of Parkinson's disease: temporal and quantitative relationship to the expression of dyskinesia. *J Neurochem.* 2010; 112: 1465-1476.
- Liou AK, Leak RK, Li L, Zigmond MJ. Wild-type LRRK2 but not its mutant attenuates stress-induced cell death via ERK pathway. *Neurobiol Dis* 2008; 32: 116-124.
- Lo Bianco C, Schneider BL, Bauer M, Sajadi A, Brice A et al. Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease. *Proc Natl Acad Sci USA* 2004; 101: 17510-17515.
- Lockhart PJ, Lincoln S, Hulihan M, Kachergus J, Wilkes K et al. DJ-1 mutations are a rare cause of recessively inherited early onset parkinsonism mediated by loss of protein function. *J Med Genet* 2004; 41: e22.
- Loos PC, Luerman GC, Hurtado-Lorenzo A, Berger Z, Mou K et al. Effects of LRRK2 on neurite outgrowth: Studies with LRRK2-in-1. Society for Neuroscience Annual Meeting 2011, Poster number: 147.13/N5.
- Lotharius J, Brundin P. Pathogenesis of Parkinson's disease: dopamine, vesicles and α -synuclein. *Nature Rev Neurosci* 2002; 3: 932-942.
- Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. *Nat Rev Mol Cell Biol* 2009; 10: 307-18.
- Mabrouk OS, Marti M, Morari M. Endogenous nociceptin/orphanin FQ (N/OFQ) contributes to haloperidol-induced changes of nigral amino acid transmission and parkinsonism: a combined microdialysis and behavioral study in naïve and nociceptin/orphanin FQ receptor knockout mice. *Neuroscience* 2010; 166: 40-48.
- Mabrouk OS, Li Q, Song P, Kennedy RT. Microdialysis and mass spectrometric monitoring of dopamine and enkephalins in the globus pallidus reveal reciprocal interactions that regulate movement. *J Neurochem* 2011; 118: 24-33.
- Macedo MG, Anar B, Bronner IF, Cannella M, Squitieri F et al. The DJ-1L166P mutant protein associated with early onset Parkinson's disease is unstable and forms higher-order protein complexes. *Hum Mol Genet* 2003; 12: 2807–2816.
- MacDonald V, Halliday GM. Selective loss of pyramidal neurons in the pre-supplementary motor cortex in Parkinson's disease. *Mov Disord* 2002; 17: 1166-1173.
- MacLeod D, Dowman J, Hammond R, Leete T, Inoue K et al. The familial Parkinsonism gene LRRK2 regulates neurite process morphology. *Neuron* 2006; 52: 587-593.
- Maj J, RogóZ Z, Skuza G, Kołodziejczyk K. The behavioural effects of pramipexole, a novel dopamine receptor agonist. *Eur J Pharmacol* 1997; 324: 31–37.

- Mangeat P, Roy C, Martin M. ERM proteins in cell adhesion and membrane dynamics. *Trends Cell Biol* 1999; 9: 187–192.
- Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S. The protein kinase complement of the human genome. *Science* 2002; 298: 1912–1934.
- Marin I. The Parkinson disease gene LRRK2: evolutionary and structural insights. *Mol Biol Evol* 2006; 23: 2423–2433.
- Marin I. Ancient origin of the Parkinson disease gene LRRK2. *J Mol Evol* 2008; 67: 41–50.
- Marin I, van Egmond WN, van Haastert PJ. The Roco protein family: a functional perspective. *FASEB J* 2008; 22: 3103–3110.
- Marks WJ Jr, Bartus RT, Siffert J, Davis CS, Lozano A et al. Gene delivery of AAV2-neurturin for Parkinson's disease: a double-blind, randomized, controlled trial. *Lancet Neurol* 2010; 9: 1164-1172.
- Marti M, Mela F, Fantin M, Zucchini S, Brown JM et al. Blockade of nociceptin/orphanin FQ transmission attenuates symptoms and neurodegeneration associated with Parkinson's disease. *J Neurosci* 2005; 25: 9591-9601.
- Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M et al. Dopaminergic loss and inclusion body formation in α -synuclein mice: implications for neurodegenerative disorders. *Science* 2000; 287: 1265-1269.
- Masliah E, Rockenstein E, Adame A, Alford M, Crews L et al. Effects of alpha-synuclein immunization in a mouse model of Parkinson's disease. *Neuron* 2005; 46: 857-868.
- Mata IF, Lockhart PJ, Farrer MJ. Parkin genetics: one model for Parkinson's disease. *Hum Mol Genet* 2004; 13: R127-R133.
- McArdle B, Hofmann A. Coronin structure and implications. *Subcell Biochem* 2008; 48: 56–71.
- McNaught KS, Olanow CW. Protein aggregation in the pathogenesis of familial and sporadic Parkinson's Disease. *Neurobiol Aging* 2006; 27: 530-545.
- Meissner WG, Frasier M, Gasser T, Goetz CG, Lozano A et al. Priorities in Parkinson's Disease research. *Nat Rev Drug Discov* 2011; 10: 377-393.
- Melrose HL, Kent CB, Taylor JP, Dachsel JC, Hinkle KM et al. A comparative analysis of leucine-rich repeat kinase 2 (Lrrk2) expression in mouse brain and Lewy body disease. *Neuroscience* 2007; 147: 1047-1058.
- Melrose HL, Dächsel JC, Behrouz B, Lincoln SJ, Yue M et al. Impaired dopaminergic neurotransmission and microtubule-associated protein tau alterations in human LRRK2 transgenic mice. *Neurobiol Dis* 2010; 40: 503-517.
- Mierau J, Schingnitz G. Biochemical and pharmacological studies on Pramipexole, a potent and selective dopamine D2 receptor agonist. *Eur J Pharmacol* 1992; 215: 161–170.
- Migliaccio E, Giorgio M, Mele S, Pelicci G, Reboldi P et al. The p66shc adaptor protein controls oxidative stress response and life span in mammals. *Nature* 1999; 402: 309-313.

Mingot JM, Bohnsack MT, Jakle U, Gorlich D. Exportin 7 defines a novel general nuclear export pathway. *Embo J* 2004; 23: 3227-3236.

Moore DJ, Zhang L, Dawson TM, Dawson VL. A missense mutation (L166P) in DJ-1, linked to familial Parkinson's disease, confers reduced protein stability and impairs homo-oligomerization. *J Neurochem* 2003; 87: 1558–1567.

Moore DJ, Dawson VL, Dawson TM. Lessons from *Drosophila* models of DJ-1 deficiency. *Sci Aging Knowledge Environ* 2006; 2006: pe2.

Mortiboys H, Johansen KK, Aasly JO, Bandmann O. Mitochondrial impairment in patients with Parkinson disease with the G2019S mutation in LRRK2. *Neurology*. 2010; 75: 2017-2020.

Munier-Lehmann H, Mauxion F, Hoflack B. Function of the two mannose 6-phosphate receptors in lysosomal enzyme transport. *Biochem Soc Trans* 1996; 24: 133-136.

Neumann M, Müller V, Kretschmar HA, Haass C, Kahle PJ. Regional distribution of proteinase K-resistant alpha-synuclein correlated with Lewy body disease stage. *J Neuropathol Exp Neurol* 2004; 63: 1225-1235.

Nichols RJ, Dzamko N, Huttu JE, Cantley LC, Deak M et al. Substrate specificity and inhibitors of LRRK2, a protein kinase mutated in Parkinson's disease. *Biochem J* 2009; 424: 47-60.

Nichols RJ, Dzamko N, Morrice NA, Campbell DG, Deak M et al. 14-3-3 binding to LRRK2 is disrupted by multiple Parkinson's disease-associated mutations and regulates cytoplasmic localization. *Biochem J* 2010; 430: 393-404.

Nielsen MS, Gustafsen C, Madsen P, Nyengaard JR, Hermey G et al. Sorting by the cytoplasmic domain of the amyloid precursor protein binding receptor SorLA. *Mol Cell Biol* 2007; 27: 6842-6851.

Nimchinsky EA, Sabatini BL, Svoboda K. Structure and function of dendritic spines. *Annu Rev Physiol* 2002; 64: 313–353.

Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 1991; 108: 193-199.

Obeso JA, Rodriguez-Oroz MC, Goetz CG, Marin C, Kordower JH et al. Missing pieces in the Parkinson's disease puzzle. *Nat Med* 2010; 16: 653-661.

Okun MS, Fernandez HH, Rodriguez RL, Foote KD. Identifying candidates for deep brain stimulation in Parkinson's disease: the role of the primary care physician. *Geriatrics* 2007; 62: 18-24.

Olanow CW, Prusiner SB. Is Parkinson's disease a prion disorder? *Proc Natl Acad Sci USA* 2009; 106: 12571-12572.

Orsini F, Migliaccio E, Moroni M, Contursi C, Raker VA et al. The life span determinant p66Shc localizes to mitochondria where it associates with mitochondrial heat shock protein 70 and regulates trans-membrane potential. *J Biol Chem* 2004; 279: 25689-25695.

Ozelius LJ, Senthil G, Saunders-Pullman R, Ohmann E, Deligtisch A et al. LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *N Engl J Med* 2006; 354: 424-425.

Paisán-Ruíz C, Jain S, Evans EW, Gilks WP, Simón J et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 2004; 44: 595-600.

Paisán-Ruíz C, Nath P, Washecka N, Gibbs JR, Singleton AB. Comprehensive analysis of LRRK2 in publicly available Parkinson's disease cases and neurologically normal controls. *Hum Mutat* 2008; 29: 485-490.

Palacino JJ, Sagi D, Goldberg MS, Krauss S, Motz C et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. *J. Biol. Chem.* 2004; 279: 18614-18622.

Pallone JA. Introduction to Parkinson's Disease. *Dis Mon* 2007; 53: 195-199.

Parisiadou L, Xie C, Cho HJ, Lin X, Gu XL et al. Phosphorylation of ezrin/radixin/moesin proteins by LRRK2 promotes the rearrangement of actin cytoskeleton in neuronal morphogenesis. *J Neurosci* 2009; 29: 13971-13980.

Park J, Lee SB, Lee S, Kim Y, Song S et al. Mitochondrial dysfunction in *Drosophila* PINK1 mutants is complemented by parkin. *Nature* 2006; 441: 1157-1161.

Parkinson J. An essay on the shaking palsy. *J Neuropsychiatry Clin Neurosci* 2002; 14: 223-236.

Paulus W, Jellinger K. The neuropathological basis of different clinical subgroups of Parkinson's disease. *J Neuropathol Exp Neurol* 1991; 50: 743-755.

Perez FA, Palmiter RD. Parkin-deficient mice are not a robust model of parkinsonism. *Proc Natl Acad Sci USA* 2005; 102: 2174-2179.

Perier C, Bové J, Wu DC, Dehay B, Choi DK et al. Two molecular pathways initiate mitochondria-dependent dopaminergic neurodegeneration in experimental Parkinson's disease. *Proc Natl Acad Sci U S A.* 2007; 104: 8161-8166.

Petit A, Kawarai T, Paitel E, Sanjo N, Maj M et al. Wild-type PINK1 prevents basal and induced neuronal apoptosis, a protective effect abrogated by Parkinson disease-related mutations. *J Biol Chem* 2005; 280: 34025-34032.

Piccoli G, Condliffe SB, Bauer M, Giesert F, Boldt K et al. LRRK2 controls synaptic vesicle storage and mobilization within the recycling pool. *J Neurosci* 2011; 31: 2225-2237.

Pisani A, Bernardi G, Ding J, Surmeier DJ. Re-emergence of striatal cholinergic interneurons in movement disorders. *Trends Neurosci* 2007; 30: 545-553.

Plowey ED, Cherra SJ 3rd, Liu YJ, Chu CT. Role of autophagy in G2019S-LRRK2-associated neurite shortening in differentiated SH-SY5Y cells. *J Neurochem* 2008; 105: 1048-1056.

Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A et al. Mutation in the α -synuclein gene identified in families with Parkinson's disease. *Science* 1997; 276: 2045-2047.

- Pramstaller PP, Schlossmacher MG, Jacques TS, Scaravilli F, Eskelson C et al. Lewy body Parkinson's disease in a large pedigree with 77 Parkin mutation carriers. *Ann Neurol* 2005; 58: 411-422.
- Ramaker C, Marinus J, Stiggelbout AM, van Hilten JJ. Systematic evaluation of rating scales for impairment and disability in Parkinson's Disease. *Mov Disord* 2002; 17: 867-876.
- Ramírez-Valle F, Braunstein S, Zavadil J, Formenti SC, Schneider RJ. eIF4GI links nutrient sensing by mTOR to cell proliferation and inhibition of autophagy. *J Cell Biol* 2008; 181: 293-307.
- Ramonet D, Daher JP, Lin BM, Stafa K, Kim J et al. Dopaminergic neuronal loss, reduced neurite complexity and autophagic abnormalities in transgenic mice expressing G2019S mutant LRRK2. *PLoS One* 2011; 6: e18568.
- Rao SS, Hofmann LA, Shakil A. Parkinson's disease: diagnosis and treatment. *Am Fam Physician* 2006; 74: 2046-2054.
- Redmond DE, Bjugstad KB, Teng YD, Ourednik V, Ourednik J et al. Behavioral improvement in a primate Parkinson's model is associated with multiple homeostatic effects of human neural stem cells. *Proc Natl Acad Sci USA* 2007; 104: 12175-12180.
- Rezak M. Current pharmacotherapeutic treatment options in Parkinson's disease. *Dis Mon* 2007; 53: 214-222.
- Rockenstein E, Mallory M, Hashimoto M, Song D, Shults CW et al. Differential neuropathological alterations in transgenic mice expressing alpha-synuclein from the platelet-derived growth factor and Thy-1 promoters. *J Neurosci Res* 2002; 68: 568-578.
- Rojas R, van Vlijmen T, Mardones GA, Prabhu Y, Rojas AL et al. Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. *J Cell Biol* 2008; 183: 513-526.
- Rozas G, Guerra M.J, Labandeira-Garcia JL. An automated rotarod method for quantitative drug-free evaluation of overall motor deficits in rat models of parkinsonism. *Brain Res Brain Res Protoc* 1997; 2: 75-84.
- Rozas G, Liste I, Guerra MJ, Labandeira-Garcia JL. Sprouting of the serotonergic afferents into striatum after selective lesion of the dopaminergic system by MPTP in adult mice. *Neurosci Lett* 1998; 245: 151-154.
- Sabatini U, Boulanouar K, Fabre N, Martin F, Carel C et al. Cortical motor reorganization in akinetic patients with Parkinson's disease: a functional MRI study. *Brain* 2000; 123: 394-403.
- Sala C. Molecular regulation of dendritic spine shape and function. *Neurosignals* 2002; 11: 213-223.
- Sanberg PR, Bunsey MD, Giordano M, Norman AB. The catalepsy test: its ups and downs. *Behav Neurosci* 1988; 102: 748-759.
- Sancho RM, Law BM, Harvey K. Mutations in the LRRK2 Roc-COR tandem domain link Parkinson's disease to Wnt signalling pathways. *Hum Mol Genet* 2009; 18: 3955-3968.

- Sann S, Wang Z, Brown H, Jin Y. Roles of endosomal trafficking in neurite outgrowth and guidance. *Trends Cell Biol.* 2009; 19: 317-324.
- Santini E, Heiman M, Greengard P, Valjent E, Fisone G. Inhibition of mTOR signaling in Parkinson's disease prevents L-DOPA-induced dyskinesia. *Sci Signal* 2009; 2: ra36.
- Schallert T, De Ryck M, Whishaw IQ, Ramirez VD, Teitelbaum P. Excessive bracing reactions and their control by atropine and L-DOPA in an animal analog of parkinsonism. *Exp Neurol* 1979; 64: 33–43.
- Seeman P. Antiparkinson therapeutic potencies correlate with their affinities at dopamine D2(High) receptors. *Synapse* 2007; 61: 1013–1018.
- Segal M, Greenberger V, Korkotian E. Formation of dendritic spines in cultured striatal neurons depends on excitatory afferent activity. *Eur J Neurosci* 2003; 17: 2573-2585.
- Sharon R, Bar-Joseph I, Frosch MP, Walsh DM, Hamilton JA et al. The formation of highly soluble oligomers of alpha-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. *Neuron* 2003; 37: 583-595.
- Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* 2000; 25: 302–305.
- Sidhu A, Wersinger C, Moussa CE, Vernier P. The role of α -synuclein in both neuroprotection and neurodegeneration. *Ann NY Acad Sci* 2004; 1035: 250-270.
- Silvera D, Arju R, Darvishian F, Levine PH, Zolfaghari L et al. Essential role for eIF4GI overexpression in the pathogenesis of inflammatory breast cancer. *Nat Cell Biol* 2009; 11: 903-908.
- Singleton AB, Farrer M, Johnson J, Singleton A, Hague S et al. α -Synuclein locus triplication causes Parkinson's disease. *Science* 2003; 302: 841.
- Siuciak JA, Fujiawara RA. The activity of pramipexole in the mouse forced swim test is mediated by D2 rather than D3 receptors. *Psychopharmacology* 2004; 175: 163–169.
- Soldner F, Hockemeyer D, Beard C, Gao Q, Bell GW et al. Parkinson's disease patient-derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 2009; 136: 964-977.
- Spillantini MG, Crowther RA, Jakes R, Hasegawa M, Goedert M. α -synuclein in filamentous inclusions of Lewy bodies from Parkinson's disease and dementia with Lewy bodies. *Proc Natl Acad Sci USA* 1998; 95: 6469-6473.
- Sriram SR, Li X, Ko HS, Chung KK, Wong E et al. Familial-associated mutations differentially disrupt the solubility, localization, binding and ubiquitination properties of parkin. *Hum Mol Genet* 2005; 14: 2571-2586.
- Surmeier DJ, Song WJ, Yan Z. Coordinated expression of dopamine receptors in neostriatal medium spiny neurons. *J Neurosci.* 1996; 16: 6579-6591.

- Tain LS, Mortiboys H, Tao RN, Ziviani E, Bandmann O et al. Rapamycin activation of 4E-BP prevents parkinsonian dopaminergic neuron loss. *Nat Neurosci* 2009; 12: 1129-1135.
- Tao X, Tong L. Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. *J. Biol. Chem.* 2003; 278: 31372–31379.
- Tapia L, Milnerwood A, Guo A, Mills F, Yoshida E et al. Progranulin deficiency decreases gross neural connectivity but enhances transmission at individual synapses. *J Neurosci* 2011; 31: 11126-11132.
- Thiruchelvam MJ, Powers JM, Cory-Slechta DA, Richfield EK. Risk factors for dopaminergic neuron loss in human α -synuclein transgenic mice. *Eur J Neurosci* 2004; 19: 845-854.
- Thobois S, Dominey P, Decety J, Pollak PP, Gregoire MC et al. Motor imagery in normal subjects and in asymmetrical Parkinson's disease: a PET study. *Neurology* 2000; 55: 996-1002.
- Tong Y, Pisani A, Martella G, Karouani M, Yamaguchi H et al. R1441C mutation in LRRK2 impairs dopaminergic neurotransmission in mice. *Proc Natl Acad Sci USA* 2009; 106: 14622-14627.
- Tong Y, Yamaguchi H, Giaime E, Boyle S, Kopan R et al. Loss of leucine-rich repeat kinase 2 causes impairment of protein degradation pathways, accumulation of alpha-synuclein, and apoptotic cell death in aged mice. *Proc Natl Acad Sci USA* 2010; 107: 9879-9884.
- Valente EM, Bentivoglio AR, Dixon PH, Ferraris A, Ialongo T et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35-p36. *Am J Hum Genet* 2001; 68: 895-900.
- Viaro R, Sanchez-Pernaute R, Marti M, Trapella C, Isacson O et al. Nociceptin/orphanin FQ receptor blockade attenuates MPTP-induced parkinsonism. *Neurobiol Dis* 2008; 30: 430-438.
- Viaro R, Marti M, Morari M. Dual motor response to l-dopa and nociceptin/orphanin FQ receptor antagonists in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) treated mice: Paradoxical inhibition is relieved by D(2)/D(3) receptor blockade. *Exp Neurol* 2010; 223: 473-484.
- Vieira SI, Rebelo S, Esselmann H, Wiltfang J, Lah J et al. Retrieval of the Alzheimer's amyloid precursor protein from the endosome to the TGN is S655 phosphorylation state-dependent and retromer-mediated. *Mol Neurodegener* 2010; 5: 40.
- Vilariño-Güell C, Wider C, Ross OA, Dachsel JC, Kachergus JM et al. VPS35 mutations in Parkinson disease. *Am J Hum Genet* 2011; 89: 162-167.
- Volta M, Mabrouk OS, Bido S, Marti M, Morari M. Further evidence for an involvement of nociceptin/orphanin FQ in the pathophysiology of Parkinson's disease: a behavioral and neurochemical study in reserpinized mice. *J Neurochem.* 2010; 115: 1543-1555.

- Von Coelln R, Thomas B, Savitt JM, Lim KL, Sasaki M et al. Loss of locus coeruleus neurons and reduced startle in parkin null mice. *Proc Natl Acad Sci USA* 2004; 101: 10744–10749.
- Weintraub D, Comella CL, Horn S. Parkinson's Disease- Part 1: pathophysiology, symptoms, burden, diagnosis and assessment. *Am J Manag Care* 2008; 14: S40-S48.
- West AB, Moore DJ, Biskup S, Bugayenko A, Smith WW et al. Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci USA* 2005; 102: 16842–16847.
- West AB, Moore DJ, Choi C, Andrabi SA, Li X et al. Parkinson's disease-associated mutations in LRRK2 link enhanced GTP-binding and kinase activities to neuronal toxicity. *Hum Mol Genet* 2007; 16: 223-232.
- Willingham S, Outerio TF, DeVit MJ, Lindquist SL, Muchowski PJ. Yeast genes that enhance the toxicity of a mutant huntingtin fragment of alpha-synuclein. *Science* 2003; 302: 1769-1772.
- Winner B, Melrose HL, Zhao C, Hinkle KM, Yue M et al. Adult neurogenesis and neurite outgrowth are impaired in LRRK2 G2019S mice. *Neurobiol Dis* 2011; 41: 706-716.
- Wishart TM, Parson SH, Gillingwater TH. Synaptic vulnerability in neurodegenerative disease. *J Neuropathol Exp Neurol* 2006; 65: 733–739.
- Yue Z, Lachenmayer ML. Genetic LRRK2 models of Parkinson's disease: Dissecting the pathogenic pathway and exploring clinical applications. *Mov Disord* 2011; 26: 1386-1397.
- Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 2004; 44: 601-607.
- Zimprich A, Benet-Pagès A, Struhal W, Graf E, Eck SH et al. A mutation in VPS35, encoding a subunit of the retromer complex, causes late-onset Parkinson disease. *Am J Hum Genet* 2011; 89: 168-175.
- Zhang L, Shimoji M, Thomas B, Moore DJ, Yu SW et al. Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis. *Hum Mol Genet* 2005; 14: 2063-2073.
- Zhou C, Huang Y, Przedborski S. Oxidative stress in Parkinson's disease: a mechanism of pathogenic and therapeutic significance. *Ann N Y Acad Sci* 2008; 1147: 93-104.