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PhD Thesis:

PHARMACOLOGICAL IMPROVEMENT OF MITOCHONDRIAL FUNCTION AS STRATEGY TO PREVENT NEURONAL DAMAGE IN PARKINSON'S DISEASE

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SUMMARY

1. INTRODUCTION

Parkinson's disease (PD) is the second most frequent neurodegenerative disorder, after Alzheimer's disease. According to the European Parkinson Disease Association (EPDA), 1,2 million of people suffer of PD and this number is expected to double in 20 years, if the incidence rate remains constant. A recent systematic review of worldwide epidemiological reports showed that, although some differences in prevalence by geographic location and sex can be detected, PD prevalence increases with age worldwide ranging from: 41 per 100,000 within 40-49 years, to 428 per 100,000 within 60-69 years, till 1,087 per 100,000 within 70-79 years and 1,903 per 100,000 in over age 80. The socio-economic relevance of PD is of extreme importance since a PD patient costs an average of 20,000 € per year in European countries. From a neuropathological point of view, PD is characterized by the demise of dopaminergic neurons in the *substantia nigra pars compacta* (SNc) and by the reduction of dopamine (DA) tone at the level of the *striatum* (Obeso *et al*., 2010; Halliday *et al*., 2011; Obeso *et al*., 2017). Specific neuropathological hallmarks of disease are represented by the presence of intraneuronal protein aggregates called Lewy bodies and Lewy neurites (Poewe *et al*., 2017), eosinophilic cellular inclusions comprising a dense core of filamentous material, which mainly consists of α-synuclein (Cookson *et al*., 2005; Spillantini, *et al*., 1997). Post mortem studies suggest that the propagation of LB pathology in the brain of affected patients correlates with disease progression (Den and Bethlem 1960; Braak and Braak 2000; Braak *et al*. 2002, 2003, 2004, 2006). However, PD is characterized by more widespread pathology in other brain regions and involves non-dopaminergic neurons as well.

Clinically, the disease is heterogeneous, and it develops in different ways for each patient. Subtypes may be recognized on the basis of age of onset, predominant clinical features and progression rate. Indeed, the clinical diagnosis of PD is based primarily on motor symptoms, such as a slowly progressive asymmetric resting tremor, rigidity, bradykinesia, and postural instability, although non-motor features, which include anosmia, constipation, depression, and REM sleep behaviour disorder, can develop years before motor deficits. During later stages of the disease, additional non-motor

features, such as autonomic dysfunction, pain, and cognitive decline, can appear (Sung *et al*., 2013). It has been estimated that approximately the 50–70% of SNpc dopaminergic neurons die by the time that clinical motor symptoms become evident (Fearnley *et a*l., 1991), with the loss of 80% of DA in the *striatum*. The selective degeneration of SNpc neurons causes a reduced stimulation of the direct pathway, mediated by D1-like receptors, and a reduced inhibition of the indirect pathway, mediated by D2-like receptors, both leading to an increased GABAergic tone in the output motor nuclei of the basal ganglia and a consequent lower activity of the thalamus with respect to facilitating the onset of movement (Obeso *et al*., 2010; Shulman *et al*., 2011).

From a pathogenetic point of view, PD can present an age-related sporadic onset or arise from genetic alterations. Moreover, compelling evidence demonstrate that PD often takes decades to develop before it can be diagnosed. This prodromal phase of PD is however characterized by the onset of various non-motor signs that can widely anticipate the onset of motor symptoms in time, encompassing olfactory dysfunctions, autonomic disturbances, such as constipation, and rapid-eye-movement sleep behaviour disorder (RBD). As these symptoms reflect PD pathogenesis years, or even decades, before the onset of the motor symptoms, it has become increasingly clear that studying these prodromal signs represents an important opportunity to shed light on the aetiology of PD. This aspect is extremely relevant especially considering that PD is devastating not only due to its economic impact on the society in terms of costs of healthcare and loss of productivity, but also because it literarily deprives individual from its own identity. Noteworthy, the therapy for this pathology, although well defined, is only aimed at the treatment of symptoms with poor results on the progression of the disease and the slowing of neurodegeneration. Moreover, the benefits of the replacement therapy with Levodopa or dopaminergic agonists wear off over time and their clinical efficacy gradually decreases as the disease progresses. Therefore, it is extremely urgent to go deep in identifying the molecular targets involved in prodromal phase of PD development since they might be diagnostically and pharmacologically engaged, to estimate, and thus to curtail, the progression of disease. Indeed, despite societies around the world invest billions of dollars in the search for drugs

that would stop, or at least significantly slow-down neurodegeneration, the results are unsatisfactory at best. Criteria for the diagnosis of prodromal PD symptoms have been defined by the Movement Disorder Society (MDS) and they provide a practical methodological framework for the calculation of individual prodromal PD risk, thus indicating a temporal window during which molecular events leading to PD-associated structural changes already occur, but no biomarker has been developed to track them to date. Pioneering studies have demonstrated a high correlation between positron emission tomography (PET)-based striatal DAT imaging or DTI and striatal terminal loss, detected by immunostaining, in models of advanced PD, such as 6-hydroxydopamine-lesioned rats and MPTP-treated monkeys. What is missing is a study in animal models able to recapitulate the prodromal phase of PD, characterized by a slow, retrograde nigrostriatal degeneration and a striatal dopamine transporter (DAT) deficit largely anticipating the cell loss. The molecular and cellular triggers of such feed forward synaptic-to-axon degeneration pattern in PD are also unknown and comparative studies aimed at evaluating whether early molecular and structural changes detected at striatal dopaminergic terminals can translate into alterations monitored by conventional brain imaging (MRI/PET) are still lacking. Furthermore, it is still lacking an effective therapeutic strategy for healing early synaptic damage.

Another aspect of PD pathogenesis that deserves to be considered is that, although most patients develop idiopathic PD (Mayeux *et al*., 1995), several factors, such as gender, mutation in specific genes, neuroinflammation, mitochondrial dysfunctions, oxidative stress, excitotoxicity, and dysfunction of the protein degradation system have all been shown to increase the risk of PD development (Shapira and Jenner, 2011; Pang *et al*., 2019). More interestingly, evidence described in the literature seem to converge on mitochondria as a primary target in the process of dopaminergic neuronal loss observed in PD. Any alteration in the mitochondrial functionality seems to deeply affect the ability of cells to bear cellular stresses, thus appearing more susceptible to additional insults. Therefore, dysfunction in mitochondria results in a deficit supply of cellular energy and in a failure in maintaining cellular homeostasis with particular regards to calcium homeostasis. These events play

a central role in apoptotic and necrotic cell death pathway leading to neurodegeneration (Ghosh *et al*., 1999; Banerjee *et al*., 2009). On these bases, a therapeutic approach aimed at reducing mitochondrial dysfunction might be considered useful to slow down dopaminergic neurons degeneration. However, the main limitation of this therapeutic strategy is to identify compounds able to selectively target mitochondria into their physiological intracellular environment. In this regard, ornithine, a non-essential amino acid, represents an interesting compound able to potentiate mitochondrial activity due to its ability to selectively reach this cellular organelle so important for neuronal metabolism. Indeed, ornithine plays a key role in the urea cycle and creates intermediate for arginine synthesis. Recent data have demonstrated the existence of the mitochondrial enzyme ornithine transcarbamylase (OTC) specifically in neurons positive for the nitric oxide synthesis enzyme (nNOS) (Bernstein *et al*., 2017), suggesting a potential role for ornithine in modulation of brain functions. This hypothesis is supported by the observation that a defect in the ornithine translocase enzyme, responsible for the transport of ornithine at the mitochondrial level, determines a hyperornithinemia associated with hyperammonemia, homocitrullinemia and neurological disorders accompanied, at the cellular level, by mitochondrial dysfunction and oxidative stress (Zanatta *et al*., 2016). However, the molecular mechanisms through which ornithine at the mitochondrial level can perform actions to improve the redox and energy properties are not entirely understood.

On these premises, the present study has been undertaken to investigate the intracellular pathways affected by ornithine treatment in *in vitro* and *in vivo* models of PD with particular regards to cellular metabolism and mitochondrial function in order to identify new and selective therapeutic strategies to prevent neuronal dysfunction occurring in PD and consequently to slow down disease progression. This aspect is extremely relevant considering the key role played by mitochondria in regulating synaptic activity. Therefore, a therapeutic strategy able to promote mitochondrial function might results useful to counteract the early synaptic dysfunction and the functional and pathological changes occurring in the brain of PD-affected patients.

To this aim *in vitro* experiments have been performed to evaluate the effect of L-Ornithine L-Aspartate (LOLA) on mitochondrial function in two cellular models of PD represented by SH-SY5Y cells treated with rotenone (ROT), an inhibitor of mitochondrial complex I, and 6-hydroxydopamine (6-OHDA), an inducer of oxidative stress. Parallel *in vivo* experiments have been performed in mice expressing the mutation A53T of human α-synuclein (A53T-α-Syn) during aging, to evaluate the relationship between α-synuclein accumulation, mitochondrial dysfunction and behavioral phenotype of A53T-α-Syn-mice compared to WT, with particular regard to the motor and non-motor symptoms. These experiments will allow to identify an animal model recapitulating the prodromal phase of PD potentially useful to investigate the effect of drugs able to improve mitochondria function in animal models of PD progression.

2. PATHOGENESIS OF PARKINSON'S DISEASE

PD is a multifactorial pathology, connected to genetic, immunologic, and environmental factors, and characterized by the progressive loss of nigral midbrain DA-neurons located in the SNpc (Fig. 1), and abnormal aggregation of many proteins, such as α-synuclein that, overall, build up Lewy bodies. Because of progressive decline in striatal DA content, PD patients experience loss of muscle control, compromised balance, bradykinesia, tremor at resting and postural instability. PD is also characterized by a variety of non-motor symptoms, including olfactory loss, depression, anxiety, and cognitive impairments that generally show up earlier or, rather, before motor deficits occur (Chaudhuri *et al*., 2003; Titova *et al*., 2017). Beside the excessive accumulation of misfolded proteins, PD pathogenesis is also characterized by mitochondria dysfunction, with a significant reduction of such functional organelles, seen in both animal models and postmortem brain from PD patients (Vila and Perier, 2008). In this line, it was found that accidental exposure of drug abusers to the mitochondrial complex I inhibitor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) caused acute and irreversible parkinsonian syndrome (Langston *et al*., 1983). Once in the brain MPTP is converted into the toxic metabolite, MPP+ that, taken up by DA neurons, accumulates into mitochondrial matrix and blocks complex I. As a consequence, ATP decline and increases of reactive oxygen species (ROS) occur within mitochondria, thus turning into cytochrome-c release, caspase 3 activation and selective loss of SNpc neurons (Langston *et al*., 1983; Dauer and Przedborski, 2003). Although most cases of PD occur sporadically, mutations in several genes have been linked to genetic forms of PD, including at least 13 gene loci and nine genes that are related to both autosomal dominant and recessive forms of PD. Mutations in three proteins encoded by these genes, namely parkin (PARK2), DJ-1 (PARK7), and PINK1 (PARK6), are associated with recessive early-onset forms of PD, whereas mutations in α-synuclein (PARK1-4) and in Leucine-rich repeat kinase 2 (LRRK2-PARK8) are responsible for dominant forms of familial PD. Recent findings suggest a possible involvement of some of the mutated proteins related to familial forms of PD in the regulation of

mitochondrial function and cellular metabolism. Indeed, PINK1 and parkin play crucial roles in the regulation of mitochondrial dynamics and function in PD (Gandhi *et al.*, 2009; Perier *et al.*, 2012), whereas, mutations in DJ-1 and parkin render mice lacking these genes more susceptible to oxidative stress (Chang *et al.*, 2014; Thomas *et al.*, 2011), and finally, mitochondrial localization of α-synuclein has been also linked to impairment of respiratory complex I activity, oxidative modification of mitochondrial proteins, and increased mitochondrial Ca^{2+} levels (Cannon *et al.*, 2013; Liu *et al.*, 2009). Interestingly, it has been demonstrated that, a dysfunction of PINK1 caused mitochondrial calcium overload due to the inhibition of the mitochondrial Na^{+}/Ca^{2+} exchanger (Gandhi *et al.*, 2009), thus confirming that mitochondrial dysfunction might play an important role in the pathogenesis of neuronal damage occurring in PD. Moreover, studies in the acute MPTP-induced PD mouse model showed that ROS overproduction also caused lysosomal membrane damage, as revealed by the accumulation of the auto phagosome protein marker LC3 II, with dramatic alterations of the autophagy system in these animals (Dehay *et al.*, 2010). Therefore, the inability of DA neurons to remove damaged long-lived proteins and organelles via autophagy and mitophagy mechanisms (Abeliovich and Gitler, 2016; Scott *et al.*, 2017) represents a major contributor in such neurodegenerative disorder.

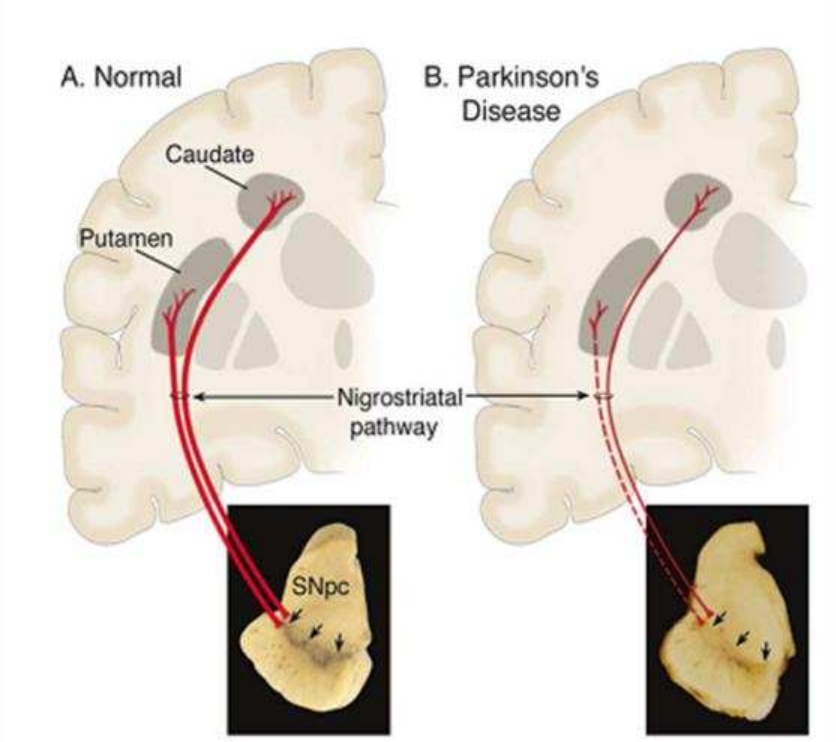


Fig. 1. **Neuropathology of Parkinson's Disease** (A) Schematic representation of the normal nigrostriatal pathway (in red). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; see arrows). These neurons project (thick solid red lines) to the basal ganglia and synapse in the striatum (i.e., putamen and caudate nucleus). The photograph demonstrates the normal pigmentation of the SNpc, produced by neuromelanin within the dopaminergic neurons. (B) Schematic representation of the diseased nigrostriatal pathway (in red). In Parkinson's disease, the nigrostriatal pathway degenerates. There is a marked loss of dopaminergic neurons that project to the putamen (dashed line) and a much more modest loss of those that project to the caudate (thin red solid line). The photograph demonstrates depigmentation (i.e., loss of dark-brown pigment neuromelanin; arrows) of the SNpc due to the marked loss of dopaminergic neurons (Dauer and Przedborski, 2003).

2.1. Parkinson's Disease-Linked Genes

Among the genes whose mutation are associated with the 5–10% of familial forms of PD the gene coding for α-synuclein (α-Syn), SNCA, was the first locus identified that linked genetics with PD (Polymeropoulos *et al*., 1997) since mutations in SNCA give rise to dominant early-onset PD (Corti *et al*., 2011). α-Syn is a small (140 amino acid) cytosolic protein that has three different domains: the N-terminal amphipathic domain; the non-amyloidogenic component (NAC) hydrophobic mid-region; and the acidic domain in the carboxyl tail. PD-associated mutations in SNCA occur within the N-terminal region: A30P, A53T, A53E, E46K, H50Q and G51D. Of these, A53T and E46K cause earlier onset and more severe manifestations, whereas A30P induces late-age onset and milder symptoms (Krüger *et al*., 2001; Zarranz *et al*., 2004; Puschmann *et al*., 2009). α-Syn accounts for 1% of the total

protein content in neurons being present in the cytosol, nucleus, mitochondria, and the mitochondria-associated membranes (MAMs) (Maroteaux *et al*., 1988; Li *et al*., 2007; Guardia-Laguarta *et al*., 2014) and with an enriched presynaptic localization (Iwai *et al*., 1995). Many physiological and pathological functions have been proposed for α-Syn, these include: a role in regulating vesicle fusion and neurotransmitter release (Abeliovich *et al*., 2000; Liu *et al*., 2004) as supported by the interaction of α-Syn with the presynaptic soluble NSF attachment protein receptor (SNARE) complex (Burré *et al*., 2010); a role in intracellular trafficking, as supported by impaired vesicular transport between the endoplasmic reticulum (ER) and Golgi when α-Syn is overexpressed in neurons (Cooper *et al*., 2006); a role in the regulation of cell death, due to the protective and antiapoptotic effects of α-Syn in the presence of caspase activation (Alves Da Costa *et al*., 2002); a role in protein clearance, since mutant α-Syn inhibits lysosomal degradation by binding to lysosome-associated membrane glycoprotein 2A (LAMP2A) and blocking protein uptake (Cuervo *et al*., 2004). In addition, α-Syn can oligomerize to form fibrils, which have been shown to spread from cell to cell; this is the most extensively studied characteristic of α-Syn and is thought to contribute to its pathogenicity (Desplats *et al*., 2009; Frost *et al*., 2010; Lee *et al*., 2010; Winner *et al*., 2011; Lashuel *et al*., 2013; Prusiner *et al*., 2015). α-Syn has also been proposed to regulate different processes associated with the maintenance of neuronal mitochondrial homeostasis (Kamp *et al*., 2010; Nakamura *et al*., 2011; Pozo Devoto *et al*., 2017).

Mutations in the parkin and PINK1 genes are responsible for the early-onset autosomal recessive PD. Both parkin and PINK1 have been linked to a cellular pathway involving the preferential degradation of dysfunctional mitochondria through lysosomes, a process termed “mitophagy” (Kitada *et al*., 1998). Loss of function of these genes leads to impaired mitophagy resulting in the accumulation of dysfunctional mitochondria. Parkin also indirectly regulates the levels of an important transcriptional regulator such as PGC-1alpha, which co-ordinately regulates the expression of genes required for mitochondrial biogenesis as well as multiple antioxidant defences (Valente *et al*., 2004) thus supporting the hypothesis that dysfunction of mitochondrial turnover might represent a pathogenetic factor related to PD development.

Mutations in the DJ-1 gene also cause autosomal recessive early-onset forms of PD (Bonifati *et al*., 2003). DJ-1 has antioxidant effects through multiple mechanisms, including regulation of NRF2, a transcription factor that upregulates multiple antioxidant defences, and by stimulating glutathione synthesis (Raninga *et al*., 2017).

Mutations in the LRRK2 gene are associated with autosomal dominant form of PD with incomplete penetrance (about 25% for the G2019S mutation, but much higher for the R144G mutation), and are present in about 2% of all PD patients and 5% in familial PD. The aberrant kinase activity of pathogenic LRRK2 mutants induces neurodegeneration by disturbing various intracellular processes, such as protein translation, endo-lysosomal pathway, autophagy, synaptic functions, and cytoskeleton dynamics, which may be mediated by the phosphorylation of several distinct putative substrates (West *et al*., 2007).

2.2. Mitochondrial Dysfunction in Parkinson's Disease

Mitochondria are ubiquitous organelles found in most eukaryotic cells, which play several important cellular functions, such as the production of energy by oxidative phosphorylation, the regulation of calcium homeostasis, and the control of programmed cell death.

Mitochondria are surrounded by a double membrane that differs in protein and lipid composition and in their functional roles. The outer mitochondrial membrane (OMM) is characterized by the presence of β-barrel pore-forming proteins, named porins, that make it permeable to solute up to 5 kDa, while the inner mitochondrial membrane (IMM) is highly selective since it is impermeable to ions and to most hydrophilic molecules. In term of composition, the IMM is characterized by an elevated proteins content, in fact, in addition to carrier proteins, it also includes the proteins of the electron transport chain (ETC) and the ATP synthase. The IMM surface is enormously enhanced by a process of membrane invagination called cristae, which can host many ETC complexes and ATP synthases, thus expanding the capacity of the cells to generate ATP in an extremely confined space (Perier and Vila, 2012; Fernandez-Vizarra *et al*., 2009). The ETC complex is composed to five multi-subunits proteins:

three of them, the complexes I, III and IV, which pump protons (H^+) across the inner membrane thus establishing an electrochemical gradient that is responsible for a membrane potential negative inside. Along with ETC complexes, electrons are transported from the reduced substrates (NADH and FADH2) to the oxygen that is converted in H_2O. Complex V, the ATP synthase, uses the electrochemical gradient generated across the IMM to produce ATP, by coupling the entry of protons to ADP phosphorylation. The electron transport, especially at complex I and III levels, also generates the free radical superoxide (O^{2-}), which, by the large majority, is converted to hydrogen peroxide by manganese superoxide dismutase (MnSOD) that in turn is converted to water (H_2O) by glutathione peroxidase and catalase. Free radicals may be also generated by the activity of mitochondrial monoamine oxidase (MAO-A and MAO-B), enzymes involved in the metabolism of serotonin, norepinephrine, and dopamine. All these reactive species are potentially dangerous because they could cause membrane lipid peroxidation and damage of protein and DNA. However, it is worth to note that the free radical superoxide (O^{2-}) and H_2O_2 also serve important signaling functions in physiological processes, thus their generation has not exclusively detrimental consequences (Angelova *et al*., 2016).

Mitochondria are the only organelles of the cell, besides the nucleus, that contain their own DNA (mitochondrial DNA, mtDNA), and their own machinery for synthesizing RNA and proteins. The human mitochondrial genome consists of a 16.6 kb multi-copy, a double-stranded, circular molecule containing 37 genes, which encodes for 13 mitochondrial proteins, all of which are subunits of respiratory chain complexes: seven subunits of complex I, one subunit of complex III, three subunits of complex IV and two of ATP synthase (DiMauro and Schon, 2003). In addition, mtDNA codes for 22 tRNAs and two rRNAs that are necessary for mitochondrial protein synthesis (Reeve *et al*., 2008). Another important feature of the mtDNA is that it replicates independently (1000-10.000 copies for mitochondrion) from the cell cycle and nuclear DNA replication. Many proteins required to build and maintain functional mitochondria are therefore encoded by nuclear DNA, synthesized in the cytosol, and imported into mitochondria (Bolender *et al*., 2008; Neupert *et al*., 2007). mtDNA is characterized

by an increased vulnerability to mutations, based on less efficient DNA repair mechanisms and on the absence of protective histones. In addition, the proximity to the respiratory chain has been suggested to favor mtDNA damage by reactive oxygen species.

Mitochondria are also dynamic organelles which actively divide (fission processes), fuse with one another (fusion processes), branch and fragment, swell and extend, exist in clusters or as individual entities, and have a regulated turnover, all of them are important for the maintenance of mitochondrial function and for quality control. Importantly, they move from the cell body to regions of the cell to deliver ATP and other metabolites where they are most required by anterograde or retrograde movements. These movements are more evident in highly elongated cells such as neurons, where mitochondria are enriched at presynaptic and postsynaptic level, because bioenergetics demand is particularly high (Fig. 2).

In the last decade, the alterations in mitochondrial function have been claimed to be central in the pathogenesis of both sporadic and familial forms of PD and have been included among the most common cell-autonomous mechanisms leading to neuronal degeneration (Cheng *et al*., 2010; Pacelli *et al*., 2015; Stanga *et al*., 2020; Wang *et al*., 2021). Observations from experimental models and human PD samples provide strong evidence for disruptions in mitochondrial dynamics, bioenergetics defects, complex I inhibition of ETC, and increased reactive oxygen species (ROS) (Ryan *et al*., 2015; Winklhofer *et al*., 2010). Many of the PD-associated genes like α-synuclein, LRRK2, beta-glucosidase 1 (GBA1; lysosomal glucocerebrosidase), Parkin, PINK1, DJ-1, Fbxo7, and VPS35, encode for components of the autophagy-lysosome pathway, mainly responsible for the control of mitochondrial homeostasis and mitophagy (Gan-Or *et al*., 2015; Verstraeten *et al*., 2015; Przedborski, 2017). Indeed, the expression of LAMP1, a glycoprotein responsible for lysosomal integrity, is reduced in the SNpc of PD patients. Similarly, the activity of the lysosomal β-glucocerebrosidase (GCase) is downregulated in different brain regions and in the cerebrospinal fluid of PD patients (Rocha *et al*., 2015). Moreover, the expression of TFEB (transcription factor EB), the master regulator of lysosomal biogenesis and autophagy, is significantly decreased in SNpc dopaminergic neurons of

PD patients and its staining co-localizes with Lewy bodies, the neuropathological hallmark of PD (Decressac *et al.*, 2013). Of relevance, the observations that either mutations of genes linked to familial forms of PD or environmental factors responsible of sporadic forms of PD both induce mitochondrial dysfunction. Because at synapses, mitochondria play a major role in energy metabolism, calcium homeostasis, signaling and neurotransmitter release, the deregulation of mitochondrial activities and their homeostasis contributes to synaptic impairment observed in PD. The molecular mechanisms involved in mitochondrial dysfunction are complex and not yet fully determined. In healthy mitochondria, PINK1 accumulates within the mitochondrial matrix (Huang *et al.*, 2017). This process requires the coordinated action of the translocase of the inner membrane complex (TIM) in conjunction with the translocase of the outer membrane complex (TOM), as well as the activity of several proteases and translocases. At the end of these events, a proteolytically processed form of PINK1 is released from mitochondria to the cytosol, where eventually undergoes to degradation. Depolarization of mitochondrial permeability transition pore (PTP) leads to accumulation of full length PINK1 at the outer mitochondrial membrane (OMM), where it forms a macromolecular complex with TOM proteins (Koyano *et al.*, 2019). At this level, PINK1 phosphorylates ubiquitin moieties and promotes phospho-Ub (pUb)-mediated recruitment of the E3 ligase parkin to the OMM and its activation by conformational changes. Once activated in neurons, parkin ubiquitylates several mitochondrial proteins, including mitofusins 1/2 (Mfn1/2), two homolog proteins involved in mitochondrial fusion, and the voltage-dependent anion channel-1 (VDAC1), a major regulator of the exchange of ions and small molecules across the OMM (Ordureau *et al.*, 2018). Ubiquitinated Mfn2 recruits the p97 segregase and, the formed complex is then released from OMM in the cytosol where Mfn2 is degraded through the proteasome. Downregulation of Mfn2 induces loss of the endoplasmic reticulum-mitochondria contact sites and membrane fusion activity, which culminates in mitochondrial fission (Tanaka *et al.*, 2010). Concomitantly, ubiquitination of VDAC1 at OMM by Parkin1 recruits the autophagy receptor optineurin (OPTN) which in turn induces auto phagosome formation and mitophagy (Ordureau *et al.*, 2018). Alternatively, vesicles containing

mitochondrial damaged proteins, known as mitochondria-derived vesicles (MDVs) can be eliminated through the selective autophagy machinery (McLelland *et al*., 2014; Matheoud *et al*., 2016). In both cases, mitophagy system plays a central role in the homeostatic control of mitochondrial clearance. A positive interplay between PINK1 and mitochondrial trafficking along neuronal dendrites and axons is critical for the homeostatic clearance of damaged mitochondria. Under stress conditions, recruitment of PINK1 to mitochondria promotes phosphorylation and parkin-mediated proteolysis of Miro, a component of the primary motor/adaptor complex that anchors kinesin motor to mitochondria and represents an essential regulator of mitochondrial transport. Indeed, the removal of Miro induces mitochondrial stalling at cell periphery and their consequent clearance through the mitophagy system (Weihofen *et al*., 2009; Wang *et al*., 2011). PINK1 also regulates mitochondrial trafficking through PINK1-induced recruitment of PKA to mitochondrial AKAP1. Here, AKAP1-bound PKA phosphorylates Miro2, another member of Miro protein family, and promotes mitochondrial trafficking. Following oxidative stress, PINK1 uncouples mitochondrial PKA signaling and induces mitophagy of damaged mitochondria. Neurons lacking PINK1 show impaired mitochondrial trafficking and dendrite outgrowth. These defects can be rescued by overexpressing AKAP1, suggesting that PKAs and PINK1 converge on mitochondria to control the mitochondrial trafficking and quality control system (Pryde *et al*., 2016; Das Banerjee *et al*., 2017). Derangement of this mechanism, as consequence of mutations involving PINK1, parkin and other genes working on the same regulatory pathway, may lead to accumulation of damaged mitochondria, oxidative stress, and neuronal cell loss. On the other hand, accumulation of α-synuclein and consequent mitochondrial dysfunction have been also observed in neurons subjected to oxidative stress. Under these conditions, high levels of oxidized dopamine impair glucocerebrosidase enzymatic activity and promotes mitochondrial/lysosomal dysfunction. These effects can be reproduced increasing the levels of dopamine in the mouse brain or overexpressing α-synuclein, implying that the interplay between mitochondria and lysosomes is crucial for the maintenance of cell homeostasis (Burbulla *et al*., 2017). The transition from monomeric to beta sheet-rich oligomers of α-synuclein leads to its accumulation

on mitochondria, affecting the organelles morphology, the oxidative phosphorylation, and the calcium buffering capacity. Specifically, α-synuclein oligomers interact with- and oxidize components of ATP synthase complex, affecting the respiratory chain activity. Moreover, oligomers strongly induce mitochondrial lipid peroxidation, leading to mitochondrial PTP opening, activation of the apoptotic pathway and neuronal cell death, as it occurs in PD (Ludtmann *et al*., 2018). Cardiolipin is an essential phospholipid of the mitochondrial membranes and has a major role in key mitochondrial processes, such as oxidative phosphorylation and energy conversion (Dudek, 2017). In α-synuclein expressing neurons, accumulation of α-synuclein on mitochondria has been linked to increased exposure of cardiolipin on the OMM, a sophisticated mechanism adopted by the cell to refold α-synuclein fibrils and, thus, counteract the abnormal formation of Levi's bodies. However, chronic exposure of mitochondria to cardiolipin promotes recruitment of components of the autophagy machinery, which culminates in excessive elimination of mitochondria with consequent neuronal cell loss (Ryan *et al*., 2018). Mutations of LRRK2 are the most common monogenic causes of familial and sporadic forms of PD (Tolosa *et al*., 2020). LRRK2 is a direct substrate of PKA, and LARRK2 phosphorylation of its Ras complex proteins (ROC) GTPase domain contributes to reduces LRRK2 kinase activity. Mutations at the ROC motif observed in PD patients dramatically affect LRRK2 phosphorylation by PKA, increasing its kinase activity. In striatal neurons, LRRK2 can also act as an AKAP-like protein targeting cAMP-dependent protein kinase type II beta regulatory subunit (PKARIIβ) at specific subcellular compartments. During synaptogenesis or in response to dopamine receptor D1 activation, LRRK2 spatially restrains PKA activity in the striatal projection neurons. Loss of LRRK2 activity or a PD-linked missense mutation within the RII α-binding domain (R1441C) of LRRK2 aberrantly induced a widespread, uncontrolled PKA phosphorylation of cellular substrates, including glutamate ionotropic receptor AMPA type subunit 1, GluR, (Muda *et al*., 2014; Parisiadou *et al*., 2014). These findings indicate the existence of a mutual control of LRRK2 and PKA activities within dopaminergic neurons, and derangement of this control circuitry may have a pathogenetic relevance for PD. LRRK2 also controls the activity of a member of RAS oncogene family (RAB10)

at mitochondrial sites. Under oxidative stress, LRRK2 phosphorylates RAB10 and induces its accumulation on depolarized mitochondria through a mechanism involving PINK1 and parkin. Mitochondria-bound RAB10 recruits the autophagy receptor OPTN and induces mitophagy. In PD neurons carrying the most common LRRK2 mutations (G2019S and R1441C), RAB10 phosphorylation at threonine 73 is enhanced, and this correlates with a marked reduction of mitochondrial activities and autophagic clearance (Wauters *et al*., 2019). These findings support a model whereby LRRK2, PINK1 and parkin in dopaminergic neurons are aligned on the same mitochondrial pathway and, mutations of each of these key elements can cause PD.

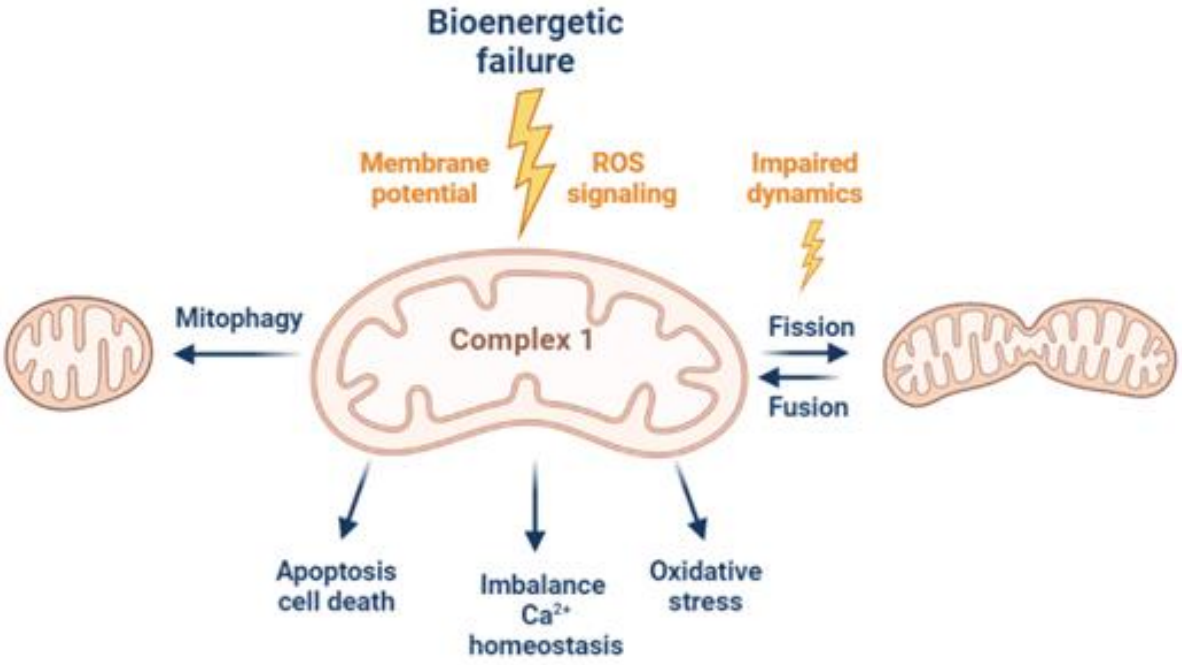


Fig. 2. Mitochondrial alterations associated with PD (Winklhofer *et al*., 2010)

2.3. *Mitochondrial calcium dysregulation in PD*

Mitochondria play a key role in the control of cytosolic Ca^{2+} buffering and cellular metabolism. Accordingly, deranged mitochondrial calcium homeostasis had been pathogenically linked to neurodegeneration occurring in PD (Bose and Beal, 2016; Verma *et al*., 2017). The maintenance of mitochondrial calcium concentrations within physiological range, necessary for neurons to regulate

aerobic ATP production and synaptic transmission and excitability, is strictly dependent by the synchronized activity of specific transporters and channels whose identity has been recently determined (De Stefani *et al*., 2011; Palty *et al*., 2012). Calcium enters into mitochondria through VDAC channel localized on the outer membrane (Tan and Colombini, 2007), and is transported across the intermembrane space and the inner membrane into the matrix by the mitochondrial calcium uniporter (MCU) and its docking/regulatory proteins MICU1/MICU2 (Tan and Colombini, 2007). Similarly, calcium is exported from the mitochondrial matrix through LETM1 or the Na^+/Ca^{2+}exchanger NCLX, in exchange for H^+ or Na^+/Li^+, respectively and then, across the outer membrane through VDAC and the isoform 3 of the nuclear encoded sodium calcium exchanger (NCX3) (Tan and Colombini, 2007; Scorziello *et al*., 2013; Anderson *et al*., 2019). In PD neurons, the activation of the nuclear encoded mitochondrial Na^+/Ca^{2+} exchanger (mNCX) is the primary mechanism by which mitochondrial calcium concentrations $[Ca^{2+}]_m$ is returned to the cytoplasm and therefore it is critical to a multitude of Ca^{2+}-dependent processes including neurotransmitters release, synaptic plasticity, bioenergetics, and mitochondrial nitric oxide (NO) and reactive oxygen species (ROS) production (Castaldo *et al*., 2009; Cali *et al*., 2013). In the absence of PINK1, mNCX activity was severely impaired, leading to mitochondrial calcium overload, permeability transition pore opening and cell death (Gandhi *et al*., 2009). Moreover, in human dopaminergic neurons plasmalemmal NCX2 and NCX3 contribute to mitochondrial Na^+/Ca^{2+} exchange and may act downstream of PINK1 in the prevention of neurodegeneration by $[Ca^{2+}]_m$ overload (Wood-Kaczmar *et al*., 2013). In primary mesencephalic neurons from A53T transgenic mice embryos, downregulation of NCX3 levels is linked to mitochondrial depolarization and mitochondrial calcium increase, compared to wild type neurons, suggesting that mitochondrial dysfunction in PD is linked to mitochondrial Ca^{2+} mishandling (Cali *et al*., 2013; Sirabella *et al*., 2018). However, the crucial question is whether the disease begins with the impairment of mitochondrial function, or if this is a consequence of derangement of other dopaminergic neuronal activities. Interestingly, SNpc dopaminergic neurons are autonomously active since they generate action potentials in the absence

of conventional synaptic input (Grace and Bunney, 1983). Due to this pacemaking activity, DA neurons are more prone to frequent transients of calcium which are necessary to allow DA release in the connected brain areas, e.g., the striatum, and consequently to the control of coordinated movements. In this scenario, the mechanisms involved in the maintenance of ionic homeostasis are forced to work under stressful conditions, including mitochondria whose calcium buffering activity is boosted to maintain cytosolic calcium within physiological ranges. Indeed, the sustained entry of Ca^{2+} into mitochondria stimulates the ATP-dependent pumps responsible for keeping Ca^{2+} concentration low, altering mitochondrial oxidative phosphorylation (Surmeier *et al.*, 2011). As consequences, overproduction of superoxide ions leads to DNA and proteins oxidation. This metabolic stress exacerbates the aging-related decline of mitochondrial function, resulting in decreased energy supply and increased sensitivity to cell death (Guzman *et al.*, 2010). Based on this evidence, Ca^{2+} homeostasis can be considered as an early feature of PD rather than a consequence of neuronal derangements. In particular, Ca^{2+} entry during the pacemaking activity of SNpc neurons can amplify the effects of genetic mutations or of the environmental factors, thus speeding forward neuronal aging and death. Interestingly, the neurons in the ventral tegmental area, which are more resistant to neurodegeneration, show a slow pacemaking activity and do not manifest Ca^{2+} oscillations (Chan *et al.*, 2007). Conversely, non-mesencephalic neurons, e.g., olfactory and some hypothalamic neurons, are characterized by spontaneously high activity associated with conspicuous Ca^{2+} currents and low buffering capacity and undergo to degeneration during PD progression (Surmeier and Schumacker, 2013). The relationship between calcium homeostasis and mitochondrial dysfunction in PD progression is further supported by the finding that α-synuclein, the main component of Lewy bodies considered a neuropathological hallmark of PD, might exert its toxicity through the engagement of the Ca^{2+} homeostatic machinery. However, the molecular mechanisms responsible for these effects are still unknown. One possibility is that α-synuclein, as aggregation-prone protein, forms fibrillary oligomers that enhance plasma membrane permeability to Ca^{2+} influx, inducing an intracellular Ca^{2+} overload and consequent neuronal toxicity (Danzer *et al.*, 2007; Schmidt, 2012;

Tsigelny *et al*., 2012). Moreover, the increase in intracellular Ca^{2+} may promote α-synuclein aggregation, thus leading to a vicious cycle which further increases intracellular Ca^{2+} levels. Recent studies also demonstrated that α-synuclein associates with mitochondria, and that its accumulation within the organelle was directly related to an increase of intramitochondrial Ca^{2+} levels (Parihar *et al*., 2008), which in turn led to a rise of NO levels, oxidative damage, and cytochrome c release from mitochondria (Parihar *et al*., 2009), leading to apoptotic neuronal death. This finding supports the model whereby α-synuclein has a major role in modulating not only the cellular, but also the mitochondrial Ca^{2+} fluxes. Accordingly, in A53T α-synuclein mesencephalic neurons, the rise of mitochondrial calcium concentration is linked to depolarization of mitochondrial membrane (Sirabella *et al*., 2018). Interestingly, this effect is accompanied to a reduction in the expression of NCX3. We recently demonstrated that the nuclear encoded NCX3 is also present on the OMM where it forms a stable complex with AKAP121 and promotes mitochondrial calcium efflux not only in physiological but also in pathological conditions like ischemia (Scorziello *et al*., 2013). More interestingly, we demonstrate that the impairment in the expression and activity of the NCX/AKAP121 mitochondrial complex causes mitochondrial dysfunction and promote mitochondrial fission (Sisalli *et al*., 2020), thus confirming the relevance of PKA-mitochondrial signaling in healthy mitochondria.

3. *SODIUM/CALCIUM EXCHANGER (NCX)*

3.1 Structural and functional features of NCXs

Na^+/Ca^{2+} exchanger (NCX) represents a major transporter assuring Ca^{2+} efflux from mammalian cells (Blaustein and Lederer, 1999). Under physiologic conditions, NCX provides the exchange of $3Na^+/1Ca^{2+}$ between the cytoplasm and extracellular medium. In most tissues, it operates in a "forward" way corresponding to inward current and thus to calcium exit from the cell (Blaustein and Lederer, 1999). Under some conditions, however, a reverse mode of Na^+/Ca^{2+} exchange can be activated coupling the extrusion of three Na^+ ions with the influx of one Ca^{2+} ion (Blaustein and Lederer, 1999; Philipson and Nicoll, 2000; Annunziato *et al*., 2004).

NCX belongs to a multigene family comprising three isoforms, named NCX1, NCX2, and NCX3. To fulfil the physiological demands of various cell types, the Na^+/Ca^{2+} exchanger isoforms and their splice variants are expressed in a tissue-specific manner (Philipson and Nicoll, 2000; Khananshvili, 2012). NCX expression is highest in cardiac muscle, skeletal muscle, and brain tissue and has also been reported in vascular smooth muscle and urinary bladder smooth muscle (Murata *et al*., 2010). NCX1 localizes at the presynaptic and postsynaptic sites and in the endoplasmic reticulum membrane of neurons (Canitano *et al*., 2002), in axons, dendrites, and growth cones (Luther *et al*., 1992). NCX1 also localizes to the inner membrane of the nuclear envelope and complexes with GM1 (Ledeen and Wu, 2007; Secondo *et al*., 2018). NCX2 and NCX3 are highly expressed in brain tissue and skeletal muscles (Li *et al*., 1994; Nicoll *et al*., 1996; Quednau *et al*., 1997) and all three NCX proteins are widely expressed throughout the rat CNS (Canitano *et al*., 2002). NCX1 and NCX3 give rise to several splicing variants that appear to be selectively expressed in different regions and cellular populations of the brain (Quednau *et al*., 1997; Yu and Colvin, 1997). In fact, NCX1 mRNA can be detected in the midbrain and in basal ganglia in which dopaminergic cell bodies are localized. Moreover, NCX1 protein isoform is present in the striatum, where the terminal projection fields of dopaminergic nigrostriatal neurons are present (Canitano *et al*., 2002; Papa *et al*., 2003).

The NCX3 gene is composed of 9 exons numbered from 1 to 9. The exons 2 and 3, also named A and B, are mutually exclusive (Quednau *et al*. 1997). Exon 4, also called exon C, is optional. Thus, in the rat, three splice variants can be detected. A variant containing exon A and C is present in skeletal muscle (NCX3-AC), while variants expressing the exon B are expressed in the brain (NCX3-B and NCX3-BC). Additionally, three truncated forms of NCX3 are expressed in humans. Two truncated variants are expressed in the foetal brain and appear to contain exons 4 to 9 and 6 to 9 (Lindgren *et al*. 2005). The third truncated variant is expressed in skeletal muscle and comprises exons 2 and 6 to 9 (Gabellini *et al*., 2002). Importantly, it has been observed that the splice variants of NCX3 have different affinity for Ca^{2+} and different binding sites in their calcium-binding domain 2 (CBD2), three for NCX3-B and two for NCX3-AC (Breukels *et al*., 2012). The X proteins have a general topology composed of 10 transmembrane domains (TM) (Ren and Philipson, 2013), with a large intracellular loop between TM5 and TM6. This loop contains the calcium-binding domain 1 (CBD1) and calcium-binding domain 2 (CBD2), which are regulatory domains required for intracellular ion sensing and binding (Fig. 3). Binding of four calcium ions to the CBD1 domain triggers a conformation change from disordered to a more rigid structure, resulting in activation of the NCX transporter (Hilge *et al*., 2006, 2009). Calcium has a low affinity for CBD2 and binds it at elevated concentration, therefore CBD2 may play a more selective role than CBD1 in regulating NCX activity (Hilge *et al*., 2009, Weber *et al*., 2001; Nicoll *et al*., 1996; Reeves and Condrescu, 2008; Boyman *et al*., 2011). Within the same intracellular loop that includes CBD1 and CBD2, there is the XIP motif, a 20 amino acid sequence located on the N-terminus of the cytosolic loop that confers sodium-dependent inactivation to the exchanger protein (Matsuoka *et al*., 1997). Domain linker between TM2 and TM3 forms the α1-repeat, and the linker between TM7 and TM8 forms the α2-repeat. The α-repeat domains contain residues essential for cation ligands and transport (Winkfein *et al*., 2003). Several factors are involved in the regulation of NCXs activity. Among them, the two transported ions, Na^+ and Ca^{2+} play a crucial role. Indeed, a rise in cytosolic [Na^+] rapidly stimulates and then inactivates the exchanger, whereas a rise in cytosolic [Ca^{2+}] activates NCX and relieves the Na^+-dependent inactivation (Hilgemann *et*

al., 1992). Moreover, NCX is extremely sensitive to cytosolic acidification, redox status, and metabolic state (DiPolo and Beauge, 1982; Doering and Lederer, 1994). These factors imply, in some cases, modifications of the exchange activity and, in others, alterations of the protein expression and docking into the membrane where, associated with other transporters such as Na^+/K^+-ATPase and Na^+/H^+ exchanger as well as enzymes like kinases and phosphatases, they form functional supramolecular complexes (Bers and Despa, 2009; Schulze *et al*., 2003; Berberián *et al*., 2009).

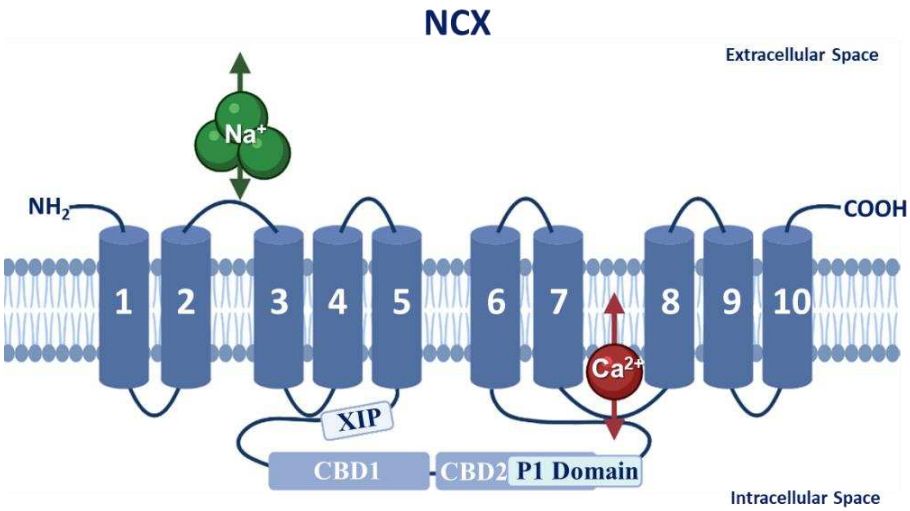


Fig. 3. Current NCX topology. The NCX protein is composed of 10 transmembrane domains, two α-repeats, and a large intracellular loop that includes two regulatory Ca^{2+} binding domains, CBD1 and CBD2.

3.2 Role of NCXs in physiological and pathological conditions

Considering the functional properties of NCX its activation is extremely important in neurons especially in some neurophysiological conditions. The level of expression of NCX in neurons is predominantly high in those sites where a large movement of Ca^{2+} ions occurs across the plasma membrane, as it happens at the level of synapses (Juhaszova *et al*., 1996; Canitano *et al*., 2002). Specifically, during an action potential or after glutamate-activated channel activity, Ca^{2+} massively enters the plasma membrane. Such phenomenon triggers the fusion of synaptic vesicles with the plasma membrane and promotes neurotransmitter release by exocytosis. After this event, outward K^+ currents repolarize the plasma membrane, thus leading to voltage-gated calcium channels closure. According to the diffusion principle, Ca^{2+} ions are distributed in the cytosolic compartment,

reversibly interacting with Ca^{2+}-binding proteins. Residual Ca^{2+} ions are then rapidly extruded by the plasma membrane Ca^{2+}-ATPase and by NCX activation. The Na^+/Ca^{2+}exchanger becomes the dominant Ca^{2+} extrusion mechanism when $[Ca^{2+}]_i$ is higher than 500nM, as it happens when a train of action potentials reaches the nerve terminals. It has been calculated that for these $[Ca^{2+}]_i$ values, more than 60% of Ca^{2+} extrusion is mediated by the Na^+/Ca^{2+}exchanger. In such physiological conditions, NCX activation is consistent with its low-affinity (Kd 500nM) and high-capacity (5x103 Ca^{2+}/s) function. In contrast, in resting conditions or after a single action potential, when $[Ca^{2+}]_i$ slightly increases, requiring, therefore, a more subtle control, the high-affinity (Kd100 nM) and low-capacity (102 Ca^{2+}/s) pump, the plasma membrane Ca^{2+}ATPase, assumes a predominant function, thus making the involvement of NCX less relevant (Blaustein and Lederer, 1999).

On the other hand, dysregulation of $[Ca^{2+}]_i$ and $[Na^+]_i$ homeostasis is involved in neuronal injury occurring in *in vitro* and in *in vivo* models of hypoxia-anoxia and in several neurodegenerative diseases. More specifically, in the early phase of neuronal anoxic insult, the Na^+/K^+-ATPase blockade causes an increase of $[Na^+]_i$, which in turn induces NCX to reverse its mode of operation. Although during this phase NCX causes an increase in $[Ca^{2+}]_i$, its effect on neurons appears beneficial for two reasons. First, by promoting Ca^{2+} influx, NCX promotes Ca^{2+} refilling into the ER, which is depleted by anoxia followed by reoxygenation, thus allowing neurons to delay ER stress (Sirabella *et al*., 2009). Second, by eliciting the decrease in $[Na^+]_i$ overload, NCX prevents cell swelling and death (Annunziato *et al*., 2007). Conversely, in the later phase of neuronal anoxia, when $[Ca^{2+}]_i$ overload takes place, the NCX forward mode of operation contributes to the lowering of $[Ca^{2+}]_i$, thus protecting neurons from $[Ca^{2+}]_i$-induced neurotoxicity (Annunziato *et al*., 2004). Moreover, further studies have demonstrated that BHK cells overexpressing NCX1 or NCX2 isoforms are more vulnerable to chemical hypoxia compared to BHK cells expressing NCX3 isoforms, thus suggesting that each of the three isoforms has different functional properties and plays a different role in the pathogenesis of a cellular damage (Secondo *et al*., 2007; Bano *et al*., 2005). Another study shows that ischemic rats treated with NCX1 or NCX3 antisense display a remarkable enlargement of the infarct

volume (Pignataro *et al*., 2004) thus suggesting a crucial role of these two isoforms in the pathogenesis of ischemic damage.

It is also demonstrated that NCX plays an important role during ageing since the impairment of Ca^{2+} homeostasis in neuronal cells is considered to be the major triggering event that leads to the development of brain ageing (Annunziato *et al*., 2002). Studies performed on the cerebro-cortex nerve endings of aged rats have shown that the activity of NCX is markedly reduced in the forward and in the reverse mode of operation (Michaelis *et al*., 1984; Canzoniero *et al*., 1992). NCX decline seems to be the consequence of a reduced affinity of the antiporter for Ca^{2+} ions (Michaelis *et al*., 1984).

In this sense, during ageing as well as during neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease in which a neuronal calcium dysfunction is described, NCX might have a relevant role. In fact, a study performed in the synaptic terminals obtained from AD patients brain cortex showed that NCX activity was increased (Colvin *et al*., 1994). Another study performed in synaptosomes prepared from cryopreserved brain of cognitively normal aged controls and late-stage Alzheimer's disease patients demonstrated that NCX2 protein expression was upregulated whereas, NCX3 native 105 kDa band was downregulated. Moreover, NCX1, NCX2, and NCX3 co-localize with Aβ in synaptic terminals (Sokolow *et al*., 2011). On the other hand, it is important to underline the role played by the mitochondrial sodium-calcium exchanger (NCXmito) during neurodegeneration with particular regard to Parkinson's disease (Castaldo *et al*., 2009; Sirabella *et al*., 2018; Di Martino *et al*., 2020). Indeed, it has been reported that during Parkinson's disease the activation of NCXmito is the primary mechanism by which mitochondrial calcium ions return into the cytoplasm and therefore it is critical to a multitude of Ca^{2+}-dependent processes including neurotransmitter release, synaptic plasticity, bioenergetics and mitochondrial NO and free radical generation (Castaldo *et al*., 2009). It was also reported that in the absence of PINK1, NCXmito activity was severely impaired, leading to mitochondrial calcium overload, permeability transition pore opening and cell death (Gandhi *et al*., 2009). It was proposed that NCXmito is entirely distinct from the characterized plasmalemmal NCX isoforms, due to the specific sensitivity of NCXmito to

the inhibitor CGP-37157 (Czyz and Kiedrowski, 2003). Recent finding demonstrated that among the three isoforms of NCX, the NCX3 isoform is the only one detected on the outer mitochondrial membrane where it plays an important role in cell survival during hypoxia (Scorziello *et al*., 2013).

Astrocytes possess several molecular entities responsible for Ca^{2+} and Na^{+} flux across the plasma membrane, including ionotropic receptors, ion channels, transient receptor potential channels and a wide variety of ion transporters and exchangers including NCXs (Verkhratsky *et al*., 2012). The plasma membrane Ca^{2+}-ATPase is a high-affinity/low-capacity transporter and is the major Ca^{2+} extruder in resting astrocytes. Similarly, the Na^{+}/K^{+}- ATPase is the major Na^{+} extruder in resting astrocytes. Plasmalemmal NCX is a high-capacity/low-affinity exchanger which appears to influence resting levels of cytosolic Ca^{2+} and Na^{+} and plays the major role in the regulation of these ions when they are elevated in an excessive or prolonged manner (Reyes *et al*., 2012). NCX is highly enriched in perisynaptic astroglial processes, which represent the third component of the tripartite synapse (Araque *et al*., 1999) and perform glutamate gliotransmission to modulate synaptic transmission and plasticity (Ni *et al*., 2007; Perea and Araque, 2007).

$[Ca^{2+}]_i$, also regulates many important functional responses in microglia, including phagocytosis, transformation, and migration (Pozner *et al*., 2015). All three NCX isoforms have been documented in cultured microglia, with NCX1 being the predominant form (Annunziato *et al*., 2013; Matsuda *et al*., 2001; Nagano *et al*., 2004; Newell *et al*., 2007). The microglial NCX expression is up-regulated by inflammatory mediators such as interferon-gamma (Nagano *et al*., 2004) and NO (Nagano *et al*., 2004). Moreover, microglial NCX upregulation has also been documented in the inflammatory phase in microglial cultured, obtained from *in vivo* stroke model, where this NCX1 up-regulation was concomitant with down-regulation of NCX2 and NCX3, an effect that accompanies post-ischemic microglial activation (Boscia *et al*., 2009).

4. *EXPERIMENTAL MODELS OF PARKINSON'S DISEASE*

To understand the pathophysiological mechanisms underlying PD and to develop disease-modifying therapies it is necessary to have adequate models for *in vitro* and *in vivo* studies.

Cellular models are instrumental in dissecting a complex pathological process into simpler molecular events. PD is multifactorial and clinically heterogeneous pathology therefore it is particularly important to identify experimental models that simplify the study of the different molecular and cellular pathways involved. Cellular models reproducing *in vitro* some of the features of degenerating neurons have contributed to many advances in the comprehension of the pathogenic flow of the disease. Indeed, the pivotal biochemical pathways like apoptosis and oxidative stress, mitochondrial impairment and dysfunctional mitophagy, unfolded protein stress and improper removal of misfolded proteins, have been widely explored in cell lines challenged with toxic insults or genetically modified. Moreover, the classical cellular models represent the correct choice for preliminary studies aimed to investigate the molecular mechanism of action of new drugs or potential toxins and, at the same time, to understand the role of single genetic factors in the pathogenetic process leading to PD developing, looking at the cellular level. Interestingly, the availability of novel cellular systems, such as cybrids or induced pluripotent stem cells (iPS), offers the chance to exploit the advantages of an *in vitro* investigation, although mirroring more closely the cell population being affected.

In general, the *in vitro* models offer the advantage of a controlled environment but may lack the cellular microenvironment critical to disease development. By contrast, this limit may be overcoming in animal models, although differences in brain structure and in the methods used to reproduce *in vivo* a condition close to the disease still represent important limitations (Blandini and Armentero, 2012). Therefore, there is no "best model of PD," as none is a true pathological copy of the human condition; these models are only approximations, each possibly holding a certain degree of relevance (Fig. 4). Thus, to increase the chance of fruitful preclinical investigations, it would be useful: first know the strengths and the weaknesses of each model, second, select models whose characteristics are most

suitable for addressing the experimental question, and third, the possible application in clinically relevant designs.

4.1 In vitro models reproducing PD

An ideal *in vitro* PD cellular model should be established in post-mitotic human dopaminergic neuronal cells susceptible to neurotoxins produced during PD in order to address questions regarding the selective loss of DA neurons in the substantia nigra. Presently, PD cell models primarily include non-neuronal tumour cell lines such as pheochromocytoma, PC12, cells (Koch *et al*., 2009), neuronal tumour cell lines represented by human neuroblastoma SH-SY5Y cells (Cheng *et al*., 2009) and primary mesencephalic neurons (Takeuchi *et al*., 2009). The treatment of these cells with neurotoxins such as 1-methyl-4-phenyl-pyridinium (MPP+), 6-OHDA, or rotenone mimics many aspects of the dopaminergic neuronal degeneration observed in PD. Since these neurotoxins are unsuitable for systemic administration because they do not cross the blood-brain barrier (BBB), they are more useful in neuronal cultures. These models are suitable for studying detailed mechanisms of dopaminergic neuronal degeneration and for screening new pharmacological agents potentially used to treat PD (Kitamura *et al*., 1998; Sawada *et al*., 1996).

The noradrenergic analogue 6-OHDA and several other synthesized analogues have been introduced as catecholaminergic neurotoxins over 30 years ago (Jonsson and Kasamatsu, 1983). Since then, these neurotoxins have been extensively used for both *in vitro* and *in vivo* investigations. 6-OHDA shares some structural similarities with dopamine and norepinephrine, exhibiting a high affinity for several catecholaminergic plasma membrane transporters such as the dopamine (DAT) and norepinephrine transporters (NET). Consequently, 6-OHDA can enter both dopaminergic and noradrenergic neurons and can inflict damage to the catecholaminergic pathways of both the peripheral and the central nervous systems (Jonsson, 1980; 1983) due to the combined effect of ROS and quinones (Cohen, 1984) produced when 6-OHDA dissolved in an aerobic and alkaline milieu readily oxidizes, yielding hydrogen peroxide (H_2O_2) and para-quinone (Saner *et al*., 1971; Heikkila and Cohen, 1971).

Another neurotoxin administered *in vitro* to mimic PD is the mitochondrial electron transport chain complex I inhibitor MPTP. This toxin is converted to MPP+, which is taken up via the DA transporter and accumulates in dopaminergic neurons. Absorbed MPP+ concentrates in mitochondria, where it inhibits complex I of the electron transport chain, thereby reducing ATP generation and causing ROS production with consequent apoptotic death of dopaminergic neurons (Kitamura *et al*., 2000, 2003; Speciale, 2002). Moreover, MPTP-induced toxicity is often presented as being "specific" for the dopamine neurons, although it is well known that MPTP induces multisystemic lesions (Bezard *et al*., 2013).

Among the toxic models of PD, rotenone represents one of the most used approaches (Betarbet *et al*., 2000). Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from various parts of Leguminosae plants. Rotenone is widely used around the world as insecticide and pesticide (Hisata, 2002). Because of its short half-life and because it does not readily leach from soil, it is not expected to be a groundwater pollutant. Consequently, the likelihood that PD might be caused by an environmental exposure to rotenone is low. Conversely, it must be underlined that many environmental neurotoxins other than rotenone have a long half-life. Thus, if rotenone appears an unlikely culprit, several others could account for the epidemiological data showing that the risk of PD increases with exposure to pesticides (Butterfield *et al*., 1993; Gorell *et al*., 1998). Like MPTP, rotenone is highly lipophilic and thus readily crosses all cellular membranes and can accumulate in subcellular organelles such as mitochondria. In mitochondria, rotenone impairs oxidative phosphorylation by inhibiting reduced nicotinamide adenine dinucleotide (NADH)-ubiquinone reductase activity through its binding to the multipolypeptide enzyme complex I of the electron transport chain (Schuler and Casida, 2001). Aside from its action on mitochondrial respiration, rotenone also inhibits the formation of microtubules from tubulin (Marshall and Himes, 1978; Brinkley *et al*., 1974). This effect may be quite relevant to the mechanism of dopaminergic neurodegeneration because excess of tubulin monomers may be toxic to cells (Burke *et al*., 1989;

Weinstein and Solomon, 1990). Rotenone has been used extensively as a prototypic mitochondrial poison in cell cultures, but less frequently in living animals. Exposure of embryonic ventral midbrain cultures to rotenone causes major neurotoxicity (Marey-Semper *et al*., 1995), especially in the presence of microglial cells (Gao *et al*., 2002).

The availability of new cellular systems, such as cybrids or iPS cells, offers the chance of exploiting the advantages of an *in vitro* investigation, although more closely mirroring the affected cell population. These systems also represent a new step towards personalized medicine, with the aim of testing each drug in a model with the same genetic background of the patients.

4.2 In vivo models reproducing PD

4.2.1 Toxic Models

Defects in complex I function, and oxidative stress have been described in the brains of patients affected by PD, thus suggesting that these mechanisms might be involved in nigral dopaminergic neuronal damage. Studies using MPTP, 6-OHDA and rotenone, as mentioned before, have provided insights into the molecular mechanisms leading to dopaminergic neuronal death (Shimohama *et al*., 2003). Although the ability of these neurotoxins to induce marked, if not total, and long-lasting lesions of the nigrostriatal pathway is widely accepted, their ability to replicate the dorso-ventral gradient of striatal denervation in PD (Kish *et al*., 1988; Brooks *et al*., 1990) is still a matter of discussion despite clear experimental evidence. Indeed, 6-OHDA and other neurotoxins poorly cross the BBB, hence failing to accumulate within the brain parenchyma at meaningful neurotoxic concentrations after systemic injections. To circumvent this problem, 6-OHDA has to be injected directly into the brain by stereotaxic approach. Several local sites of injection have been used to damage the central dopaminergic pathways including intraventricular, intracisternal, and intracerebral (Jonsson *et al*., 1983) injections. Discrete intrastriatal administrations of 6-OHDA in the rat can achieve such replication (Kirik *et al*., 1998).

Striking examples of such preferential dorsoventral striatal denervation have been produced with the MPTP in the marmoset (Iravani *et al*., 2005), the African green monkey (Jan *et al*., 2003) and the macaque monkeys (Bezard *et al*., 2001; Guigoni *et al*., 2005; Moratalla *et al*., 1992) suggesting that chronic exposures consistently allow to mimic the human PD specific pattern of nigrostriatal lesion while earlier intracarotid and acute regimen did not. Interestingly, various MPTP intoxication regimens have been reported to affect α-synuclein levels and aggregation. Indeed, α-synuclein aggregates have been observed in a chronic MPTP model (Fornai *et al*., 2005). Such phenomenon has been better documented in the MPTP monkey models in which a clear relationship between dopaminergic cell loss, α-synuclein upregulation, neuritic α-synuclein pathology and α-synuclein aggregation has been established (McCormack *et al*., 2010; McCormack *et al*., 2008; Purisai *et al*., 2005). Interestingly, such changes occurred mostly in neuromelanin-positive neurons (Purisai *et al*., 2005) (Fig. 4).

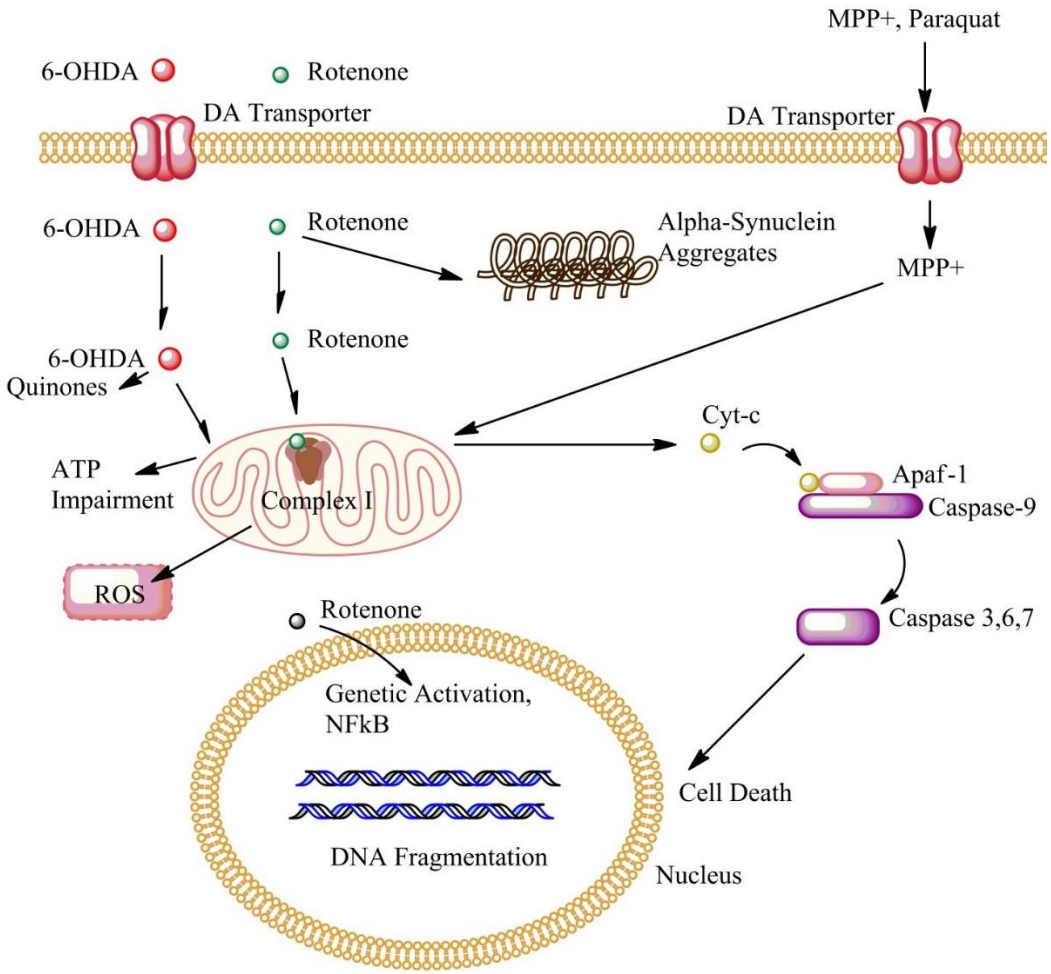


Fig. 4. Experimental *in vitro* models in PD. Many molecules are currently used in cellular and animal models of PD, including pesticides as paraquat or rotenone and neurotoxins such as 6-OHDA and MPP+. Paraquat, 6-hydroxydopamine (6-OHDA) and MPP+ easily cross cell membrane through the dopamine transporter (DA) thus inducing the formation of α-synuclein aggregates and mitochondrial impairment with the subsequent production of ROS and quinones. Compounds, as rotenone, are extremely hydrophobic and penetrate easily the cellular membrane of neurons and astrocytes. Rotenone may promote processes such as the formation of α-synuclein aggregates, and the genetic activation through the nuclear translocation of NF-κB. Additionally, as an inhibitor of mitochondrial complex I, rotenone causes the impairment of ATP, the generation of ROS and the release of proapoptotic molecules, such as cytochrome c that activates caspase 9, which triggers caspases 3, 6 and 7, and induces apoptosis (Cabezas *et al.*, 2013).

4.2.2 Genetic Models

Recent gene-targeting and transgenic strategies have been applied in order to generate several mouse lines with mutations in the dopaminergic system (Jankowsky *et al.*, 2002). Mice that lack the gene encoding *Nurr1* (Zetterstrom *et al.*, 1997) or tyrosine hydroxylase (Zhou *et al.*, 1995), or are deficient in DA production (Zhou and Palmiter, 1995), die at a late embryonic stage or shortly after birth, suggesting that DA is essential for embryonic development. By contrast, mice in which the D2 receptor is deleted survive but show decreased spontaneous movement, as with drug-induced

parkinsonism (Baik *et al*., 1995), and mice that lack D1 or D3 receptors show increased locomotor activity (Xu *et al*., 1994; Accili *et al*., 1996). Thus, D2-receptor-deficient mice exhibit PD-like symptoms without neuronal death and Lewy body formation.

In the last decades, the discovery of familiar mutations in PD patients, like mutations in the gene encoding α-synuclein (Vila *et al*., 2001) contributed to generate a series of genetic models to study PD. In this sense, a model of PD expressing mutant and normal forms of α-synuclein in Drosophila was produced (Feany *et al*., 2000). In this model an age-dependent loss of dorsomedial neurons positive to TH was reported. However, other subsets of dopaminergic neurons showed no abnormalities, consistent with the differential vulnerability of dopaminergic neurons in human PD. However, α-synuclein-stained inclusions that resembled Lewy bodies have been also observed in neurons by light microscopy and EM. The flies developed locomotor dysfunction with age, and this was more evident in transgenic flies overexpressing A30P-mutant α-synuclein than in those with A53T-mutant or wild type α-synuclein. Nevertheless, transgenic flies recapitulate most of the criteria for a relevant PD model, including progression, age dependence, selective loss of DA neurons and the formation of Lewy-body-like inclusions. Because invertebrates have simple neuronal networks and motor functions, they might be useful as PD models.

Additionally, overexpression of human wild type α-synuclein in mice (Masliah *et al*., 2000) led to the formation of cytoplasmic and nuclear micro-inclusions with α-synuclein and ubiquitin, and a reduction in striatal TH activity and motor performance. However, wild type α-synuclein mouse was natively expressed, and the decrease in TH activity and locomotion were relatively low. Some of the transgenic mice expressing full-length α-synuclein with the A53T or A30P mutations show abnormal motor behaviour associated with granular or filamentous material. However, in some cases these alterations appear to be mainly linked to the pathology in the spinal cord and not to a dysfunction in the *substantia nigra* (Giasson *et al*., 2002; Rieker *et al*., 2011).

The identification of LRRK2 (Leucine-rich repeat kinase 2) mutations as the genetic cause for PARK8 (Familial Parkinson's disease type 8) (Zimprich *et al*., 2004; Paisan-Ruiz *et al*., 2004) has triggered another wave of PD model generation. LRRK2 encodes a large complex protein consisting of multiple conserved domains such as N-terminal ankyrin repeat, Leucine-rich repeat domain, ROC (GTPase) domain, MAPKKK-like kinase domain, and C-terminal WD40 domain. At least 6 mutations in LRRK2, G2019S, R1441C/G/H, I2020T and Y1699C are believed to be pathogenic. G2019S mutation alone accounts for the most common inherited form of PD (Mata *et al*., 2006; Healy *et al*., 2008) (~4%). In fact, mutations of LRRK2 are also linked to some sporadic forms and the clinical symptoms and neuropathology of LRRK2-associated PD are indistinguishable from idiopathic PD (Zimprich *et al*., 2004; Paisan-Ruiz *et al*., 2004). The autosomal dominant transmission of LRRK2 mutations makes transgenic expression of pathogenic LRRK2 species suitable for modelling disease process in PD. Indeed, various invertebrate transgenic models producing LRRK2 PD mutants have been quickly generated after the discovery of LRRK2 mutations in PD; the phenotypes of the models range from no change to apparent neuronal loss or deficits in dopaminergic systems and motor behaviour (Yue, 2009). These models are useful for understanding the early pathogenic events in PD. Current evidence suggests that the striatal DA abnormality often precedes the frank motor function deficits in G2019S PD patients and perhaps the loss of dopaminergic neurons. Therefore, the LRRK2 rodent models may recapitulate the specific disease stage that presents an early pathological alteration prior to the loss of nigral neurons. Thus, the LRRK2 models can be used to explore the interactions between genetic risk and environmental factors that underlie the PD etiology (Bezard *et al*., 2013).

5. *AIM OF THE STUDY*

The present study has been undertaken to investigate the hypothesis that pharmacological strategies able to improve mitochondrial function might prevent neuronal dysfunction in *in vitro* and *in vivo* models of Parkinson's Disease. To this aim, the attention was focused on the amino acid ornithine that is capable of reaching and selectively penetrating inside the mitochondria thanks to its mitochondrial transporter. Inside mitochondria, ornithine takes part to the urea cycle with consequent production intermediate that can affect cellular oxidative metabolism. Among them, citrulline which participates to arginine production in the cytosol, polyamines (synthesized primarily from ornithine) via ornithine decarboxylase (ODC) and proline through the ornithine amino transferases (OAT). This aspect is extremely relevant considering the key role played by mitochondria in regulating synaptic activity. Therefore, a therapeutic strategy able to promote mitochondrial function might result useful to counteract the early synaptic dysfunction and the functional and pathological changes occurring in the brain of PD-affected patients. Specifically, the intracellular pathways affected by ornithine treatment have been explored *in vitro* in two cellular models largely used to investigate the contribution of mitochondrial-induced metabolic impairment in PD pathogenesis. They are represented by SH-SY5Y cells treated with rotenone (ROT), an inhibitor of mitochondrial complex I, or with 6-OHDA, an inducer of oxidative stress. Parallel *in vivo* experiments have been also performed in mice expressing the mutation A53T of human α-synuclein (A53Tα-syn), a model of familial form of PD largely characterized in the Lab in which I performed my PhD program, to investigate *in vivo* the pathogenetic mechanisms involved in dopaminergic degeneration during aging and to evaluate the relationship between α-synuclein accumulation, mitochondrial dysfunction and behavioral phenotype of A53Tα-syn-mice compared to WT, with particular regard to the motor and non-motor symptoms (Sirabella *et al*., 2018; Di Martino *et al*., 2021; Costa *et al*., 2021). The *in vivo* experiments have been realized in order to identify the experimental condition more appropriate to explore the potential effects of ornithine on the phenotypic manifestation associated with the

progression of dopaminergic neuronal degeneration. To this aim behavioral and biochemical experiments have been performed in 4- and 12-months old WT and A53Tα-syn-transgenic-mice.

The results of the *in vitro* experiments demonstrated that the co-exposure of cells to L-Ornithine L-Aspartate (LOLA) and ROT for 24h significantly increased mitochondrial redox activity in SH-SY5Y compared to cells exposed to ROT alone. Conversely no effects of LOLA were observed in SH-SY5Y cells co-exposed to 6-OHDA for 48h compared to SH-SY5Y cells treated with 6-OHDA alone. These effects were not related to a difference in the expression of the mitochondrial ornithine transporter 1 (ORNT1) in cells exposed to the toxins in the presence of LOLA, since no change in ORNT1 protein expression was detected in the two above mentioned experimental conditions. In order to clarify the intracellular events involved in the effect of LOLA on mitochondria, confocal microscopy experiments were performed to measure mitochondrial membrane potential, calcium content and free radical production in SH-SY5Y co-exposed to ROT or 6-OHDA and LOLA. The results obtained demonstrated that the treatment of the cells with LOLA was able to counteract ROT- and 6-OHDA-induced mitochondrial depolarization in SH-SY5Y cells by promoting a significant hyperpolarization of the mitochondrial membrane potential in both tested experimental conditions. However, this effect was associated with a reduction in LOLA-induced mitochondrial calcium levels in SH-SY5Y cells exposed to ROT compared to cells treated with ROT alone, and to an increase in LOLA-induced mitochondrial calcium levels in cells exposed to 6-OHDA with respect to 6-OHDA-treated cells. In order to verify whether this effect might be related to changes in cytosolic calcium concentration, further experiments were performed to evaluate the effect of LOLA on cytosolic calcium concentrations in SH-SY5Y co-exposed to the treatment with ROT or 6-OHDA and LOLA. The results of these experiments demonstrated that LOLA significantly reduced cytosolic calcium levels in SH-SY5Y cells exposed to ROT, bringing them closer to the levels observed in control untreated cells, whereas it did not affect intracellular calcium concentration in cells exposed to 6-OHDA. Further experiments aimed to evaluate the effect of LOLA on free radical production demonstrated

that LOLA treatment in SH-SY5Y exposed to ROT caused a reduction in ROS production compared to ROS levels produced after ROT treatment alone. Conversely, LOLA treatment was unable to decrease ROS levels in SH-SY5Y cells co-exposed to 6-OHDA. In order to understand the intracellular events involved in the different effect of LOLA treatment on mitochondrial function in cells treated with ROT or 6-OHDA the attention was focused on those proteins responsible for the regulation of intracellular calcium homeostasis and, specifically, on the sodium calcium exchangers (NCXs) isoforms 1 and 3 (NCX1 and NCX3), since previously data reported in the literature demonstrated their role in the regulation of calcium concentration in cytosol and in the mitochondria respectively. Therefore, western blot experiments were performed to evaluate the expression of NCX1 and NCX3 in SH-SY5Y cells exposed to the treatment with ROT or 6-OHDA in the presence and in the absence of LOLA. The results of these experiments demonstrated that ROT induced an increase in NCX1 protein expression that was not affected by LOLA, whereas 6-OHDA did not affect NCX1 protein expression both when it was administered alone and when it was administered in the presence of LOLA. On the contrary, both ROT and 6-OHDA alone stimulated NCX3 protein expression levels in SH-SY5Y treated cells, and the co-treatment with LOLA was able to counteract the effect of 6-OHDA on NCX3 expression without affecting ROT-induced NCX3 increased expression. In order to clarify the effects of LOLA on intracellular calcium homeostasis in cells exposed to ROT and 6-OHDA treatments further experiments were performed to investigate the effects of the above indicated treatments on NCX activity. These experiments were performed by using Fura 2 and a microfluorimetric approach by exposing the cells to a Na^+ free medium, an experimental condition able to activate the reverse mode of operation of NCX. The results of these experiments demonstrated that LOLA alone was able to potentiate the reverse mode of operation of NCX as well as ROT and 6-OHDA. However, when the cells were co-exposed to LOLA and ROT or 6-OHDA, the activity of NCX was reduced in cells treated with ROT whereas, it was potentiated in cells treated with 6-OHDA. Because it was reported that both ROS and NO are able to modulate NCX activity, the hypothesis that the effect of LOLA on NCX activation might be related to its ability to

regulate the level of mitochondrial ROS and RNS, thus modulating mitochondrial function, was also explored in cells co-treated with LOLA and alternatively with ROT or 6-OHDA. In these experimental conditions, western blot experiments, firstly performed to measure nNOS expression levels in SH-SY5Y cells, demonstrated that neither ROT nor 6-OHDA were able to improve nNOS protein expression both alone or in presence of LOLA. However, either ROT or 6-OHDA were able to increase NO production as demonstrated by microfluorimetry experiments performed in SH-SY5Y cells loaded with fluorescent dye DAF that selectively reveal the amount of NO produced by the single cell. Moreover, this effect was more pronounced in cells exposed to ROT than to 6-OHDA. Interestingly, the co-treatment with LOLA in ROT- or 6-OHDA-exposed cells was able to reduce NO production in both conditions as compared to the effect induced by the single toxin alone. However, the amount of NO produced in SH-SY5Y cells exposed to ROT in the presence of LOLA was still elevated compared to that observed in control untreated or LOLA-exposed cells. Conversely, in cells treated with LOLA and 6-OHDA the amount of NO produced was similar to that observed in control and in LOLA treated cells. These results allowed to confirm the hypothesis that LOLA might exert its effects on mitochondria by interfering with those mitochondrial mechanisms related to ROS and RNS production thus favoring mitochondrial functional recovery. However, in cells treated with ROT, the increased ROS production was accompanied to an increase in intracellular calcium concentration probably due to the stimulation of ROS-sensitive NCX1 activity in the reverse mode of operation. These effects, associated whit the block of mitochondrial complex I caused by ROT, contributed to mitochondrial membrane depolarization and consequently mitochondrial dysfunction. In these conditions the treatment with LOLA, by reducing ROS and promoting RNS production was able to improve mitochondrial function by stimulating mitochondrial calcium efflux trough NCX3 activation. Conversely, in 6-OHDA-treated SH-SY5Y cells the stimulation of NCX1 activity, by promoting mitochondrial metabolic activation, induced a massive ROS production that was not counteracted by LOLA treatment. However, the reduction of RNS production observed in SH-SY5Y cells co-treated with LOLA and 6-OHDA might represent the mechanism by which LOLA, without

affecting mitochondrial calcium efflux mechanisms, might prevent mitochondria depolarization and, in turn, mitochondrial induced 6-OHDA dysfunction.

Parallel *in vivo* experiments performed in A53T-α-syn mice have been performed in order to identify the experimental condition more appropriate to explore the potential effect of LOLA on the phenotypic manifestation associated with the progression of dopaminergic neuronal degeneration. To this aim western blot experiments were performed in midbrain and striatum of 4- and 12- month-old WT and A53Tα-syn-transgenic-mice to confirm the alteration of dopaminergic synaptic dysfunction, by measuring the expression synapsine 3 (SYN3), a synaptic protein which play a key role in the dopamine release and that is able to interact with α-synuclein and, the expression of dopamine transporter (DAT), since α-synuclein directly interacts with the dopamine transporter and this interaction modulate DAT activity by decreasing its expression in the membrane of DA-neurons. These experiments would help to identify the early stage of the dopaminergic dysfunction and would represent the starting point to verify whether, LOLA treatment, by affecting neuronal metabolic function, might slow down the progression of dopaminergic damage and consequently, the related phenotypic disease manifestations.

Western blot experiments performed in midbrain and striatum of 4- and 12-month-old WT and A53T-α-syn transgenic mice showed that SYN3 expression increased in 4-month-old A53T-α-syn transgenic mice compared to wild type, and that this effect was more pronounced in the midbrain than in the striatum. Conversely, no significant changes occurred in the protein expression in the midbrain and striatum of adult 12-month-old A53T-α-syn transgenic mice compared to WT. However, when compared each other 4- and 12-month-old A53T-α-syn mice a significant impairment in SYN3 expression was observed in striatum compared to midbrain, an effect more evident in 12- than in 4-month-old A53T-α-syn mice, thus confirming that alteration in DA transmission occurred in transgenic mice. Similar results were obtained by measuring DAT protein expression. Indeed, an increase in DAT expression was detected in 4-month-old A53T-α-syn transgenic mice compared to

WT both in the midbrain and striatum although more evident in the midbrain, whereas a reduction in DAT expression occurred only in the midbrain of 12-month-old A53T-α-syn transgenic mice. Once again, when compared each other 4- and 12-month-old A53T-α-syn transgenic mice, a significant reduction in DAT expression was revealed in the midbrain compared to striatum of older mice. In order to verify whether these alterations in DA-synaptic activity might be related to the phenotypic manifestations of PD, behavioral experiments were performed to evaluate motor and non-motor symptoms in 4- and 12-month-old WT and A53T-α-syn transgenic mice. To this aim Pole test, Open field test, and Rotarod test, were used to evaluate the alterations in motor symptoms, whereas Olfactory and One-hour stool collection tests were performed to evaluate the presence of peripheral deficits related to olfactory or intestinal dysfunctions. The results obtained revealed that 4-month-old A53T-α-syn transgenic mice showed a greater spontaneous exploratory locomotor activity, measured by Open field test, compared to 12-month-old A53T-α-syn transgenic and to 4- and 12-month-old-WT mice as confirmed by the greater distance travelled. Similarly, 4-month-old A53T-α-syn transgenic mice displayed a better balance and motor coordination compared to WT, during the Rotarod test, since they spent more time on the wheel whereas, no differences were observed between 12-month-old-Tg and WT-mice. Interestingly, 4- and 12-month-old A53T-α-syn transgenic mice exhibited a progressive imbalance and bradykinesia, measured by Pole test, since they spent more time climbing down the pole compared to WT. All together, these data confirmed that 4-month-old A53T-α-syn transgenic mice displayed an anxiety-like and hyperactive behavior compared to older and WT mice.

Regarding the non-motor symptoms, the One-hour stool collection test indicated that 12-month-old A53T-α-syn transgenic mice displayed a lower dry stool weight and a lower content of stool water compared to WT and to 4-month-old A53T-α-syn transgenic mice. Conversely, the Olfactory test revealed an impairment of olfaction already present in 4-month-old A53T-α-syn transgenic mice.

The results of *in vivo* model demonstrated that 4-month-old A53T-α-syn transgenic mice represent a good stage of disease in which verify the effects of LOLA treatment on mitochondrial function, DA synaptic dysfunction and the amelioration of motor and non-motor symptoms in a *in vivo* model of PD.

6. *MATERIALS AND METHODS*

6.1 In vitro experiments

Materials and Methods

Ornithine (Esseti Farmaceutici, Italy) is a non-essential amino acid, which plays a central role in the urea cycle. It is produced by the enzymatic action of arginase on arginine, which results in the production of urea and generation of ornithine. It has been shown to reduce blood ammonia concentrations by increasing ammonia detoxification in the muscle and reducing the severity of hepatic encephalopathy in cirrhosis. L-Ornithine L-Aspartate (LOLA) is a peptide formed by the two amino acids ornithine and aspartic acid. This conformation makes ornithine more absorbable by the digestive system. This peptide has the benefits of ornithine with the advantage that even aspartic acid, participating in the urea cycle, favors the elimination of ammonia. For these reasons, ornithine is commercialized in this form, because the two amino acids taken together are more effective in detoxification processes. Cells were exposed to 5 mM LOLA for 24h or 48h. The substance was dissolved in H_2O.

Rotenone (Sigma-Aldrich, Italy) is an inhibitor of mitochondrial electron transport at nicotinamide adenine dinucleotide (NADH)-ubiquinone oxidoreductase. Rotenone acts as a neurotoxic agent which can produce parkinson-like condition both *in vitro* and *in vivo* models. Cells were exposed to 500 nM rotenone for 24h. The substance was dissolved in DMSO (0.5 mg/ml).

6-OHDA (Sigma-Aldrich, Italy) is a neurotoxin, commonly used to induce PD both in *in vitro* and *in vivo* models. 6-OHDA is highly oxidable and exerts cytotoxicity by generating reactive oxygen species, initiating cellular stress and cell death. Cells were exposed to 30 µM 6-OHDA for 48h. The substance was dissolved in H_2O.

Cell culture

Human SH-SY5Y cells were cultured as monolayers in polystyrene dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. The culture medium was replaced every 2 days.

Determination of mitochondrial oxidative activity

Mitochondrial activity was assessed by measuring the level of mitochondrial dehydrogenase activity using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyltetrazolium bromide (MTT) as the substrate (Hansen *et al*., 1989; Amoroso *et al*., 1999). The assay is based on the ability of living mitochondria to convert dissolved MTT into insoluble formazan. Briefly, after treatments the medium was removed, and the cells were incubated in 500 µM of MTT solution (0.5 mg/ml) for 1h in a humidified 5% CO2 incubator at 37°C. The incubation was then stopped by removing the medium and adding 1 ml of DMSO to solubilize the formazan. The absorbance was read at 540 nm. Data are expressed as the percentage of cell viability to sham-treated cultures.

Confocal Microscopy and Mitochondrial Function

To assess the mitochondrial calcium concentrations $[Ca^{2+}]_m$, cells were loaded with X-Rhod-1 (0.2 µM) for 15 min in a medium containing: 156 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.35 with NaOH. At the end of the incubation, cells were washed 3 times in the same medium. An increase in the mitochondria-localized intensity of fluorescence was indicative of mCa^{2+} overload.

Cytosolic calcium concentrations $[Ca^{2+}]_c$ was measured by using the fluorescent dye Fluo-3 acetoxymethyl ester (Fluo-3AM). The advantage to use Fluo-3AM was that this calcium indicator can be loaded into the cells together with the mitochondrial calcium indicator X-Rhod-1, thus allowing a simultaneous comparison of calcium levels in the cytoplasmic and mitochondrial compartments. Cells were loaded with Fluo-3AM (5 nM) for 30 min at room temperature in the same

medium described above. At the end of incubation, cells were washed 3 times in the same medium. An increase in $[Ca^{2+}]_c$ intensity of fluorescence was indicative of cytosolic Ca^{2+} overload.

Mitochondrial membrane potential ($\Delta\Psi m$) was assessed using the fluorescent dye tetramethylrhodamine ethyl ester (TMRE) in the "redistribution mode" (Livigni *et al*., 2006). Cells were loaded with TMRE (20 nM) for 30 min in the above-described medium. At the end of the incubation, cells were washed in the same medium containing TMRE (20 nM) and allowed to equilibrate. A decline in the mitochondria-localized intensity of fluorescence was indicative of mitochondrial membrane depolarization.

ROS levels were measured by using the fluorescent dye MitoSox (5 μM for 20 min) that is rapidly oxidized by superoxide. The oxidized product is highly fluorescent upon binding to nucleic acid. Cells were loaded with MitoSox (5 μM) for 20 min at room temperature in the same medium described above. At the end of incubation, cells were washed 3 times in the same medium. An increase in MitoSox intensity of fluorescence was indicative of increased ROS production.

Confocal images were obtained using Zeiss inverted 700 confocal laser scanning microscopy and a 63× oil immersion objective. The illumination intensity of 543 Xenon laser, used to excite X-Rhod-1, TMRE and MitoSox, and the illumination intensity of 488 Argon laser, used to excite Fluo-3AM fluorescence, were kept to a minimum of 0.5% of laser output to avoid phototoxicity.

Western blot Analysis

SH-SY5Y cells were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5); 10 mM NaF; 150 mM NaCl; 1 mM phenylmethylsulphonyl fluoride (PMSF); 1% NONIDET P-40, 1%; 1 mM Na_3VO_4; 0.1% aprotinin; 0.7 mg/ml pepstatin; and 1 μg/ml leupeptin. Homogenates were centrifuged at 12.000 rpm for 30 min at 4 °C. Supernatant was collected and used for protein content quantification using the Bradford method. The total protein amount used for each sample was 50 μg. Proteins were separated on 10% sodium dodecyl sulphate polyacrylamide gels with 5% sodium dodecyl sulphate

stacking gel (SDS-PAGE), and subsequently transferred to nitrocellulose membranes and incubated overnight at 4 °C in the blocking buffer containing 1:1000 antibody for ORNT1 (polyclonal rabbit antibody), 1:1000 antibody for NCX1 (polyclonal rabbit antibody), 1:1000 antibody for NCX3 (polyclonal rabbit antibody), 1:1000 antibody for nNOS (polyclonal rabbit antibody). Next, all membranes were washed 3 times with a solution containing Tween 20 (0.1%) and subsequently incubated with the secondary antibodies for 1 h (1:2000) at room temperature. The immunoreactive bands were visualized by enhanced chemiluminescence. The optical density of the bands was normalized with those of β-actin and measured by ImageJ.

$[Ca^{2+}]_i$ measurement

SH-SY5Y, grown on glass coverslips, were loaded with 5 μM Fura-2 acetoxymethyl ester (Fura-2AM) for 1h at room temperature in normal Krebs solution containing the following (in mM): 5.5 KCl, 160 NaCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes–NaOH, pH 7.4. At the end of the Fura-2AM loading period, the coverslips were placed into a perfusion chamber mounted onto the stage of an inverted Nikon Diaphot fluorescence microscope. A 100-Watt Xenon lamp, with a computer-operated filter wheel bearing two different interference filters (340 and 380 nm), illuminated the microscopic field with UV light every 3 sec, alternating the wavelengths at an interval of 500 ms. The light emitted by Fura-2AM loaded cells was passed through a 400 nm dichroic mirror filtered at 510 nm and collected with an intensified camera. Images were digitized and analysed with a Magiscan image processor driven by the AUTOLAB software (Secondo *et al.*, 2007). NCX activity, was evaluated as Ca^{2+} uptake through the reverse mode by switching the normal Krebs medium to Na^+- deficient NMDG+ medium (Na^+- free) (in mM): 5.5 KCl, 147 N-methyl glucamine, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 10 glucose, and 10 Hepes–NaOH (Ph 7.4)

Nitric Oxide Detection

Cells were loaded with 10 μM 4,5-diaminofluorescein-2-diacetate (DAF-2DA) in a humidified 5% CO_2 atmosphere at 37°C for 20 min in Normal Krebs' solution (5.5 mM KCl, 160 mM NaCl, 1.2 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, and 10 mM HEPES-NaOH, pH 7.4) containing the drugs or their vehicles (Melisi *et al*., 2006). Thereafter, fluorescent cells were fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for 5 min at 4°C. This procedure permits a subsequent densitometric analysis with the fluorescence microscope Nikon Eclipse E400 (Nikon, Torrance, CA) set at an excitation/emission wavelength of 495/515 nm. Fluorescent images were then stored and analysed with Pro-Plus software (Media Cybernetics, Silver Spring, MD). Data were calculated as the percentage of sample fluorescence compared with that of controls.

6.2 In vivo experiments

Transgenic mice bearing α-synuclein A53T human mutation

Mice expressing the human A53T α-synuclein (A53T-α-syn) mutation under the control of a prion promoter (Pmp-SNCA*A53T) were obtained from The Jackson Laboratory. Mice hemizygous for the α-synuclein A53T mutation were bred on a mixed C57Bl/6 × C_3H background to produce transgenic and non-transgenic littermates. To identify A53T mice, PCR was performed according to the protocol provided by The Jackson Laboratory. All mice were housed in groups of 1–5, in temperature and humidity-controlled rooms under a 12h light/dark cycle and fed an ad libitum diet of standard mouse chow. Experiments were performed according to the international guidelines for animal research.

Pole Test

The pole test was used to evaluate the agility of A53T-α-syn-transgenic and WT mice. This test has been previously used to assess basal ganglia-related motor impairment in mice, since it involves

skilled forelimb grasping and maneuvering which require an intact basal ganglia and activation of the rubrospinal pathway (Ogawa *et al*., 1985, 1987; Matsuura *et al*., 1997; Fleming *et al*., 2004, Hwang et al., 2005). Mice received 2 days of training before testing, during which they were placed head upward at the top of a vertical rough-surfaced pole (diameter 1 cm; height 55 cm). On the test day, the time spent by each mouse to reach the floor was recorded during three trials and the average score was expressed in seconds.

Rotarod Test

Motor coordination and balance were assessed using a five-station mouse rotarod apparatus. Mice were trained for two days to maintain balance at increasing speed from 4 to 40 rpm for five consecutive trials. On the third day mice were tested and the cut-off of the performance was set at 180 sec. Each mouse undergone to three trials on the rod, and the latency to fall was measured for each trial. The maximum latency of 180 sec was assigned to the mice that did not fall at all (Giampa *et al*., 2010)

Open Field Test

Spontaneous exploratory locomotor behavior was evaluated by means of the open field test. The Open Field test provides a highly efficient paradigm for phenotype characterization of A53T mice (Hinkle *et al*., 2012). Indeed, the test has been successfully used with other familial PD mouse models (Zhu *et al*., 2007). Behavioral activity was evaluated in A53T and WT mice. The open field apparatus, that is a Plexiglas square arena (45 × 45 cm, 40 cm high), was placed in a homogenously lit experimental room. For each test, mice were placed individually in the center of the square and allowed to explore it for 15 min. Fifteen minutes is a long enough time to evaluate the impairment in locomotor activity once the mice have become familial to the new environment. Total travelled distance was measured with a video-tracking software.

One-Hour Stool Collection Test

A53T and WT mice were individually placed in a clean cage and monitored throughout the 1h collection period. Fecal pellets were collected immediately after expulsion and placed in sealed 1.5 ml tubes to avoid evaporation. Tubes were weighed to obtain the wet weight of the stool, which was then dried overnight at 65°C and reweighed to obtain the dry weight. The stool water content was calculated from the difference between the wet and dry stool weights (Li *et al*., 2006).

Olfactory Test

Mice were food-deprived for 20h before testing, which was conducted in a clean plastic cage (length 42 cm, width 24 cm, height 15 cm). Each mouse was individually placed in the center of the cage and had to retrieve an odorous pellet that was buried under the bedding (at a depth of 1 cm). The amount of time required to retrieve the pellet and bite it was measured for each mouse tested (Lehmkuhl *et al*., 2014).

Western blot Analysis

Mouse brain tissues were lysed in a buffer containing 20 mM Tris-HCl (pH 7.5); 10 mM NaF; 150 mM NaCl; 1 mM phenylmethylsulphonyl fluoride (PMSF); 1% NONIDET P-40, 1%; 1 mM Na3VO4; 0.1% aprotinin; 0.7 mg/ml pepstatin; and 1 µg/ml leupeptin. Homogenates were centrifuged at 12.000 rpm for 30 min at 4 °C. Supernatant was collected and used for protein content quantification using the Bradford method. The total protein amount used for each sample was 50 µg. Proteins were separated on 10% sodium dodecyl sulphate polyacrylamide gels with 5% sodium dodecyl sulphate stacking gel (SDS-PAGE), and subsequently transferred to nitrocellulose membranes and incubated overnight at 4 °C in the blocking buffer containing 1:1000 antibody for SYN3 (polyclonal rabbit antibody), and 1:1000 antibody for DAT (monoclonal rat antibody). Next, all membranes were washed 3 times with a solution containing Tween 20 (0.1%) and subsequently incubated with the secondary antibodies for 1h (1:2000) at room temperature. The immunoreactive

bands were visualized by enhanced chemiluminescence. The optical density of the bands was normalized with those of α-tubulin and measured by ImageJ.

6.3 Statistical Analysis

Data were generated from a minimum of 3 independent experimental sessions for the *in vitro* studies. Calcium and mitochondrial membrane potential measurements were performed at least in 200 cells for each of the 3 independent experimental sessions. Data were expressed as mean percentage ± S.E.M. Statistical comparisons between control and treated-cells were performed using the one-way ANOVA test followed by Newman–Keuls test. For the *in vivo* study the relationship between mice's genotype and ages was analysed by using two-way ANOVA test followed by Newman-Keuls test, while unpaired Student's t-Test was used to analyse the trend inside the same group. The analysis was performed in 120 animals (60 WT, including 30, 4-months old WT mice and 30, 12-months old WT mice; 60 A53T-α-syn Tg including 30, 4-months old A53T-α-syn Tg mice and 30, 12-months old A53T-α-syn Tg mice). p-value < 0.05 was considered statistically significant.

7. *RESULTS*

In vitro experiments

Effect of LOLA treatment on mitochondrial function in SH-SY5Y cells in basal conditions and after exposure to rotenone or 6-OHDA

In order to investigate the pharmacological profile of L-Ornithine L-Aspartate (LOLA) the first experiments were performed in SH-SY5Y cells in basal conditions and after exposure to rotenone (ROT 500nM/24h) an inhibitor of mitochondrial complex I, or 6-OHDA (30µM/48h), an inducer of oxidative stress, two experimental conditions largely validated to reproduce *in vitro* a mitochondrial dysfunction useful to investigate the contribution of mitochondria to the metabolic impairment involved in PD pathogenesis. To this aim SH-SY5Y cells were first exposed to a dose-response treatment with LOLA (500nM – 5mM) for 24h, added to the culture medium, in order to verify whether long exposures to LOLA in basal conditions were able to affect mitochondrial redox activity and consequently to impair cell survival. These experiments were also useful to identify the more appropriate LOLA concentration to use in the subsequent experiments. As reported in Figure 5 the dose response experiments performed in SH-SY5Y cells allowed excluding any deleterious effect of LOLA on cell survival after prolonged exposure (Fig. 5). Moreover, this experiment also suggested that 5mM LOLA was the candidate concentration to perform all the other experiments reported in the study.

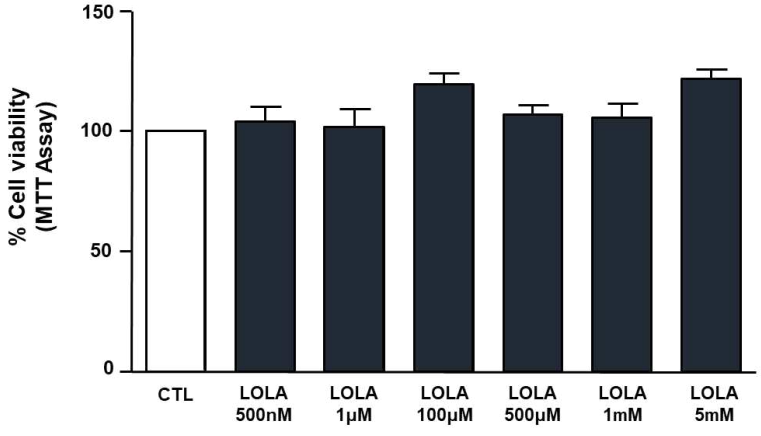


Fig. 5. Dose-response effect of LOLA on mitochondrial redox activity in SH-SY5Y cells: LOLA does not affect mitochondrial redox activity of SH-SY5Y cells. The values for each column represent the mean percentage ± SEM.

Indeed, as described in Figure 6 the co-exposure of cells to LOLA 5mM and 500mM ROT for 24h significantly increased mitochondrial redox activity in SH-SY5Y compared to cells exposed to ROT alone (Fig. 6A). Conversely no effect of LOLA was observed in SH-SY5Y cells co-exposed to 30µM 6-OHDA for 48h compared to SH-SY5Y cells treated with 6-OHDA alone (Fig. 6B).

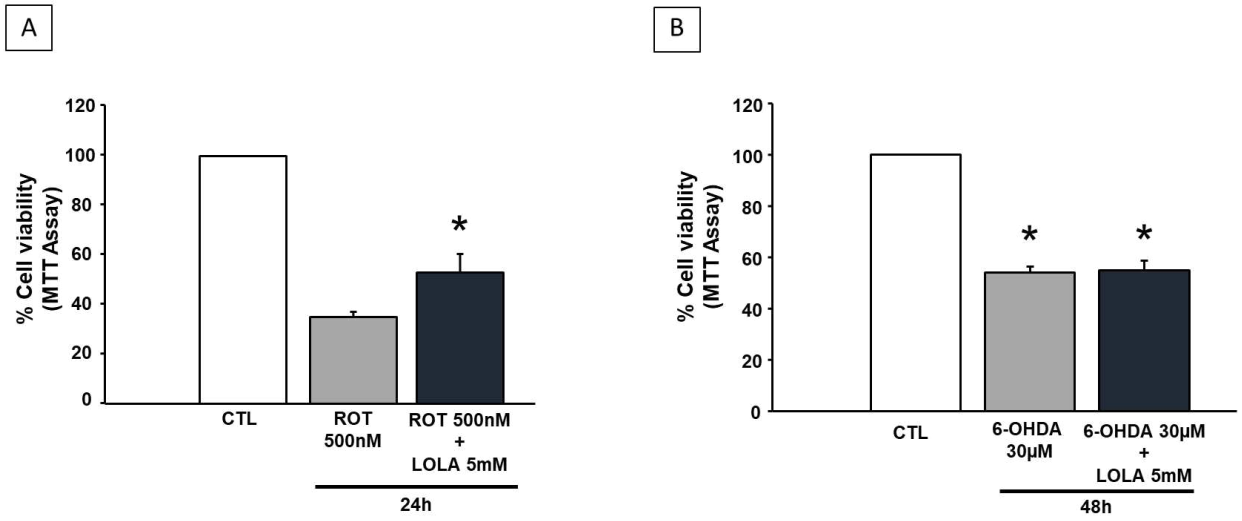


Fig. 6. Effect of LOLA 5mM/24h on mitochondrial redox activity: (A) The co-exposure of cells to LOLA 5mM/24h and ROT 500mM/24h increases mitochondrial redox activity in SH-SY5Y compared to cells exposed to ROT 500mM/24h alone; * p <0.05 vs ROT 500nM. (B) No effect of LOLA is observed in SH-SY5Y cells co-exposed to 6-OHDA 30µM/48h compared to SH-SY5Y cells treated with 6-OHDA 30µM/48h alone; * p <0.05 vs CTL. The values for each column represent the mean percentage ± SEM.

These effects were not related to a difference in the expression of the mitochondrial ornithine transporter 1 (ORNT1) in cells exposed to the two toxins in the presence of LOLA, since no change in ORNT1 protein expression was detected in the two above mentioned experimental conditions (Fig. 7).

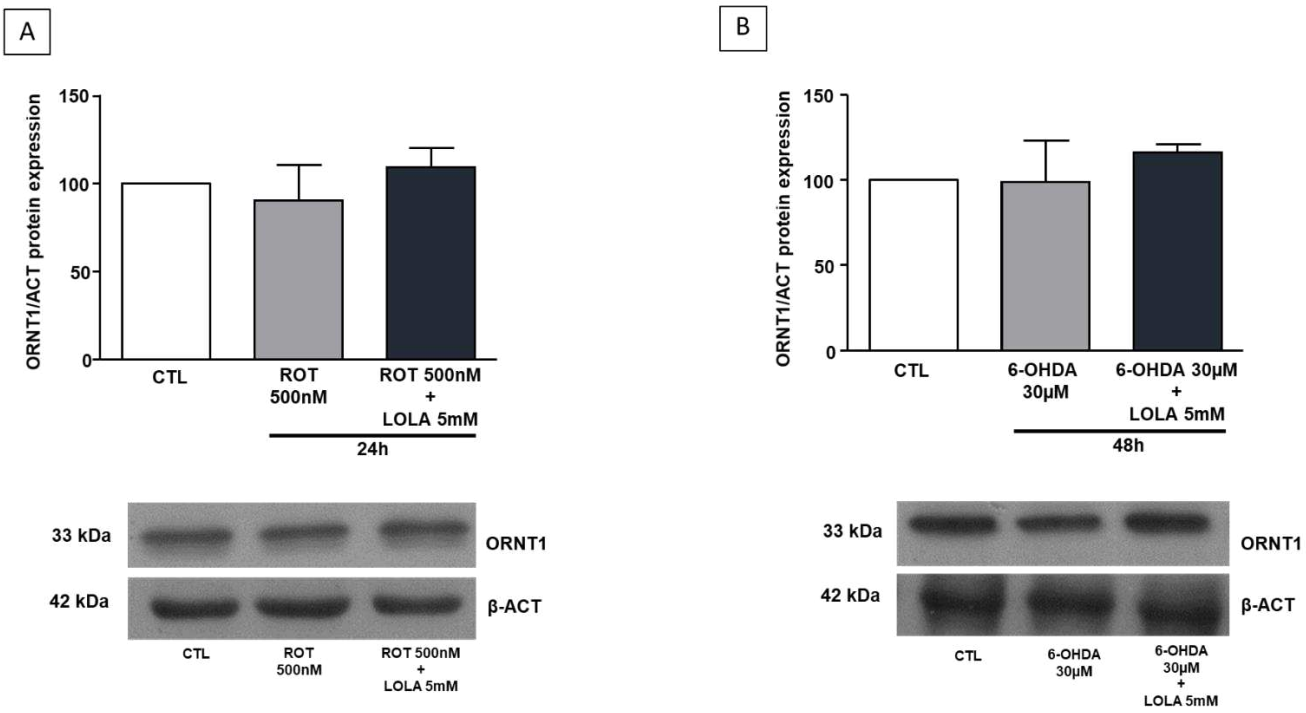


Fig. 7. Ornithine transporter 1 expression after the exposure to ROT 500nM/24h and 6-OHDA 30μM/48h: (A) The expression of the ornithine transporter 1 (ORNT1) is not altered following exposition to ROT 500nM/24h in SH-SY5Y cells. (B) The expression of the ornithine transporter 1 (ORNT1) is not altered following exposure to 6-OHDA 30μM/48h in SH-SY5Y cells. The values for each column represent the mean percentage ± SEM.

In order to go deeper in the comprehension of the intracellular events involved in the effect of LOLA on mitochondria, confocal microscopy experiments were performed to measure mitochondrial membrane potential, calcium content and free radical production in SH-SY5Y exposed to ROT or 6-OHDA in the presence and in the absence of LOLA. As reported in Figure 8 the treatment of the cells with LOLA was able to counteract ROT- and 6-OHDA- induced mitochondrial depolarization in SH-SY5Y cells by promoting a significant hyperpolarization of the mitochondrial membrane potential in both tested experimental conditions (Fig. 8A and 8B).

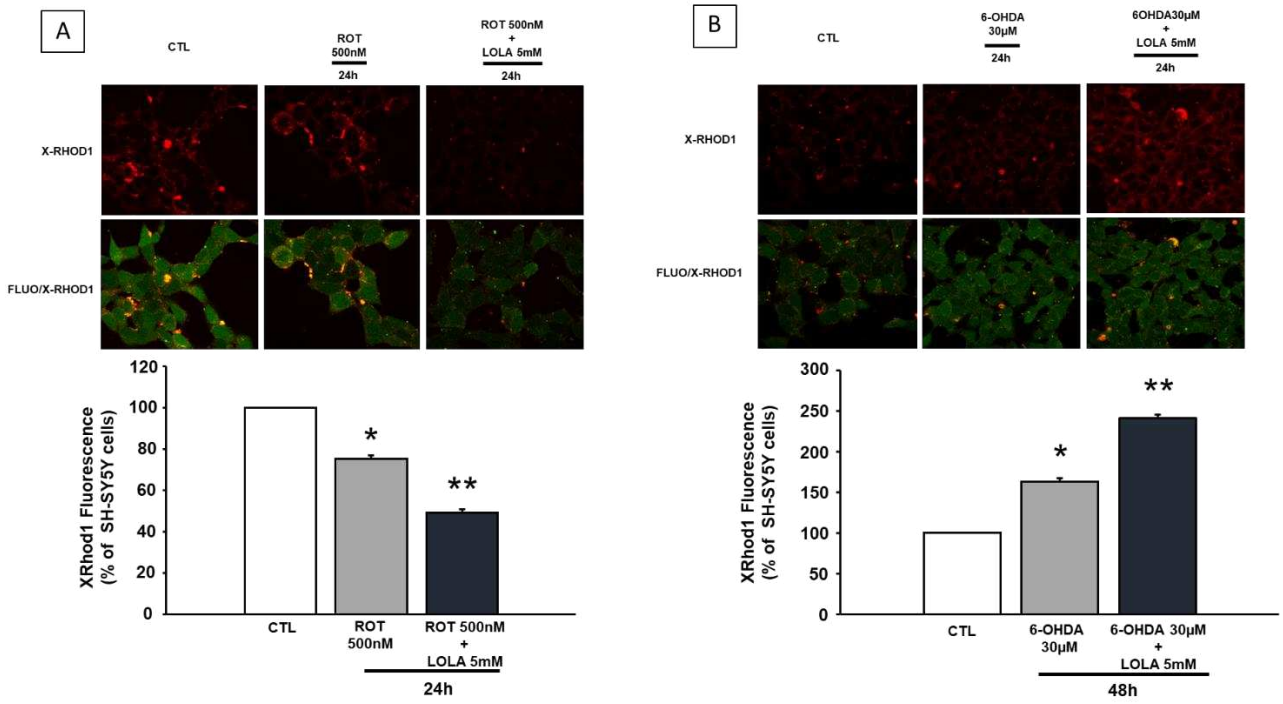


Fig. 8. Mitochondrial Δψ in SH-SY5Y cells exposed to ROT 500nM/24h and 6-OHDA 30µM/48h: (A) LOLA 5mM/24h induces mitochondrial depolarization in SH-SY5Y cells exposed to ROT 500nM/24h; * p <0.05 vs CTL, ** p <0.05 vs ROT. (B) LOLA 5mM/24h induces mitochondrial depolarization in SH-SY5Y cells exposed to 6-OHDA 30µM/48h; * p <0.05 vs CTL, ** p <0.05 vs 6-OHDA. The values for each column represent the mean percentage ± SEM.

However, this effect was associated with a reduction in LOLA-induced mitochondrial calcium levels in SH-SY5Y cells exposed to ROT compared to cells treated with ROT alone (Fig. 9A), and to an increase in LOLA-induced mitochondrial calcium levels in cells exposed to 6-OHDA with respect to 6-OHDA-treated cells (Fig. 9B).

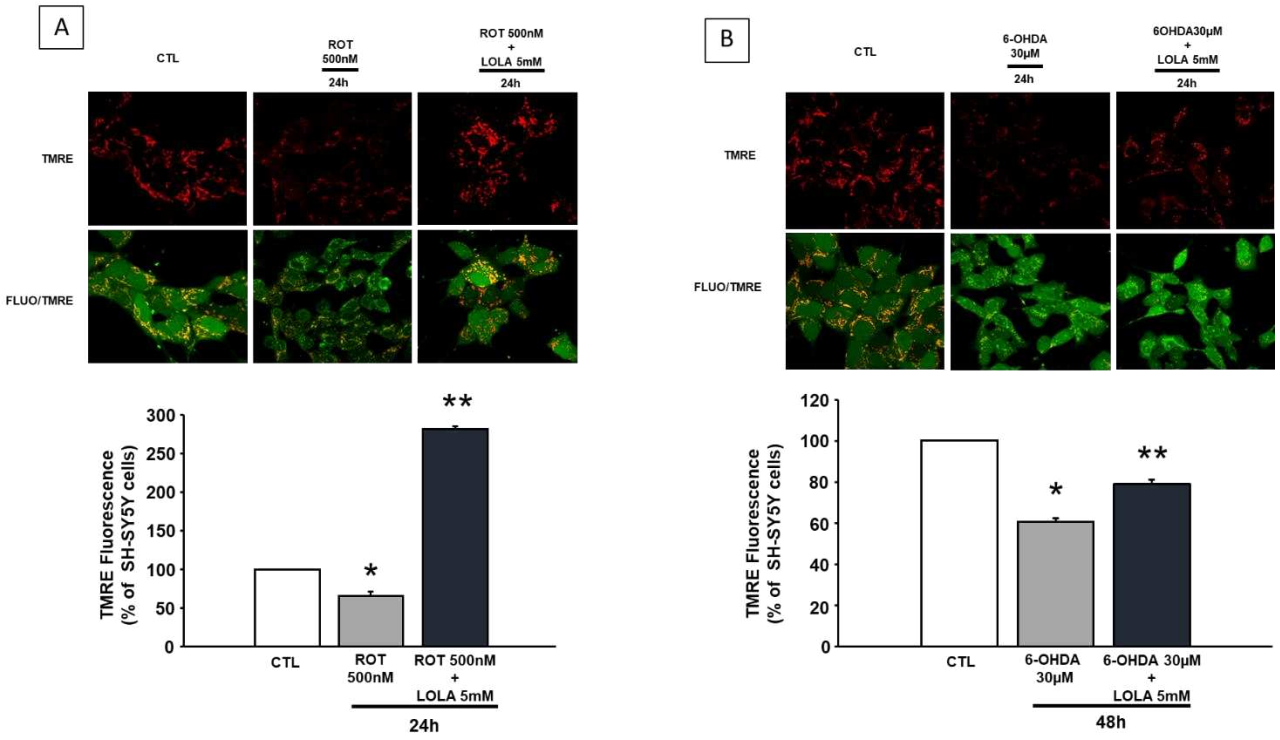


Fig. 9. $[Ca^{2+}]_m$ in SH-SY5Y cells exposed to ROT 500nM/24h and 6-OHDA 30µM/48h: (A) LOLA 5mM/24h reduces mitochondrial calcium levels in SH-SY5Y cells exposed to ROT 500nM/24h; * p <0.05 vs CTL, ** p <0,05 vs ROT. (B) LOLA increases mitochondrial calcium levels in SH-SY5Y cells exposed to 6-OHDA 30µM/48h; * p <0.05 vs CTL, ** p <0,05 vs 6-OHDA. The values for each column represent the mean percentage ± SEM.

Since mitochondrial calcium, is tightly regulated by cytosolic calcium concentration as well as by the mitochondrial membrane potential (Berridge *et al*., 2000; Berridge *et al*., 2003), further experiments were performed in order to evaluate the effect of LOLA on cytosolic calcium concentrations in SH-SY5Y exposed to the treatment with ROT or 6-OHDA in the presence and in the absence of LOLA. The results of these experiments demonstrated that LOLA significantly reduces cytosolic calcium levels in SH-SY5Y cells exposed to ROT, bringing them closer to those observed in control untreated cells (Fig. 10A), whereas it does not affect intracellular calcium concentration in cells exposed to 6-OHDA (Fig. 10B), thus suggesting that LOLA effect on mitochondrial function might be related to its ability to interfere with the intracellular events affecting mitochondrial membrane potential.

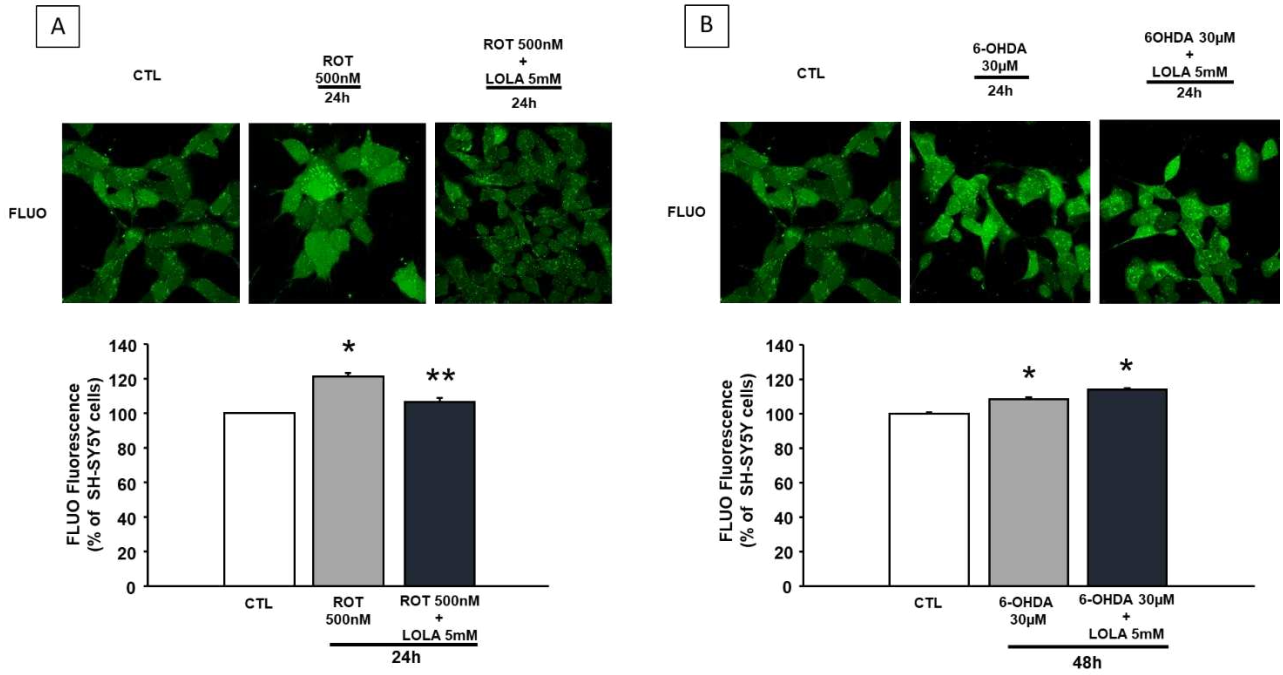


Fig. 10. $[Ca^{2+}]_c$ in SH-SY5Y cells exposed to ROT 500nM/24h and 6-OHDA 30µM/48h: (A) LOLA 5mM/24h reduces cytosolic calcium levels in SH-SY5Y cells exposed to ROT 500nM/24h; * p <0.05 vs CTL; ** p <0.05 vs ROT. (B) No effects are detected in SH-SY5Y cells exposed to 6-OHDA 30µM/48h; * p <0.05 vs CTL. The values for each column represent the mean percentage ± SEM.

This hypothesis was supported by further experiments aimed to evaluate the effect of LOLA on free radical production (Fig. 11). Indeed, in SH-SY5Y exposed to ROT in the presence of LOLA, a reduction in ROS production was observed in comparison to ROS levels produced after ROT treatment (Fig. 11A). Conversely, LOLA treatment was unable to decrease ROS levels in SH-SY5Y cells co-exposed to 6-OHDA and LOLA as reported in Figure 11B.

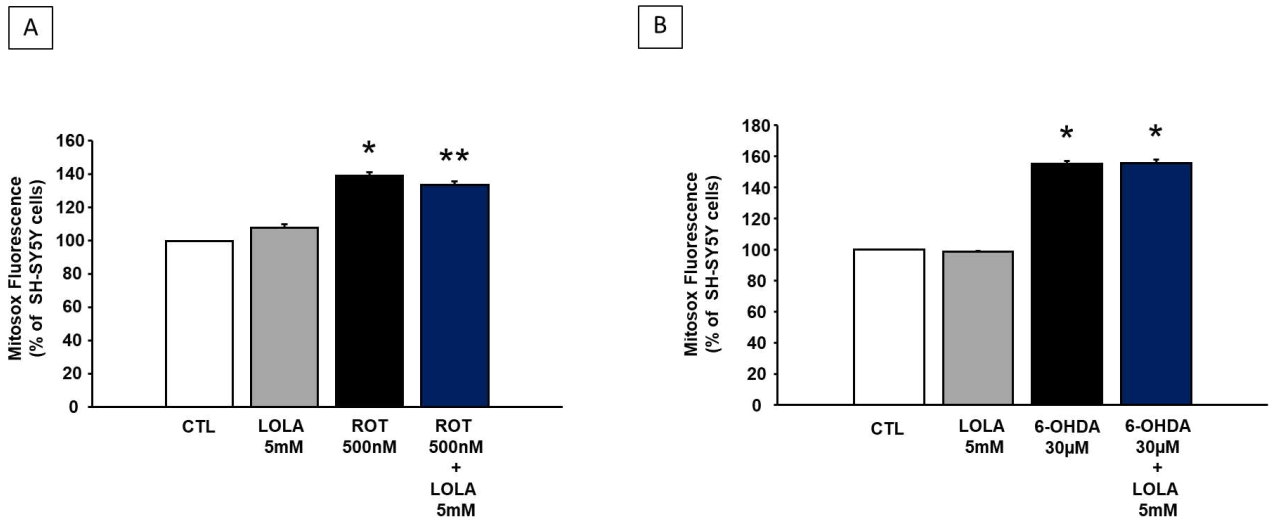


Fig.11. ROS levels in SH-SY5Y cells exposed to ROT 500nM/24h and 6-OHDA 30µM/48h: (A) LOLA 5mM/24h reduces ROS levels in SH-SY5Y cells exposed to ROT 500nM/24h; * p <0.05 vs CTL; ** p <0.05 vs ROT. (B) LOLA does not modulate ROS levels in SH-SY5Y cells exposed to 6-OHDA 30µM/48h; * p <0.05 vs CTL, LOLA. The values for each column represent the mean percentage ± SEM.

This finding was in line with data reported in the literature supporting the hypothesis that ROS production is strictly related to the metabolic activity of mitochondria that, in turn, depends on intracellular calcium concentration due to its ability to activate mitochondrial oxidative metabolism and to promote mitochondrial respiration (Denton, 2009; Denton and McCormack, 1980; McCormack *et al.*, 1990). Therefore, it is possible to speculate that 6-OHDA by promoting intracellular calcium accumulation in SH-SY5Y stimulates mitochondrial calcium increase and ROS production and consequently induces mitochondrial membrane depolarization that is the only effect to be counteracted by LOLA treatment in cells exposed to 6-OHDA. On the contrary, ROT by blocking mitochondrial complex I activity primarily induces a massive mitochondrial membrane depolarization and free radical production both stimulating mitochondrial calcium efflux and increasing cytosolic calcium concentrations, all effects that are prevented by LOLA treatment in cells co-exposed to ROT.

Effect of LOLA treatment on ROT- and 6-OHDA-induced NCXs expression and activity in SH-SY5Y cells

In order to understand the molecular intracellular events involved in the different effect of LOLA treatment on mitochondrial function in cells treated with ROT or 6-OHDA the attention was focused on those proteins responsible for the regulation of intracellular calcium homeostasis and specifically, among them, on the sodium calcium exchangers (NCXs) isoforms 1 and 3 (NCX1 and NCX3), since previously data reported in the literature demonstrated their role in the regulation of calcium concentration in cytosol and in the mitochondria respectively (Secondo *et al*. 2007; Scorziello *et al*., 2013). Therefore, western blot experiments were performed to evaluate the expression of NCX1 and NCX3 in SH-SY5Y exposed to the treatment with ROT or 6-OHDA in the presence and in the absence of LOLA. As reported in Figure 12A, ROT induced an increase in NCX1 protein expression that was not affected by LOLA, whereas 6-OHDA did not affect NCX1 protein expression both when it was administered alone and when it was administered in the presence of LOLA (Fig. 12B).

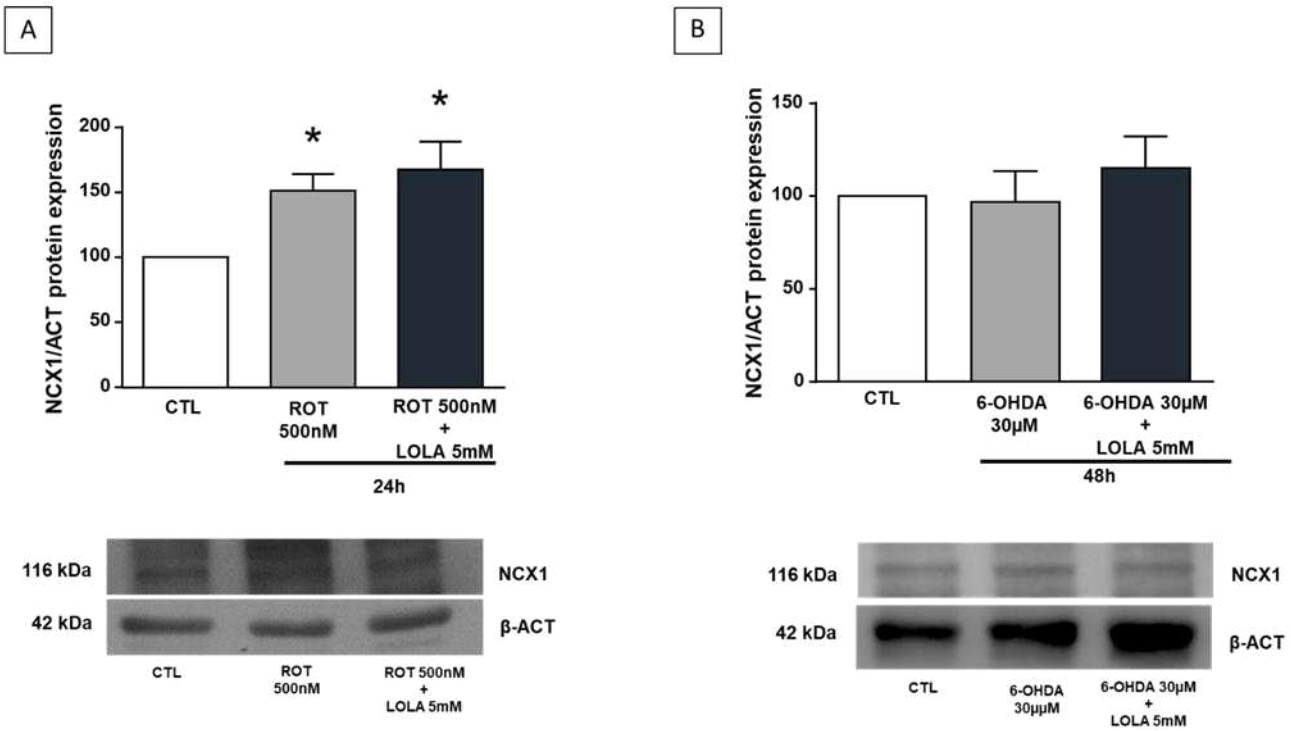


Fig. 12. NCX1 expression after the exposure to ROT 500nM/24h and 6-OHDA 30μM/48h: (A) ROT 500nM/24h induces an increase in NCX1 protein expression in SH-SY5Y cells; * $p < 0.05$ vs CTL. (B) No differences are detected in SH-SY5Y cells exposed to 6-OHDA 30μM/48h. The values for each column represent the mean percentage ± SEM.

On the contrary, both ROT and 6-OHDA alone stimulated NCX3 protein expression levels in SH-SY5Y treated cells, and the co-treatment with LOLA was able to counteract the effect of 6-OHDA on NCX3 expression without affecting ROT-induced NCX3 increased expression (Fig. 13).

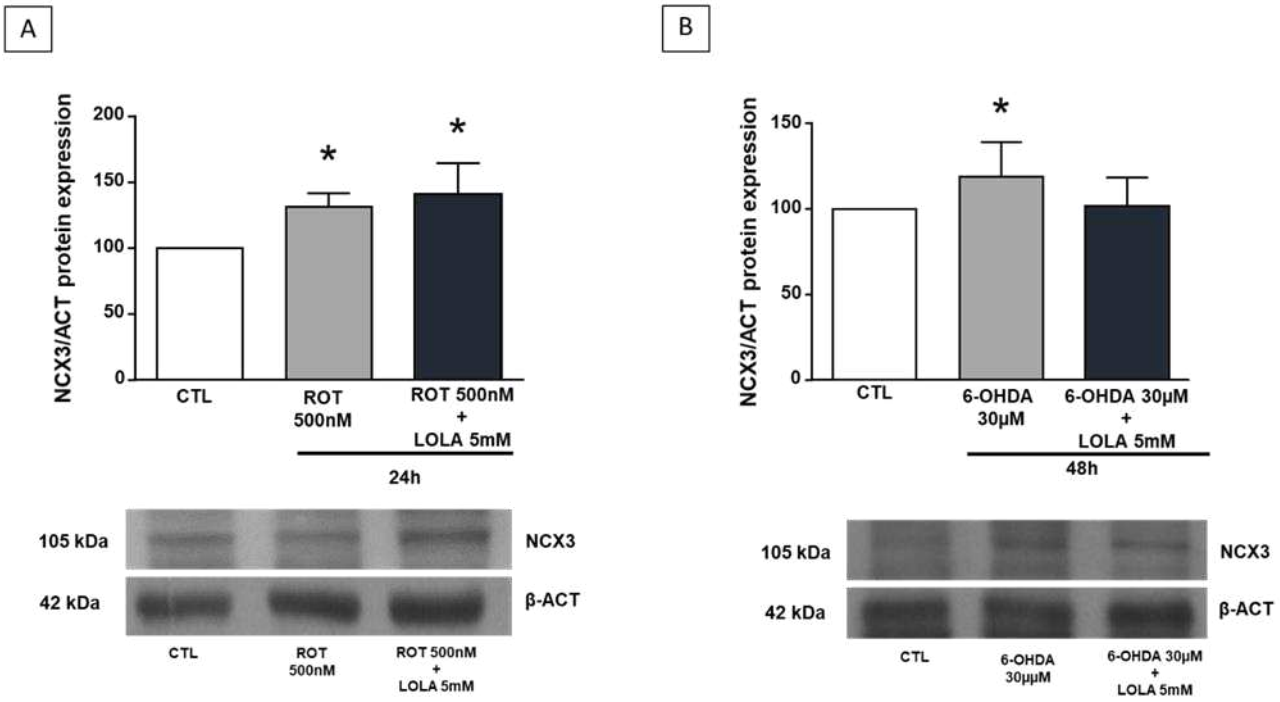


Fig. 13. NCX3 expression after the exposure to ROT 500nM/24h and 6-OHDA 30μM/48h: (A) ROT 500nM/24h induces an increase in NCX3 protein expression in SH-SY5Y cells; * $p < 0.05$ vs CTL. (B) LOLA is able to counteract the 6-OHDA-induced NCX3 increased expression; * $p < 0.05$ vs CTL. The values for each column represent the mean percentage ± SEM.

In order to clarify the effects of LOLA on intracellular calcium homeostasis in cells exposed to ROT and 6-OHDA treatments further experiments were performed to investigate the effects of the above indicated treatments on the activity of NCXs. These experiments were performed by using Fura 2 and a microfluorimetric approach by exposing the cells to a Na^+ free medium, an experimental condition able to activate the reverse mode of NCX (Secondo *et al.*, 2007). The results of these experiments demonstrated that LOLA alone was able to potentiate the reverse mode of operation of NCX as well as ROT and 6-OHDA, as indicated in Figure 14. However, when the cells were co-exposed to LOLA and ROT or 6-OHDA, the activity of NCX was reduced in cells treated with ROT whereas, it was potentiated in cells treated with 6-OHDA (Fig. 14B) thus confirming that LOLA treatment affected

mitochondrial function with a mechanism depending on the type of toxin used to induce mitochondrial injury.

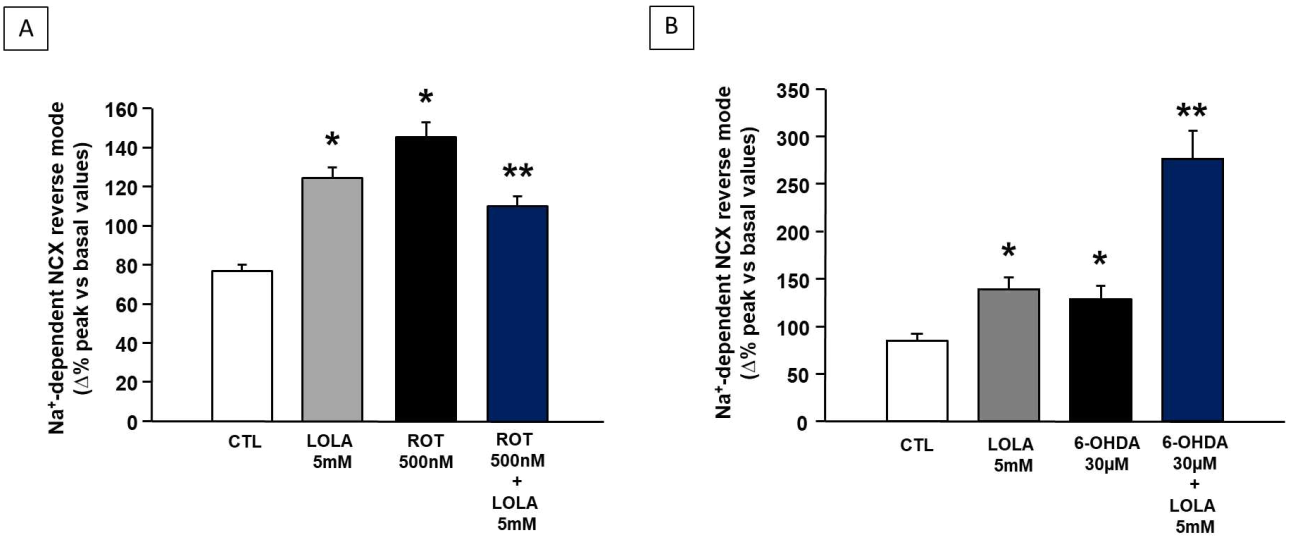


Fig. 14. Effect of LOLA 5mM/24h on NCX activity: (A) LOLA 5mM/24h increases the reverse mode of operation of the sodium calcium exchanger, whereas in combination with ROT reduces the Na^+-dependent activity of the protein in SH-SY5Y; * p <0.05 vs CTL, ** p <0.05 vs ROT. (B) LOLA 5mM/24h significantly increases the exchanger activity both alone and in combination with 6-OHDA; * p <0.05 vs CTL, ** p <0.05 vs 6-OHDA. The values for each column represent the mean percentage ± SEM.

This hypothesis might be in line with the effect of ornithine on ROS production as well as on its mechanism of action at mitochondrial level, since it has been reported that ornithine is able to decrease ROS levels and to increase the production of nitric oxide (NO) through the inhibition of mitochondrial arginase (El-Bassossy *et al*., 2013). Because it has also reported that both ROS and NO are able to modulate NCX activity (Annunziato *et al*., 2004), the hypothesis that the effect of LOLA on NCX activation in the experimental conditions tested in the present study might be related to its ability to regulate the level of mitochondrial ROS and RNS, thus modulating mitochondrial function, was also explored in cells co-treated with LOLA and ROT or 6-OHDA. In these experimental conditions, western blot experiments were firstly performed to measure nNOS expression levels in SH-SY5Y cells. As reported in Figure 15, neither ROT nor 6-OHDA were able to improve nNOS protein expression both alone and in presence of LOLA compared to control.

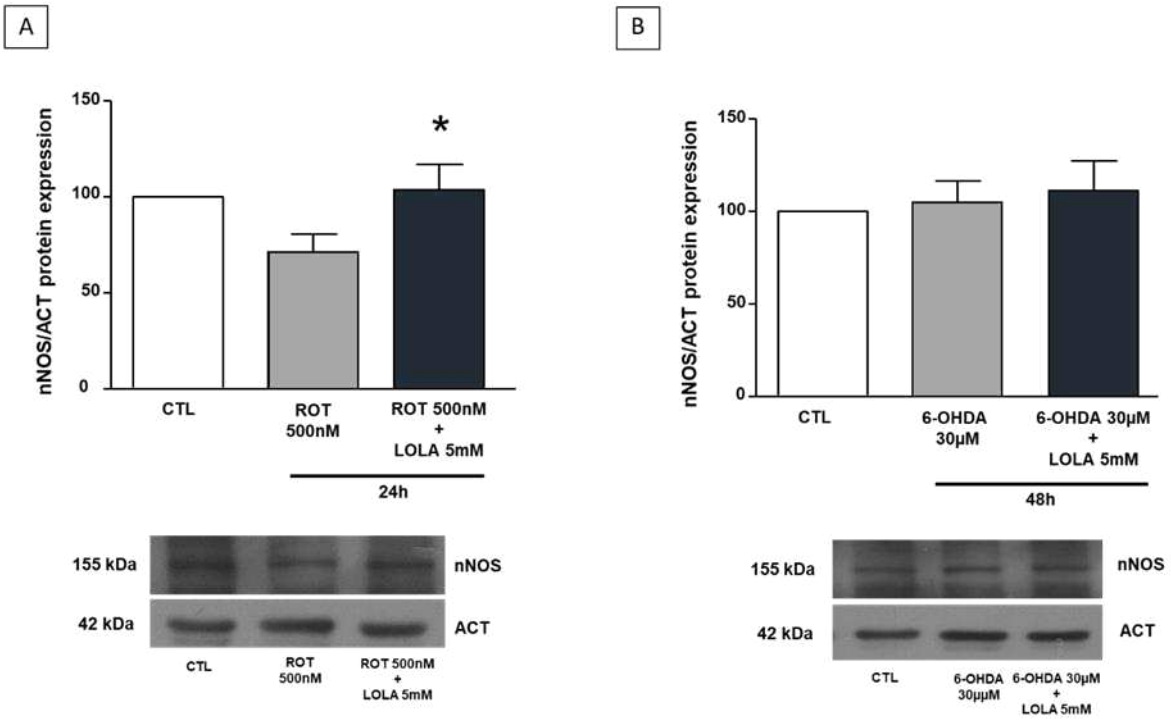


Fig. 15. nNOS expression after the exposure to ROT 500nM/24h and 6-OHDA 30µM/48h: (A) ROT 500nM/24h does not improve nNOS expression both alone and in presence of LOLA 5mM/24h in SH-SY5Y cells compared to control; * p <0.05 vs ROT. (B) 6-OHDA 30µM/48h does not improve nNOS expression both alone and in presence of LOLA 5mM/24h in SH-SY5Y cells**.** The values for each column represent the mean percentage ± SEM.

However, either ROT or 6-OHDA were able to increase NO production as demonstrated by microfluorimetry experiments performed in SH-SY5Y cells loaded with fluorescent dye DAF that selectively reveal the amount of NO produced by the single cell (Fig. 16). Moreover, this effect was more pronounced in cells exposed to ROT than to 6-OHDA. Interestingly, the co-treatment with LOLA in ROT- or 6-OHDA-exposed cells was able to reduce NO production in both conditions as compared to the effect induced by the single toxin alone. However, the amount of NO produced in SH-SY5Y cells exposed to ROT in the presence of LOLA was still elevated compared to that observed in control untreated or LOLA-exposed cells (Fig. 16A). Conversely, in cells treated with LOLA and 6-OHDA the amount of NO produced was similar to that observed in control and in LOLA treated cells (Fig. 16B).

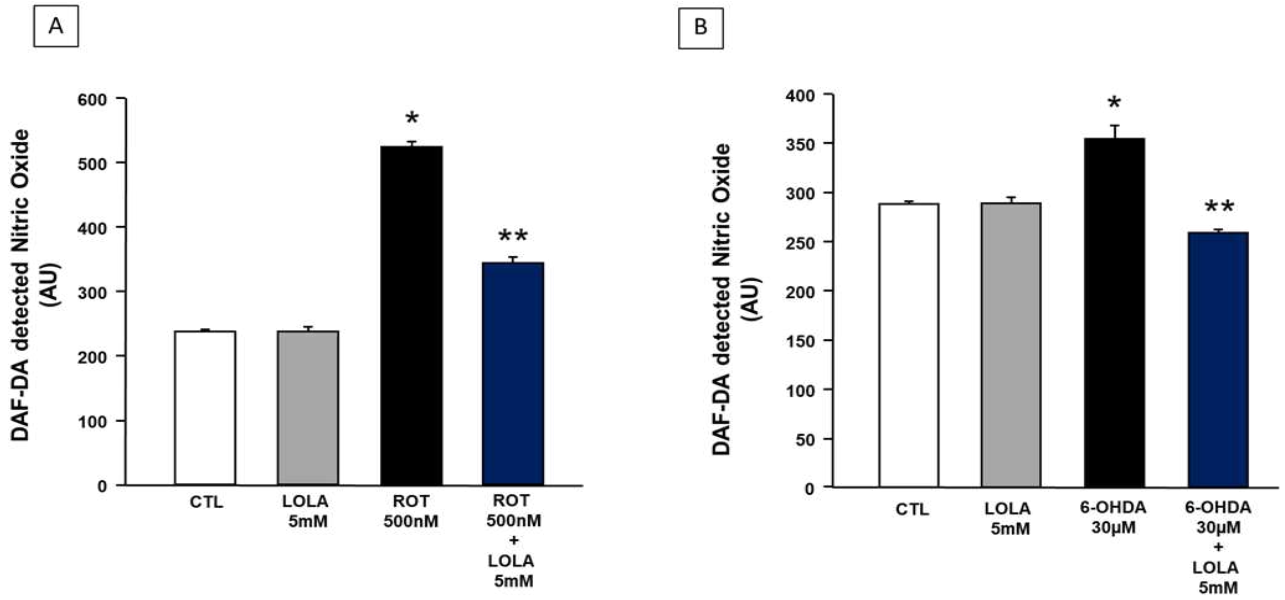


Fig. 16. Effect of LOLA 5mM/24h on NO production: (A) LOLA 5mM/24h reduces NO production in SH-SY5Y cells exposed to ROT 500nM/24h; * p <0.05 vs CTL; ** p <0.05 vs ROT. (B) LOLA 5mM/24h reduces NO production in SH-SY5Y cells exposed to 6-OHDA 30µM/48h; * p <0.05 vs CTL; ** p <0.05 vs 6-OHDA. The values for each column represent the mean percentage ± SEM.

These results allowed to confirm the hypothesis that LOLA might exert its effects on mitochondria by interfering with those mitochondrial mechanisms related to ROS and RNS production thus favoring mitochondrial functional recovery. However, in cells treated with ROT, the increased ROS production was accompanied to an increase in intracellular calcium concentration probably due to the stimulation of ROS-sensitive NCX1 activity in the reverse mode of operation. These effects, associated with the block of mitochondrial complex I caused by ROT, contributed to mitochondrial membrane depolarization and consequently mitochondrial dysfunction. In these conditions the treatment with LOLA, by reducing ROS and promoting RNS production was able to improve mitochondrial function by stimulating mitochondrial calcium efflux trough NCX3 activation. Conversely, in 6-OHDA-treated SH-SY5Y cells the stimulation of NCX1 activity, by promoting mitochondrial metabolic activation, induced a massive ROS production that was not counteracted by LOLA treatment. However, the reduction of RNS production observed in SH-SY5Y cells co-treated with LOLA and 6-OHDA might represent the mechanism by which LOLA, without affecting

mitochondrial calcium efflux mechanisms, might prevent mitochondria depolarization and, in turn, mitochondrial induced 6-OHDA dysfunction.

In vivo experiments

Parallel in vivo experiments have been performed in mice expressing the mutation A53T of human α-synuclein (A53T-α-syn), a model of familial form of PD, mainly utilized to investigate *in vivo* the pathogenetic mechanisms involved in dopaminergic degeneration during aging and, largely characterized in the Lab in which I performed my PhD program. The *in vivo* experiments have also been undertaken in order to identify the experimental condition more appropriate to explore the potential effect of LOLA on the phenotypic manifestation associated with the progression of dopaminergic neuronal degeneration. To this aim behavioural and biochemical experiments have been performed in 4- and 12-month-old WT and A53T-α-syn-trensgenic-mice.

Behavioural and biochemical analysis in mice bearing the human mutation A53T of α-synuclein during aging

Previous experiments performed in A53T-α-syn-transgenic-mice demonstrated a progressive accumulation of α-synuclein in different brain areas included midbrain and striatum (Gaisson *et al*. 2002; Sirabella *et al*., 2018) accompanied to a reduction in TH expression in those regions occurring already in 4-month-old transgenic mice compared to WT. Interestingly, mitochondrial dysfunctions in nigrostriatal dopaminergic neurons has been also described in these mice during aging (Di Martino *et al*., 2021; Sirabella *et al*., 2018). On these basis, further western blot experiments were performed in midbrain and striatum of 4- and 12-month-old WT and A53T-α-syn-transgenic-mice in order to confirm the alteration of dopaminergic pathway, by measuring the expression of proteins known to be marker of synaptic dysfunction. In particular the attention was focused on synapsin 3 (SYN3), a synaptic protein which play a key role in the dopamine release (Faustini *et al.,* 2018) and that is able to interact with α-synuclein and dopamine transporter (DAT), since α-synuclein directly interacts with the DAT and this interaction modulates the transporter activity by decreasing its expression in

the DA-neuronal membrane (Longhena *et al.,* 2019). These experiments allowed to identify the early stage of the dopaminergic dysfunction and might represent the starting point to verify whether LOLA treatment, by affecting neuronal metabolic function, could slow down the progression of dopaminergic damage and consequently, the related phenotypic disease manifestations.

As reported in Figure 17, western blot experiments performed in midbrain and striatum of 4- and 12-month-old WT and A53T-α-syn transgenic mice showed that SYN3 expression increased in 4-month-old A53T-α-syn transgenic mice compared to wild type, and that this effect was more pronounced in the midbrain than in the striatum (Fig. 17A).

Interestingly, SYN3 expression decreased significantly in 12-month-old A53T-α-syn transgenic mice compared to wild type, both in midbrain and striatum (Fig. 17B).

However, when compared each other 4- and 12-month-old A53T-α-syn mice, a significant reduction in SYN3 expression was observed both in midbrain and striatum of 12-month-old A53T-α-syn mice compared to 4-month-old transgenic mice, thus confirming that alteration of neurotransmission occurred in A53T mice during aging (Fig. 17C).

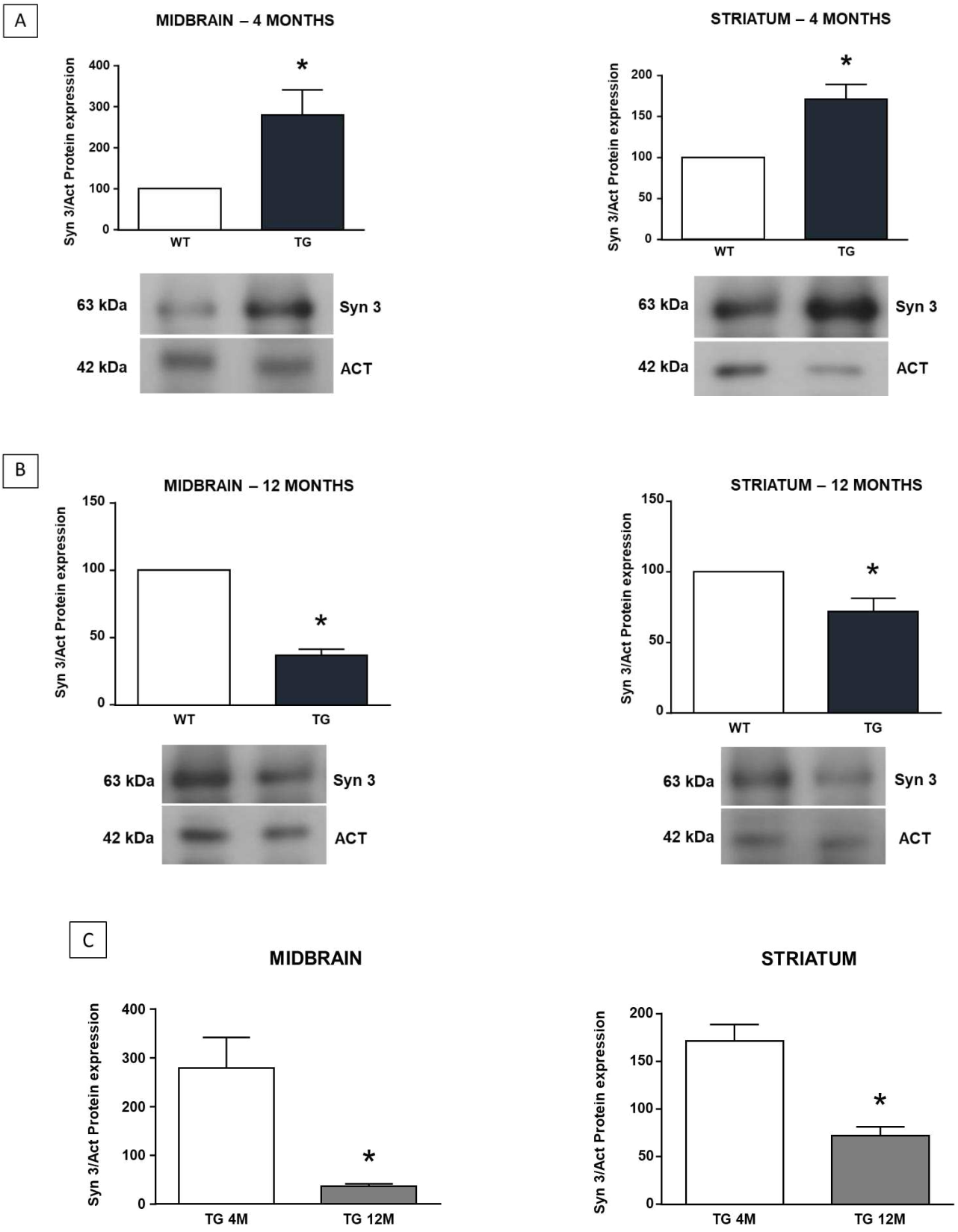
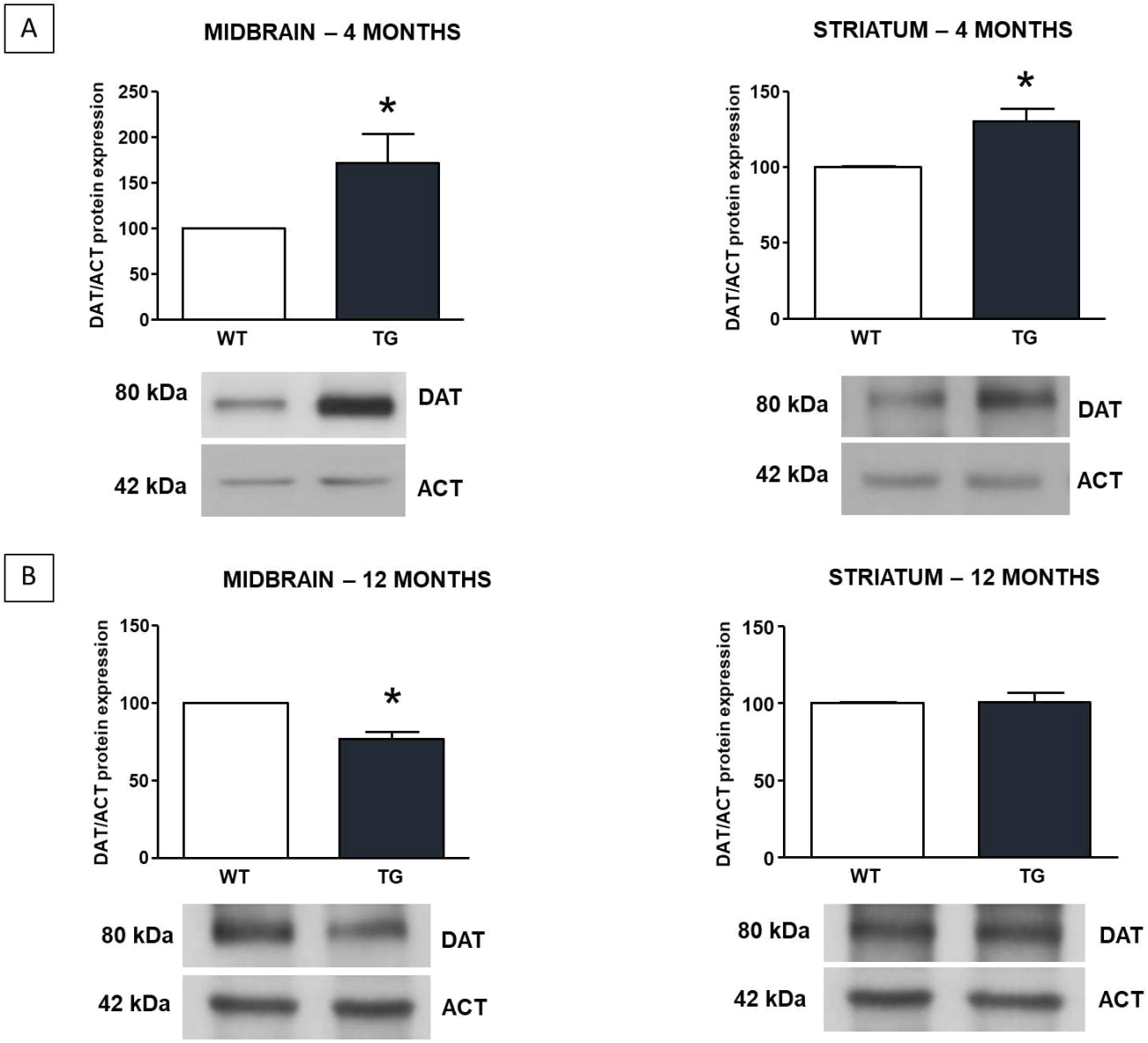


Fig. 17. Synapsin 3 expression in 4- and 12-month-old transgenic mice both in midbrain and striatum: (A) Synapsin 3 expression increases in 4-month-old transgenic mice compared to wild type both in the midbrain and striatum; * $p < 0.05$. (B) Synapsin 3 expression decreases in 12-month-old transgenic mice compared to wild type both in the midbrain and striatum; * $p < 0.05$. (C) Synapsin 3 expression decreases in 12-month-old transgenic mice compared to young mice both in the midbrain and striatum; * $p < 0.05$. The values for each column represent the mean percentage ± SEM.

Similar results were obtained by measuring DAT protein expression. Indeed, an increase in DAT transporter expression was detected in 4-month-old A53T-α-syn transgenic mice compared to WT both in the midbrain and striatum (Fig. 18A) although more evident in the midbrain, whereas a reduction in DAT expression occurred only in the midbrain of 12-month-old A53T-α-syn transgenic mice (Fig. 18B). Once again, when compared each other 4- and 12-month-old A53T-α-syn transgenic mice, a significant reduction in DAT expression was revealed in midbrain (more pronounced) and striatum of older mice (Fig. 18C).



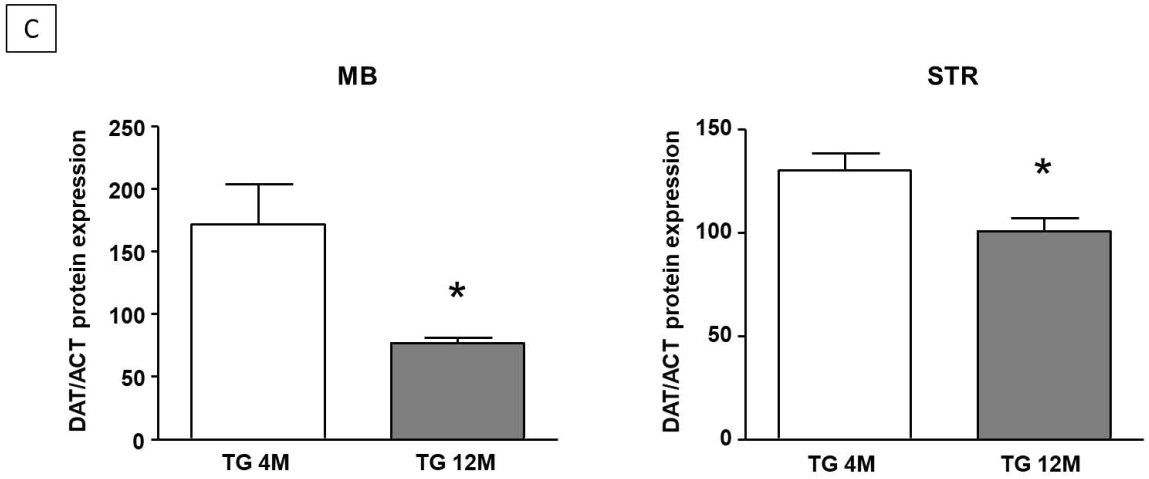


Fig. 18. DAT expression in 4- and 12-month-old transgenic mice both in midbrain and striatum: (A) DAT expression increases in 4-month-old transgenic mice compared to wild type both in the midbrain and striatum; * $p < 0.05$ (B) DAT expression increases in 12-month-old transgenic mice compared to wild type only in the midbrain; * $p < 0.05$. (C) DAT expression decreases in 12-month-old transgenic mice compared to young mice both in the midbrain and striatum; * $p < 0.05$. The values for each column represent the mean percentage ± SEM.

Collectively, these results demonstrated that: (1) a dysfunction in synaptic activity was detectable in transgenic mice compared to WT, (2) this dysfunction was more evident in the midbrain than in the striatum, and more interestingly, (3) this dysfunction was associated with an increase in SYN3 and DAT protein levels in 4-month-old A53T-α-syn transgenic mice, and to a decrease in the above mentioned proteins expression in 12-month-old A53T-α-syn transgenic mice, thus confirming that a progression in synaptic dysfunction might be detected during the disease progression. In order to verify whether these alterations in DA-synaptic activity might be related to the phenotypic manifestations of PD, behavioral experiments were performed to evaluate motor and non-motor symptoms in 4- and 12-month-old WT and A53T-α-syn transgenic mice. To this aim Pole test, Open field test, and Rotarod test, were used to evaluate the alterations in motor symptoms, whereas Olfactory test and One-hour stool collection test were performed to evaluate the presence of peripheral deficits related to olfactory or intestinal dysfunctions.

The results reported in Figure 19 revealed that 4-month-old A53T-α-syn transgenic mice showed a greater spontaneous exploratory locomotor activity, measured by Open Field test, compared to 12-

month-old A53T-α-syn transgenic and to 4-month-old WT mice as confirmed by the greater distance travelled (Fig. 19).

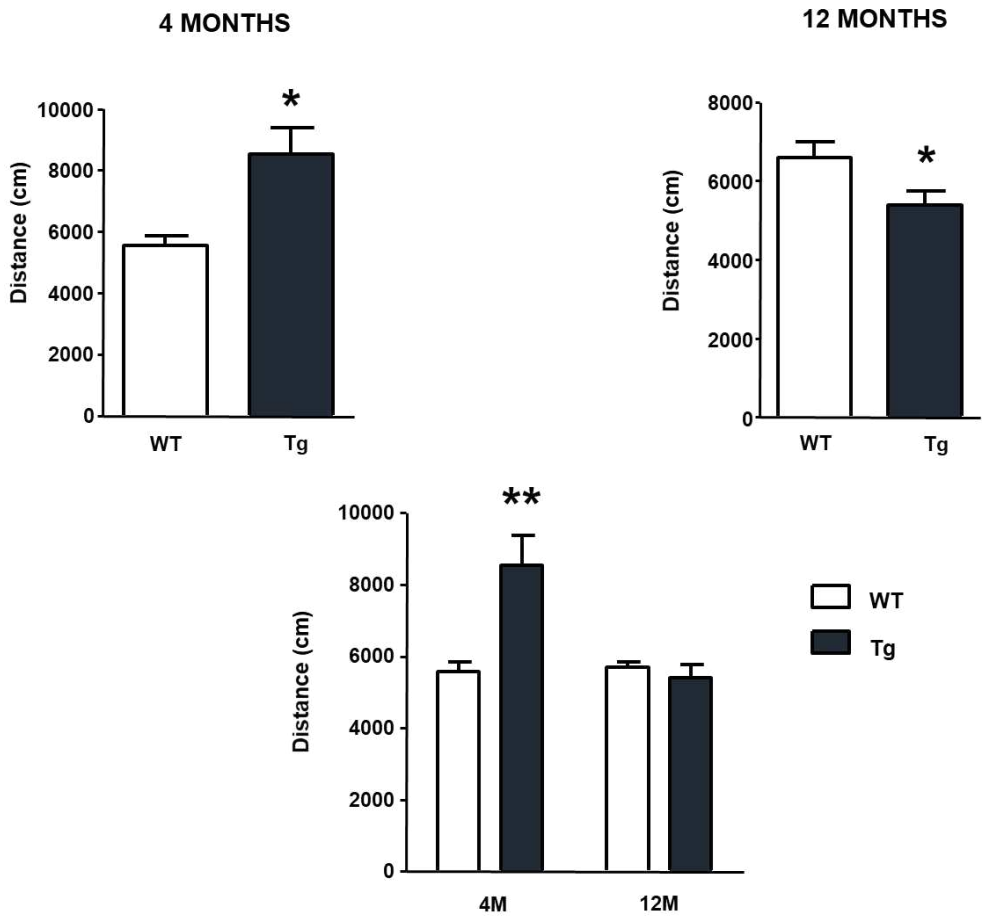


Fig. 19. Locomotor activity in 4- and 12-month-old A53T mice: young transgenic mice travel greater distance compared to 4-month-old WT mice and 12-month-old A53T mice. * p <0.05 vs WT, ** p<0,05 vs 12M Tg. The values for each column represent the mean percentage ± SEM.

Similarly, 4-month-old A53T-α-syn transgenic mice displayed a better balance and motor coordination compared to WT during the Rotarod test since they spent more time on the wheel, whereas no differences were observed between 12-months-old Tg and WT mice (Fig. 20).

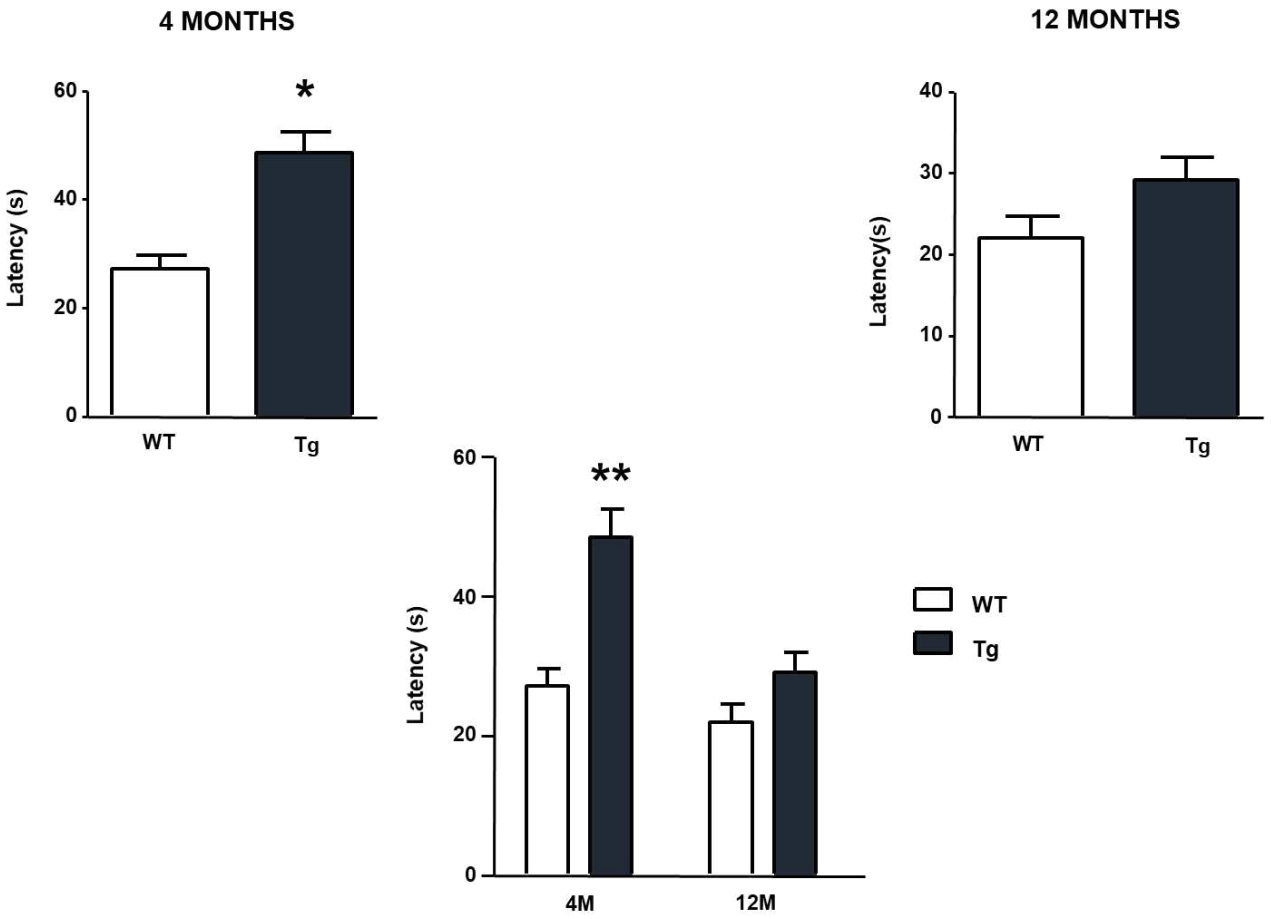


Fig. 20. Balance and motor coordination in 4- and 12-month-old A53T mice: 4-month-old A53T-α-syn transgenic mice display a better balance and motor coordination compared to young WT mice and to 12-month-old A53T mice; * p <0.05 vs WT, ** p<0,05 vs 12M Tg. The values for each column represent the mean percentage ± SEM.

Interestingly, 4- and 12-month-old A53T-α-syn transgenic mice exhibited a progressive decrease of imbalance and bradykinesia, measured by Pole test, since they spent more time climbing down the pole compared to WT (Fig. 21).

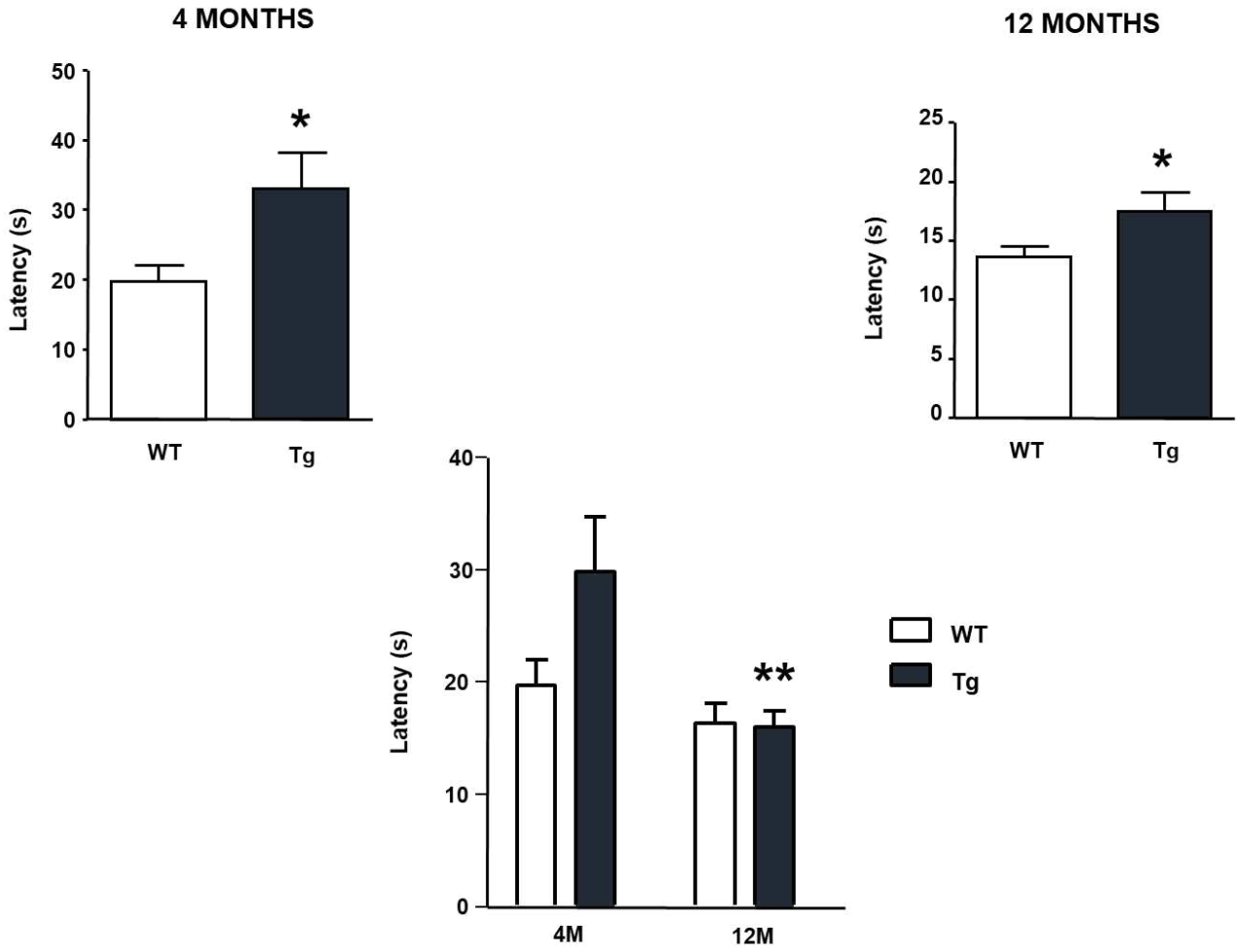


Fig. 21. Fine motor ability in 4- and 12-month-old A53T mice: 4- and 12-month-old A53T-α-syn transgenic mice exhibited a progressive decrease of imbalance and bradykinesia; * p <0.05 vs WT, ** p<0,05 vs 4M Tg. The values for each column represent the mean percentage ± SEM.

All together, these data confirmed that 4-month-old A53T-α-syn transgenic mice displayed an anxiety-like and hyperactive behavior compared to older and WT mice.

Regarding the non-motor symptoms, the One-hour stool collection test indicated that 12-month-old A53T-α-syn transgenic mice displayed a lower dry stool weight and a lower content of stool water compared to WT and to 4-month-old A53T-α-syn transgenic mice (Fig. 22).

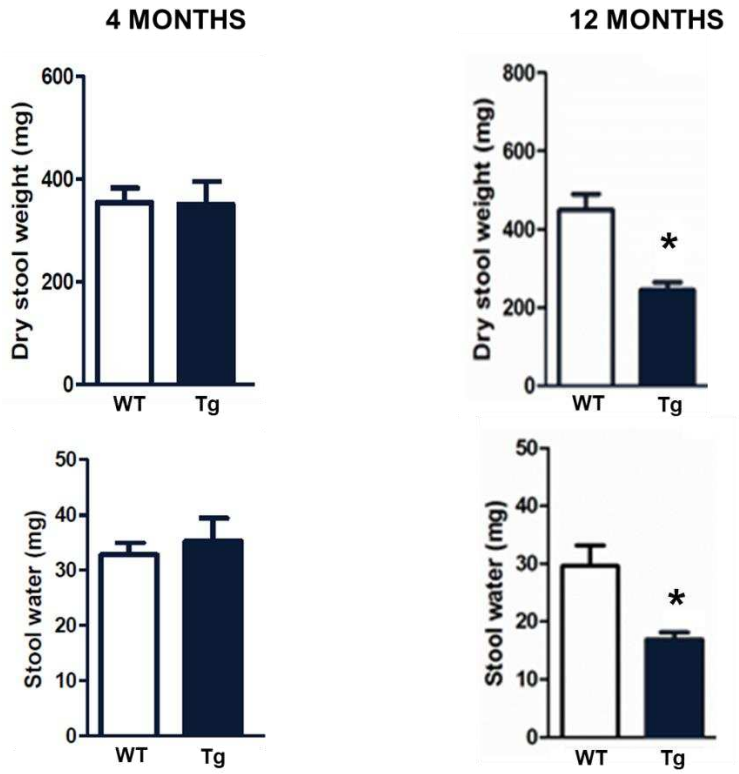


Fig. 22. Constipation in 4- and 12-month-old A53T mice: 12-month-old transgenic mice displayed a lower dry stool weight and a lower content of stool water compared to 12-month-old WT mice and to 4-month-old transgenic mice. The values for each column represent the mean percentage ± SEM. * p <0.05 vs WT.

Conversely, the Olfactory test revealed an impairment of olfaction during aging, already at 4 months of age (Fig. 23).

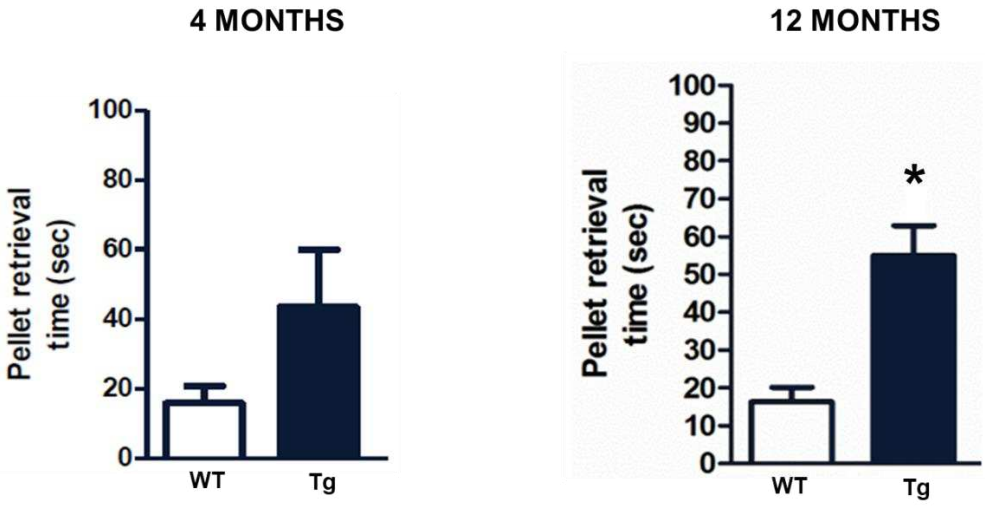


Fig. 23. Impaired olfaction in 4- and 12-month-old A53T mice: 4- and 12-month-old transgenic mice displayed an impairment of olfaction compared to WT. The values for each column represent the mean percentage ± SEM. * p <0.05 vs WT.

8. *DISCUSSION*

The results of the present study demonstrate that LOLA treatment is able to prevent mitochondrial dysfunction and cell survival induced by ROT and 6-OHDA exposure, two experimental conditions mimicking in vitro the pathogenetic mechanisms leading to PD. Interestingly, these data also suggest that LOLA ability in protecting damaged mitochondria seems to be related to the severity of the injuring insult, since it appears able to preserve mitochondrial function with a mechanism that is different for the two experimental conditions explored. Indeed, although ROT- or 6-OHDA-induced mitochondrial membrane depolarization is counteracted by LOLA co-exposure, however, LOLA effect on both mitochondrial calcium content and ROS production is different in SH-SY5Y cells treated with ROT or 6-OHDA respectively. LOLA induces a reduction of mitochondrial calcium content in cells exposed to ROT and an increase of mitochondrial calcium levels in cells exposed to 6-OHDA. Moreover, LOLA can reduce ROS production in cells treated with ROT, whereas it is unable to decrease ROS levels in SH-SY5Y cells co-exposed to 6-OHDA. These findings are in line with data reported in the literature supporting the hypothesis that ROS production is strictly related to the metabolic activity of mitochondria that, in turn, depends on intracellular calcium concentration due to its ability to activate mitochondrial oxidative metabolism and to promote mitochondrial respiration (Denton, 2009; Denton and McCormack, 1980; McCormack *et al*., 1990). On the other hand, this discrepancy in the effects of LOLA on ROT- or 6-OHDA- injured mitochondria might be explained considering the effects that LOLA plays on cytosolic calcium concentrations in cells treated with the two different toxins. Indeed, LOLA co-exposure significantly reduced cytosolic calcium levels in SH-SY5Y cells treated with ROT, bringing them closer to the levels observed in control untreated cells, whereas it does not affect intracellular calcium concentration in cells treated with 6-OHDA. Because previously data reported in the literature demonstrated a tight relationship between calcium concentration in cytosol and in mitochondria respectively (Berridge *et al*., 2000; 2003), and considering that the sodium calcium isoforms NCX1 and NCX3 are among the cellular players

involved in the regulation of this phenomenon (Scorziello *et al*., 2013, Secondo *et al*., 2007; Sirabella *et al*., 2009), it is possible to speculate that LOLA may interfere with the molecular events controlling the expression and the activity of these two exchangers. This hypothesis is further supported by the finding that NCX3, apart its expression at plasma membrane level, is also detectable on the outer mitochondrial level, where it plays a role in the regulation of mitochondrial calcium efflux in physiological and in pathological conditions like ischemia and PD (Scorziello *et al*., 2013; Sisalli *et al.* 2020; Sirabella *et al*., 2018; Di Martino *et al*., 2021), and that NCX1 activation represents a neuroprotective mechanism in ischemic conditions (Pignataro *et al*., 2004; Sisalli *et al*., 2014). Moreover, NCX1 and NCX3 have been considered as contributing factors to neurodegeneration and neuroinflammation in *in vivo* model of PD (Sirabella *et al*., 2018; Di Martino *et al.,* 2021). The results obtained in the present study demonstrate that an increase in NCX1 and NCX3 expression is detectable in cells treated with ROT, whereas in cells exposed to 6-OHDA a rise in the expression of NCX3 occurs without any change in NCX1 expression. Moreover, both ROT and 6-OHDA are able to improve the reverse mode of operation of sodium calcium exchanger thus contributing to the increase in cytosolic calcium concentration in both experimental conditions. Interestingly, the co-exposure of cells to LOLA can prevent the effects of ROT on NCX3 expression without affecting 6-OHDA-induced effects on NCX1 and NCX3 expression. On a functional point of view, it is important to underline that the experiments performed in this study demonstrate that LOLA alone is able to potentiate the reverse mode of operation of the exchanger like it occurs in cells treated with ROT and 6-OHDA alone. Interestingly, when the cells are co-exposed to LOLA and ROT or 6-OHDA, the activity of the exchanger is reduced in cells treated with ROT whereas, it is potentiated in cells treated with 6-OHDA. Because it is reported that both ROS and NO can modulate NCX activity (Annunziato *et al*., 2004; Sisalli *et al*., 2014), these effects may be related to the ability of LOLA to regulate the level of mitochondrial ROS and RNS, thus modulating mitochondrial function. This hypothesis is supported by the finding that in cells treated with ROT, the increased ROS production was accompanied to an increase in intracellular calcium concentration probably due to the stimulation of

ROS-sensitive NCX1 activity in the reverse mode of operation. These effects, associated with the block of mitochondrial complex I caused by ROT, contribute to mitochondrial membrane depolarization and consequently to mitochondrial dysfunction. In these conditions the treatment with LOLA, by reducing ROS and promoting RNS production can improve mitochondrial function by stimulating mitochondrial calcium efflux trough NCX3 activation. Conversely, in 6-OHDA-treated SH-SY5Y cells the stimulation of NCX1 activity, by promoting mitochondrial metabolic activation, induces a massive ROS production that is not counteracted by LOLA treatment. However, the reduction of RNS production observed in SH-SY5Y cells co-treated with LOLA and 6-OHDA might represent the mechanism by which LOLA, without affecting mitochondrial calcium efflux mechanisms, might prevent mitochondria depolarization and, in turn, mitochondrial 6-OHDA-induced dysfunction. Therefore, depending on the mechanism by which each toxin induces mitochondria dysfunction, LOLA may contribute to the recovery of mitochondrial functional properties by affecting two different intracellular pathways: one direct, to counteract ROT-induced mitochondrial calcium increase and one indirect, aimed to reduce 6-OHDA-induced cytosolic calcium content. These findings are supported by the effects of LOLA on RNS and ROS production and are in line with the hypothesis that these radical species are able to stimulate NCXs activity, thus playing a role in the regulation of intracellular calcium homeostasis (Annunziato *et al.*, 2004; Sisalli *et al.*, 2014; Sisalli *et al.*, 2020). This effect, although needs to be further confirmed, seems to suggest a novel emerging mechanism of action of LOLA that is not directly related to its ability to selectively target mitochondria, but may depend on the activation of NO production, as already reported in some brain nitrergic neurons (Bernstein *et al.*, 2017). Moreover, the results of the present study confirm the tight relationship existing between cytosolic ionic homeostasis and cellular metabolism and underline the importance of a finely regulation of calcium fluxes among different cellular compartments like mitochondria, cytosol, and plasma membrane in the regulation of cellular vulnerability. Finally, the results of the *in vitro* experiments, allow to conclude that pharmacological strategies able to improve mitochondrial function have a role in preventing cellular demise, and may

reveal helpful in those pathological conditions in which mitochondrial dysfunction represents a pathogenetic factor in disease development like it occurs in PD.

The results of *in vivo* study in A53T-α-syn transgenic mice, a familial model of PD, provide appropriate indications to explore the potential effect of LOLA on the phenotypic manifestation associated with the progression of dopaminergic neuronal degeneration. Behavioural and western blotting experiments performed in 4- and 12-month-old WT and A53T transgenic mice reveals a dysfunction in synaptic activity in transgenic mice, more evident in the midbrain than in the striatum. This effect is associated with an increase in SYN3 and DAT protein levels in 4-month-old A53T-α-syn transgenic mice, and with a decrease in these two proteins expression in 12-month-old A53T-α-syn transgenic mice, thus confirming that a progression in synaptic dysfunction might be detected in these mice during aging. Moreover, the greater spontaneous locomotor activity, the better balance and motor coordination, and the progressive imbalance and bradykinesia observed in behavioural and motor tests performed in young mice, suggest that 4-month-old A53T transgenic mice display an anxiety-like and hyperactive behaviour compared to older and WT mice, in line with the alteration of DA-system activity (Unger *et al*., 2006; Zhuang *et al*., 2001; Graham *et al*., 2010; Guerriero *et al*., 2017). Collectively, the results related to the *in vivo* experiments allow to identify an animal model in which the early stage of the dopaminergic dysfunction are detectable, and represent the starting point to verify whether, LOLA treatment, by affecting neuronal metabolic function, might slow down the progression of dopaminergic damage and consequently, the related phenotypic disease manifestations.

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