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Identification of new molecular targets involved in pathogenesis of Parkinson's Disease: new perspectives for therapeutic intervention.

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SUMMARY

1. INTRODUCTION

1.1. BACKGROUND

Parkinson's Disease (PD) is a neurodegenerative disease related to the dopaminergic neuron loss of the *Substantia Nigra pars compacta* (SNpc) (Scalzo et al., 2010), which is clinically characterized by bradykinesia, hypokinesia, resting tremor, rigidity and postural instability (Hausdorff, 2009). The annual incidence rate of disease has been estimated to range from 16 to 19 per 100,000 persons affected, mainly over 50 years, with prevalence and incidence rates increasing with age (Pahwa and Lyons, 2010).

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. There are two principal clinical subtypes: a tremor predominant form that is often found in younger people, and a type characterized by a postural imbalance and a gait disorder that is often observed in older people (>70 years old) distinguished by akinesia, rigidity, and gait and balance impairment. The first subtype leads to a slow decline of motor function, whereas the second subtype worsens more rapidly (Obeso et al., 2010). The clinical diagnosis of PD is typically based on the presence of cardinal motor features, absence of atypical findings suggestive of an alternative diagnosis, and response to levo-3, 4-dihydroxyphenylalanine (L-DOPA) (Perfeito et al., 2013).

In addition to the loss of dopaminergic neurons in the SNpc, PD is neuropathologically characterized by the presence of Lewy bodies, intracytoplasmic eosinophilic inclusions found in injured or fragmented neurons, (Burch, Sheerin, 2005) with α-synuclein as the major fibrillar component (Spillantini et al., 1997; Baba et al., 1998). However, the exact mechanism underlying selective dopaminergic neurodegeneration is still not understood. It is believed that many cellular mechanisms may be involved, including oxidative stress, intracellular Ca^{2+} homeostasis impairment, and mitochondrial dysfunctions (Cali et al., 2011).

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 LRRK2 (PARK8) are responsible for dominant forms of familial PD. The activity and cellular distribution of the proteins encoded by these genes are different and still not completely elucidated. PINK1 and LRRK2 are kinases, DJ-1 is a multifunctional protein that acts as peptidase, chaperone redox/oxidative sensor, transcriptional modulator, and oncogene, parkin is a E3-ubiquitin ligase and α-synuclein, in addition to be the principal component of Lewy bodies, plays important role in the regulation of vesicles trafficking (Cali et al., 2011). Among these proteins, PINK1 and Parkin play crucial roles in the regulation of mitochondrial dynamics and function (Gandhi et al., 2009). Mutations in DJ-1 and Parkin render animals more susceptible to oxidative stress (Gandhi et al., 2009). Moreover, mitochondrial association of α-synuclein in cells was also linked to impairment of respiratory complex I activity, oxidative modification of mitochondrial proteins, and increased levels of Ca^{2+} (Cannon et al., 2013; Liu et al., 2009; Li et al., 2013). Alterations in the mitochondrial complex I are also found in autoptic samples of *substantia nigra* from patients affected with the disease in which the activity of complex I was shown to be reduced (Parker et al., 2008).

As mentioned above, many evidences 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 to support cellular stresses, thus making the cells 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). Indeed, perturbations of calcium homeostasis are common denominator in several neurodegenerative disorders including PD. In fact, SNpc dopaminergic neurons have a singular characteristic in respect to the other groups of neurons. Dopaminergic neurons are autonomously active, being

characterized by a pace making activity responsible to generate action potentials also in the absence of synaptic input. This activity exposes dopaminergic neurons to large Ca^{2+} influx (Surmeier et al., 2009). Furthermore, it has been reported that an increased activity of several classes of Ca^{2+} channels including L-type voltage-dependent-and cyclic nucleotide-sensitive channels seem to play a key role in the pathogenesis of Parkinson's disease, leading to an alteration of intracellular Ca^{2+} homeostasis. Moreover, the recent observation that cells deficient in complex-I showed an alteration in the cytosolic calcium handling, reduced mitochondrial calcium accumulation and consequent ATP synthesis (Visch et al., 2004), suggested a possible relationship between mitochondrial dysfunction and perturbation of intracellular calcium homeostasis in the pathogenesis of PD.

1.2 Pathogenesis of Parkinson's Disease

1.2.1 Mitochondrial dysfunction during Parkinson's Disease

Mitochondrion is intracellular membrane enclosed organelle found in most eukaryotic cells, which plays 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. Furthermore, mitochondria are dynamic organelles that actively divide, fuse with one another, and undergo regulated turnover, all of which is important for the maintenance of mitochondrial function and quality control (Perier and Vila, 2011).

According to a widespread concept, neurons are critically dependent on mitochondrial integrity based on their specific morphological, biochemical, and physiological features. Indeed, neurons are characterized by high rates of metabolic activity and need to respond promptly to activity-dependent fluctuations in bioenergetic demand. The dimensions and polarity of neurons require efficient transport of mitochondria to hot spots of energy consumption, such as presynaptic and postsynaptic sites. Consequently, alterations in any of these mitochondrial features can potentially cause disease and have been linked to the pathogenesis of neurodegenerative diseases including Parkinson's disease (Exner et al., 2012). In particular, defective mitochondrial respiration, at the level of complex I, has long been associated with the pathogenesis of PD.

Evidence of this involvement first emerged after the observation that accidental exposure of drug abusers to 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP), an inhibitor of mitochondrial complex I, resulted in an acute and irreversible parkinsonian syndrome almost indistinguishable from PD (Langston et al., 1983). It was subsequently shown that MPTP, when injected into non human primates and mice, selectively kills dopaminergic neurons of the substantia nigra pars compacta (SNpc), the type of cells that preferentially degenerate in PD (Dauer and Przedborski 2003). Similarly, chronic infusion of the potent complex I inhibitor rotenone to rats has been reported to produce nigrostriatal dopaminergic neurodegeneration (Betarbet et al., 2000). A link between complex I dysfunction and PD was further established when several groups reported that a reduction in complex I activity occurs in the brain, platelets, and skeletal muscle of patients with sporadic PD (Parker et al., 1989; Schapira et al., 1990).

As a consequence of complex I dysfunction, it was found a reduction in ATP production and subsequent bioenergetic failure. Supporting this view, MPP+ (MPTP's active metabolite) causes a rapid and profound depletion of cellular ATP levels in hepatocytes (Di Monte et al., 1986), in brain synaptosomal preparations (Scotcher et al., 1990) and in whole mouse brain tissues (Chan et al., 1991). Another consequence of complex I inhibition is the formation of reactive oxygen species (ROS), which damage mitochondria DNA (mtDNA), components of the respiratory chain and other mitochondrial factors, thereby triggering a vicious circle between mitochondrial impairment and oxidative stress (Exner et al., 2012).

The subsequent discovery of hereditary forms of PD caused by dominant and recessive mutations in nuclear genes encoded proteins functionally related to mitochondria has added additional importance to the study of mitochondrial dysfunction in PD. The products of two dominantly inherited genes, α-synuclein and LRRK2, and of several autosomal recessive inherited genes, DJ-1, parkin, PINK1 and Omi/HTRA2, have been found to be localized in, and/or to interfere with mitochondria (Liu et al., 2009; Cali et al., 2012). In addition, PD-like protein DJ-1 has been identified as a mitochondrial peroxi-redoxin-like peroxidase, able to scavenge mitochondrial ROS; its deficiency in mutant mice results in increased mitochondrial ROS production (Perier and Vila, 2012). Indeed, PINK1

has been linked to a number of a physiological role such as, the phosphorylation of mitochondrial proteins in response to cellular stress and the protection against mitochondrial dysfunction (Gandhi et al., 2009; Ziviani et al., 2010; Akundi et al., 2011). There is evidence that wild type PINK1, in contrast to mutant PINK1, may protect neurons from stress-induced mitochondrial dysfunction and apoptosis (Perfeito et al., 2013). Furthermore, loss of PINK1 function in human cell lines produced morphological changes in mitochondria impaired energy metabolism, and decreased mitochondrial membrane potential (Exner et al., 2007). Recently, few studies considered the possibility that these mutations may be also involved in mitochondrial Ca^{2+} signaling. There is evidence that α-synuclein modulates Ca^{2+} influx (Buttner et al., 2013), suggesting that its oligomers promote alterations in calcium dynamics via interference with intracellular buffering mechanisms. In another study it was found that overexpression of Parkin increased mitochondrial Ca^{2+} transients by enhancing endoplasmic reticulum-mitochondria interactions (Cali et al., 2013). Furthermore, Ca^{2+} accumulation in the mitochondrial matrix results at an energetic cost, as it dissipates the electrochemical gradient created by respiratory metabolism along the electron transport chain (ETC) (Chan et al., 2010) and also leads to a disruption of mitochondrial membrane integrity, permeability transition, irreversible oxidative damage and consequently cell death (Cali et al., 2012).

1.2.2 Dysregulation of calcium concentration and neurodegeneration

Ca^{2+} is the main second messenger that helps to transmit depolarization status and synaptic activity to the biochemical machinery of a neuron. In neurons, Ca^{2+} have multiple complex and integrated functions, including the control of dendritic responses to neurotransmitters, signalling to the nucleus to regulate gene expression, and initiation of neurotransmitter release from presynaptic axon terminals (Gleichmann and Mattson, 2011). In these ways Ca^{2+} plays pivotal roles in controlling neuronal excitability. Thus, Ca^{2+} functions as a key regulator of electrochemical signalling, not only within individual neurons, but also among large populations of neurons that comprise neuronal networks (Gleichmann and Mattson, 2011).

The influx of Ca^{2+} through voltage-dependent and ligand-gated channels in the plasma membrane is a critical signal for the release of the neurotransmitters from presynaptic terminals and for responses of the postsynaptic neuron (Mattson 2007). Glutamate, an excitatory neurotransmitter in the central nervous system, induces local and general increases of cytoplasmic Ca^{2+} through the activation of AMPA and NMDA receptors in the plasmamembrane, with consequent activation of voltage-dependent Ca^{2+} channels (VDCC) (Cali et al., 2011). In addition, the activation of metabotropic glutamate receptors coupled to the GTP-binding protein G_{q11} stimulates the release of inositol triphosphate (IP_3), which activates Ca^{2+} channels in the endoplasmic reticulum (Mattson, 2007).

The cost for extensive neuronal Ca^{2+} signalling is an increased energy demand because all the Ca^{2+} that enters in neurons must be removed from the cytoplasm by ATP-dependent membrane calcium pumps in order to maintain Ca^{2+} homeostasis (Gleichmann and Mattson, 2011). Moreover, Ca^{2+} is removed from the cytoplasm thank to the activity of the plasma membrane Na^{2+}/Ca^{2+} Exchanger (NCX), the plasma membrane and endoplasmatic reticulum (ER) Ca^{2+}-ATPases, and the Ca^{2+}-bindings proteins such as calbidin and parvalbulmin. Finally, Ca^{2+} can also be transported into and released from mitochondria (Mattson, 2007).

In the dopaminergic neurons of the SNpc, that have most of Ca^{2+}channels open much of the time due to their pace making activity, the magnitude of the Ca^{2+} influx appears to be much larger and the charge to the cell much greater compared to neurons in other different brain regions (Chan et al., 2010). Because of the slow kinetics of the plasma membrane transporters and their restriction to the cellular surface, Ca^{2+} entering neurons must be rapidly sequestered either in organelles lying below the plasma membrane or through ionic interactions with mobile buffering proteins before being escorted out of the cell. Mitochondria and the endoplasmic reticulum (ER) are the principal organelles involved in sequestering Ca^{2+} in neurons (Rizzuto and Pozzan, 2006; Verkhratsky, 2005). The ER uses high-affinity ATP-dependent transporters to take Ca^{2+} from the cytoplasm into the ER lumen. As this store fills up, Ca^{2+} triggers the opening of ER Ca^{2+} channels that let the Ca^{2+} flow back into the cytoplasm. These channels are often found in close apposition to mitochondria

and their opening creates a region of high-local Ca^{2+} concentration that drives influx of Ca^{2+} into the matrix of mitochondria through Ca^{2+} uniporters (Rizzuto and Pozzan, 2006). Ca^{2+} accumulation in the mitochondrial matrix again comes at an energetic cost, as it dissipates the electrochemical gradient created by respiratory metabolism along the electron transport chain (ETC). In fact, an important feature of the mitochondrial Ca^{2+} transport pathway is that this organelle contains low calcium in resting cells, but is able to accumulate large amounts of calcium in condition stimulating Ca^{2+} entry, and to release this calcium loaded during the recovery phase (Nicholls, 2005). This is due to the ability of specific transporters localized on the inner mitochondrial membrane that allow calcium to cycle from mitochondrial matrix to the cytosol and from the cytosol to the mitochondrial matrix. Ca^{2+} is removed from the matrix through the mitochondrial NCX (Kim and Matsuoka, 2008; Rizzuto and Pozzan, 2000), the Ca^{2+} proton exchanger (Williams and Fry, 1979), and the transient opening of the mitochondrial permeability transition pore (mPTP) (Hüser and Blatter, 1999). These events allow the maintenance of mitochondrial calcium concentrations within physiological range that are necessary for the neurons to adjust aerobic ATP production, to regulate synaptic transmission and excitability, to promote organelle dynamics and trafficking, to mediate signalling to nucleus, to control the generation of ROS, and to preserve neuronal survival (Nicholls, 2005; Starkov, 2002; Chinopoulos and Adam-Vizi, 2010; Duchen, 2004; Mattson et al., 2008).

ER is another important organelle responsible to maintain Ca^{2+} homeostasis into neurons. The ER is able to store Ca^{2+} within the cell thanks to its intralumial Ca^{2+} capacity of about 0.5 mM. Ca^{2+} is pumped into the ER by sarcoplasmic endoplasmic reticulum Ca^{2+} ATPase (SERCA) and is extruded by IP_3-RS and RyRs channels (Gleichmann and Mattson, 2011). The regulation of Ca^{2+} release from this organelle is responsible for many neuronal functions, from plasmalemmal excitability to synaptic plasticity. Together with mitochondria, ER forms junctions that support signal transduction and biosynthetic pathways and affect distribution of the organelles. These junctions have a pivotal role in mediating Ca^{2+} signal propagation to the mitochondria (Zundorf and Reiser, 2011).

During normal physiological activity, the intracellular Ca^{2+} concentration increases only transiently and has no adverse effects on the neurons. However, in pathological conditions, and in normal aging, the ability of neurons to control Ca^{2+} effluxes and recover from a Ca^{2+} load is compromised (Mattson, 2007).

Perturbations in calcium homeostasis are common denominator in several neurodegenerative disorders including PD (Surmeier, 2007). However, the molecular mechanisms responsible for the selective loss of neuronal population in the different neurodegenerative disorders are less clear. Several hypothesis have been made, originally based on dopamine implication and proposing that dopamine cytosolic oxidation (and its metabolites) may cause toxic effects through the generation of free radicals (Greenamyre and Hastings, 2004). However, this pathway cannot be the only responsible for the selective neuronal loss as, not all dopaminergic neurons in the brain die in PD. Recently, an interesting hypothesis has been provided to explain the reason why SNpc dopaminergic neurons are particularly vulnerable and it takes into account their peculiar physiology. In fact, SNpc dopaminergic neurons, with difference to the large majority of other neurons, are autonomously active, being characterized by a pace making activity responsible to generate action potential also in the absence of synaptic input (Grace and Bunney, 1983). This characteristic depends on the structural properties of L-Type Ca^{2+} channels, characterized by the presence of Cav1.3 pore forming subunit, that confers to this type of Ca^{2+}channels the property to be open at relatively hyperpolarized potentials and thus exposing SNpc dopaminergic neurons to larger Ca^{2+} influx than other neurons (Wilson and Callaway, 2000).

Several evidences underline the importance of maintaining a correct Ca^{2+} buffering capacity and suggest the perturbations of Ca^{2+} channels activity as responsible for PD associated Ca^{2+} dyshomeostasis. Indeed, a dramatic reduction in the Ca^{2+} buffering protein calbindin levels has been described in brains of PD patients (Iacopino and Christakos, 1990) and dopaminergic neurons expressing higher levels of calbindin, or other Ca^{2+} buffering proteins as calretinin and parvalbumin, are resistant to degeneration in PD (Yamada et al., 1990; Kim et al., 2000). Furthermore, pharmacological inhibition of glutamatergic neurotransmission ameliorates motor abnormalities in experimental models of PD (Bonsi et al., 2007) and the administration of

dihydropiridine, an L-type channel blocker, confers protection against toxins that induce Parkinsonism in experimental animal models (Chan et al.,, 2007). Ca^{2+} dyshomeostasis may be also exacerbated by environmental insults such as pesticides, neurotoxins, or inflammation (Mrak and Griffin, 2007). According to these findings, rotenone has been shown to induce apoptosis through enhanced intracellular Ca^{2+} influx that can be blocked by nifedipine, a voltage-gated Ca^{2+}channel-inhibitor (Wang and Xu, 2005). Dopamine itself appears to affect the activity of Ca^{2+} channels in neurons and PC12 cells (Missale et al., 1998), and recently, it has been proposed that the dopamine metabolism induces Ca^{2+} signaling in astrocytes by stimulating phospholipase C and subsequent release of Ca^{2+} from the ER (Vaarmann et al., 2010).

The control of sustained Ca^{2+} transients, in addition to need the action of Ca^{2+} buffering proteins, requires functional mitochondria. Mitochondria directly participate in the buffering action, but they are also largely responsible in producing ATP indispensable for the action of the active Ca^{2+} transport systems.

In addition to the aforementioned considerations, further support for a Ca^{2+} dyshomeostasis link in PD comes from the rapidly expanding literature on genetic mutation associated with familial forms of PD. Few papers have started to explore the possibility of Ca^{2+} handling by the PD-related proteins. As matter of fact, DJ-1 is a multifunctional protein and, despite its predominant role as antioxidant (Taira et al., 2004), it has been shown essential to maintain cytosolic basal Ca^{2+} concentration values and to permit depolarization-induced Ca^{2+} release from the sarcoplasmic reticulum in muscle cells (Shtifman et al., 2011) as well as to protect dopaminergic neurons by Ca^{2+}-induced mitochondrial uncoupling and ROS production during physiologic pace making (Guzman et al., 2010). An augmented phospholipase C activity with consequent increase of cytosolic basal Ca^{2+} concentration and increased vulnerability to 6-hydroxydopamine (6-OHDA) have also been shown in parkin-deficient human neuroblastoma cells (Sandebring et al., 2009).

More compelling evidences come from studies on α-syn and PINK1. α-Syn dependent modulation of Ca^{2+} influx from the extracellular milieu has been described suggesting that the α-syn oligomers could enhance the plasma membrane ion permeability (Danzer et al., 2007) either through their direct insertion into the plasmamembrane and/or the formation of a pore (Lashuel et

al.,, 2002) or through the modulation of plasma membrane Ca^{2+} permeability (Furukawa et al., 2006). However, the link with the pathogenesis is still missing and controversial as it has been reported that PD-related α-syn mutants, characterized by an enhanced aggregation propensity, play a minor role in intracellular Ca^{2+} increase (Danzer et al., 2007) but also that A53T mutant oligomers may increase the plasma membrane Ca^{2+} permeability (Furukawa et al., 2006). The mechanisms through which α-syn aggregation and Ca^{2+} dysfunction influence each other are not clear, but interestingly a functional interplay has been reported: intracellular Ca^{2+} increase promotes α-syn aggregation, that in turn could promote intracellular Ca^{2+} increase. Recent studies have shown that α-syn can be associated to mitochondria and that its accumulation was directly related to an increase of intramitochondrial Ca^{2+} levels (Parihar et al., 2008), which in turn led to a raise of nitric oxide levels, oxidative damage, and cytochrome c release (Parihar et al., 2009), supporting the idea of a major role of α-syn in modulating not only the cellular but also the mitochondrial Ca^{2+} fluxes. However, these observations were not constantly reported as other studies showed no changes in cellular Ca^{2+} homeostasis following overexpression of WT or mutant α-syn (Hettiarachchi et al., 2009). As to PINK1, its direct role in regulating cellular, and most specifically mitochondrial Ca^{2+} fluxes, has been recently proposed starting by the seminal observation that the coexpression of mutant, but not WT, PINK1 in a cellular model of PD expressing mutated A53T α-syn exacerbated the observed mitochondrial defects leading to loss of mitochondrial membrane potential, increased mitochondrial size with loss of cristae and reduced ATP levels. The proposed mechanisms of PINK1 action was based on a dysregulation of mitochondrial Ca^{2+} influx, as by blocking mitochondrial Ca^{2+} uptake it was possible to restore the original phenotype (Marongiu et al., 2009), thus suggesting that mutant PINK1 could reinforce α-syn pathology by acting on converging pathways affecting mitochondrial function.

1.2.3 Role of alpha synuclein in pathogenesis of Parkinson's Disease

α-syn is a 140 amino-acid protein that was originally identified in association with synaptic vesicles in the presynaptic nerve terminal (Maroteaux et al., 1988) and has been shown to interact with membranes both in vitro and in vivo (Davidson et al., 1998; Jo et al., 2000; Fortin et al., 2004). It is highly abundant in the brain and also present in other tissues including red blood cells (Jakes et al., 1994; Iwai et al., 1995; Nakai et al., 2007; Barbour et al., 2008). α-syn is a member of a protein family of synucleins, together with beta (β)-and gamma (γ)-synuclein. These proteins share a characteristic consensus sequence (KTKEGV) that is repeated about six times at the N-terminal part of the protein. β-synuclein shares the closest homology (90% homology in the N-terminus and 33% homology in the C-terminus) with α-syn (Jakes et al., 1994).

Point mutations in the SNCA gene, encoding for α-syn, and multiplications of the SNCA locus have been identified in families with autosomal-dominant forms of PD (Hardy et al., 2009). Genome-wide association studies linked single-nucleotide polymorphisms in the SNCA gene with increased susceptibility to sporadic PD (Nalls et al., 2011). Moreover, SNCA gene polymorphisms have also been associated with increased risk of multiple system atrophy (MSA) (Scholz et al., 2009).

In PD, α-syn is found as a major component of Lewy bodies and Lewy neurites, the hallmark protein inclusions made up primarily of insoluble and fibrillar α-syn protein (Spillantini et al., 1998a). α-syn also accumulates in dementia with Lewy bodies (DLB) and MSA (Spillantini et al., 1998b). In MSA, α-syn is found predominantly within oligodendrocytes as cytoplasmic inclusions (Wenning and Gesser, 2003). These disorders share the accumulation of α-syn aggregates, as a pathological feature and are collectively known as synucleinopathies. Additionally, α-syn was also identified as a component of amyloid from brain tissues of Alzheimer's disease (AD) patients (Ueda et al., 1993).

The presence of a hydrophobic 12 amino-acid sequence in the central part of the protein is required for the oligomerization and fibrillization of α-syn (Giasson et al., 2001). Deletion or disruption of this domain blocks the capacity of α-syn to form amyloid fibrils. The process of α-syn aggregation (Figure 1) has

been studied in detail in an attempt to identify the toxic species responsible for neuronal dysfunction and death. However, it is still unclear what is/are the toxic forms of the protein. There is evidence showing that inhibition of α-syn aggregation process is associated with a decrease of α-syn toxicity (Hashimoto et al., 2001; Periquet et al., 2007). However, similarly to the case of amyloid-beta (Aβ) plaques in AD, it was suggested that the fibrillar forms of α-syn might not represent the most toxic α-syn species. Instead, pre-fibrillar, soluble oligomeric species (comprising multiple α-syn molecules) are now suggested to be the main toxic α-syn species, with amyloid aggregates possibly serving as a reservoir for these oligomeric species. In vitro studies showed that the acceleration of oligomerisation, and not fibrilization, is the distinctive shared property of the A53T and A30P α-syn mutations linked to early-onset PD (Conway et al., 2000). The neurotoxic effects of α-syn oligomers were also studied in vivo, using animal models of synucleinopathies. In these studies, α-syn mutant variants that promote oligomer formation were designed and tested for toxicity in vivo. On the other hand, in *C. elegans* and Drosophila models the overexpression of α-syn variants with impaired capacity to form fibril caused increased toxicity (Karpinar et al., 2009). Similarly, the inability of the mutants to form fibrils was directly correlated with toxicity and neurodegeneration (Karpinar et al., 2009). In another study, α-syn variants that were shown to promote oligomer formation caused most prominent dopaminergic cell death upon lentiviral injection into rat substantia nigra (SN) (Winner et al., 2011). Together, these studies provide evidence for the importance of soluble oligomers as the prominent toxic species in synucleinopathies, although the precise size and type of the toxic oligomeric species remains to be determined.

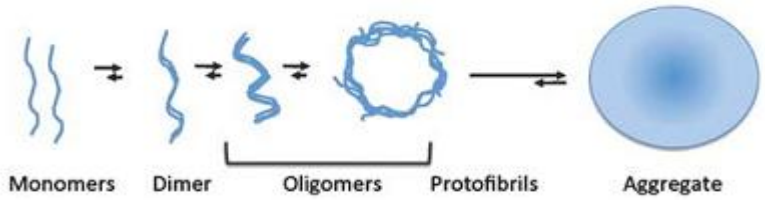

Figure 1. Schematic representation of the α-syn aggregation process. Monomeric forms of α-syn associate to form dimers and oligomers that grows into protofibrils and, finally, form mature fibrillar strutucters (Marques and Outeiro, 2012).

Recently, it was hypothesized that PD, as well as other neurodegenerative disorders associated with protein misfolding and aggregation, might be a prion-like disease where pathological forms of α-syn spread throughout the brain. In particular, the spreading of α-syn pathology could contribute to the progression of neurodegeneration and clinical symptoms (Braak et al., 2003; Li et al., 2008; Hansen et al., 2011).

The mechanisms that underlie the aberrant functions of α-synuclein and how these impacts on disease pathogenesis remain poorly understood, but some possibilities have been suggested.

Data largely derived from α-syn knockout mice suggest that α-syn normally mediates negative control of neurotransmitter release and has a possible role in assembly of SNARE (Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) family complexes (Burre et al., 2010; Abeliovich et al., 2000; Larsen et al., 2006; Chandra et al., 2005). Whether these apparently physiological functions at the presynaptic terminal contribute to the pathogenic effects of α-synuclein has been investigated. It was described an abnormal localization of the protein at presynaptic terminals (Iwai et al., 1995). It is believed that this accumulation leads to the synaptic dysfunction associated with amyloid plaques. In the brains of patients with dementia with Lewy bodies, α-synuclein aggregates were located at presynaptic terminals and resulted in severe synaptic pathology, leading to almost complete loss of dendritic spines at the postsynaptic area (Kramer et al., 2007). As far as α-Syn role in neurotransmitter release it has been reported that slight increases in α-syn, inhibited neuroansmitter release in glutamatergic hippocampal pyramidal and dopaminergic mesencephalic neurons, potentially by a reduction in the pool of readily releasable synaptic vesicles (Nemani et al., 2010). In another study, overexpression of α-syn in cultured neurons was associated with low concentrations of several critical presynaptic proteins involved in exocytosis and endocytosis (Scott et al., 2010) and substantial reductions in the frequency of excitatory postsynaptic currents, diminished exocytosis, and altered vesicular size. A different perspective was provided by a study in another model, in which α-syn with C-terminal truncation was transgenically expressed in mouse nigral dopaminergic neurons. Functional deficits were related to severe reductions in

dopamine release, which were in turn probably due to redistribution, but not loss, of presynaptic proteins (Garcia-Reitbock et al., 2010).

The role of α-syn in neurotransmitter release is mainly based on its regulation of synaptic vesicle recycling. α-syn knockout mice display altered DA release in response to paired stimuli, reduction in striatal DA and an attenuation of DA locomotor response to amphetamine (Abeliovich et al., 2000). Another *in vivo* study showed that lack of α-syn leads to a permanent increase of the vesicle refilling rate in the DA readily releasable pool, maintaining stable DA release during stimulation in contrast to decline of DA release observed in normal conditions (Yavich et al., 2004). Together, these findings suggest that α-syn is an activity-dependent, negative regulator of DA neurotransmission.

A prevailing hypothesis suggests that oligomeric species of α-syn can promote the formation of ion-permeable pores on membranes and alter cellular homoeostasis (Lashuel et al., 2002; Volles et al., 2001). Increased Ca^{2+} influx is thought to be the main resulting toxic effect (Danzer et al., 2007; Kostka et al., 2008; Tsigelny et al., 2007; Kayed et al., 2004; Hettiarachchi et al., 2009), and could be further augmented via glutamate AMPA receptors (Huls et al., 2011). Catecholaminergic neurons might be especially vulnerable to oscillations in Ca^{2+} concentrations because L-type voltage-gated calcium (Cav1.3) channels help to maintain their spontaneous pacemaker activity (Chan et al., 2007). Although neurons might compensate for high cytosolic concentrations of free Ca^{2+} through a functional sarcoendoplasmic reticulum pump, this system is thought to break down in conditions of energy depletion or oxidative stress, which might be caused by mitochondrial dysfunction in PD (Chan et al., 2009; Guzman et al., 2010). The same pore-forming mechanism is thought to enable oligomeric α-syn to attack synaptic vesicles, which leads to leakage of neurotransmitter into the cytosol: for dopamine, high cytosolic concentrations could lead to oxidative stress, intracellular interactions between Ca^{2+}, dopamine, and α-syn, and the triggering of a neurodegenerative cascade (Mosharov et al., 2006). Another contributing factor could be calcium-mediated activation of calpains, which might lead to C-terminal truncation and oligomerisation of α-synuclein, (Mishizen-Eberz et al., 2005) further Ca^{2+} influx, and neurotoxic effects.

1.3 Sodium Calcium Exchanger

The Na^+/Ca^{2+} exchanger (NCX) represents a major transporter assuring Ca^{2+} efflux from mammalian cells (Blaustein and Lederer, 1999). Under physiologic conditions NCX is a ubiquitous mechanism providing the exchange of $3Na^+/1Ca^{2+}$ between 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 proteins consist of nine transmembrane segments (TMSs) with a large cytoplasmic f-loop between TMSs 5 and 6 composed of two adjacent Ca^{2+} -binding domains, CBD1 and CBD2 (Philipson and Nicoll, 2000; Levitsky et al., 1994; Hilge et al., 2006). Two CBD domains are connected through a short linker to form a “head-to-tail”-oriented two-domain tandem, CBD1/2 (Hilge et al., 2006, 2009). Allosteric regulation of NCX requires Ca^{2+} to interact with the CBD domains (Weber et al., 2001; Nicoll et al., 2007; Reeves and Condrescu, 2008; Boyman et al., 2011) (Fig. 2).

Figure 2. Schematic representation of the sodium calcium exchanger (NCX) (Modified from Iwamoto et al., 2004).

NCX belongs to a multigene family comprising three isoforms, named NCX1, NCX2, and NCX3. To fulfill 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; Lytton, 2007; Khananshvili, 2012). NCX1 is ubiquitously expressed in all tissues, NCX2 is mainly restricted to the brain, and NCX3 is expressed exclusively in brain and skeletal muscles (Quednau et al., 1997). In addition, 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 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 found (Canitano et al., 2002; Papa et al., 2003).

Several factors are involved in the regulation of NCX 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., 1992a, b). Moreover, NCX is extremely sensitive to cytosolic acidification, redox status and metabolic state (DiPolo and Beauge, 1982, 2006; Doering and Lederer, 1994; Doering et al., 1996). 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 supra molecular complexes (Bers and Despa, 2009; Schulze et al., 2003; Hilgemann, 2007; McLaughlin et al., 2002; Berberián et al., 2009).

The activity of NCX is important especially in some neurophysiological conditions. In fact, 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 plasmamembrane and promotes neurotransmitter 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 Na^+/Ca^{2+}exchanger families. In such physiological conditions, NCX activation is consistent with its low-affinity (K_d 500nM) and high-capacity ($5x10^3Ca^{2+}/s$) 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 (K_d100 nM) and low-capacity ($10^2Ca^{2+}/s$) pump, the plasmamembrane 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 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 increases $[Na^+]_i$, 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, 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 NCX3 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 might play 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 aging, 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 aging (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 action (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 aging and also during neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease in which a neuronal calcium dysfunction is found, NCX might have a relevant role. In fact, a study performed in the synaptic terminals obtained from the brain cortex of AD patients 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 (Sokolow et al., 2011). Moreover, NCX1, NCX2, and NCX3 colocalize 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 (NCX_{mito}) during neurodegeneration with particular regard to Parkinson Diseases (Castaldo et al., 2009). Indeed, it has been reported that during Parkinson's disease the activation of NCX_{mito} 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 neurotransmitter release, synaptic plasticity, bioenergetics and mitochondrial NO and free radical generation (Castaldo et al., 2009). Recently, It was found that in the absence of PINK1, NCX_{mito} activity was severely impaired, leading to mitochondrial calcium overload, permeability transition pore opening and cell death (Gandhi et al., 2009). It was proposed that NCX_{mito} is entirely distinct from the characterized plasmalemmal NCX isoforms, due the specific sensitivity of NCX_{mito} to the inhibitor CGP-37157

(Czyz and Kiedrowski, 2003). Recently, it has been shown 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). Moreover, a recent study showed that the plasmalemal NCX2 and NCX3 contribute to mitochondrial Na^+/Ca^{2+}exchange in human DA neurons and may act downstream of PINK1 in the prevention of neurodegeneration by $[Ca^{2+}]_m$ accumulation (Wood-Kaczmar et al.,, 2013). These studies have potentially revealed a new molecular target in Parkinson's disease pathologenesis, which ultimately may open up new avenues for future therapeutic intervention.

1.4 Experimental models for Parkinson's disease

1.4.1 In vitro models

Over the last two decades significant advances have been made to improve the knowledge of both the etiology and pathogenesis of PD. Experimental models are of paramount importance to obtain greater insights into the pathogenesis of the disease (Bezard et al., 2013). In this sense, great progresses have been made in the experimental studies of PD, especially through the use of cellular models. The development of a stable and reliable dopaminergic neuronal cell model is particularly necessary for studying the pathogenesis of PD and developing therapeutic strategies. An ideal *in vitro* PD cellular model should be established in post-mitotic human dopaminergic neuronal cells susceptible to neurotoxins produced during PD so as to address questions regarding the selective loss of DA neurons in the substantia nigra. Presently, PD cell models primarily include non-neuronal tumor cell lines such as pheochromocytoma, PC12, cells (Koch et al., 2009), neuronal tumor 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-hydroxydopamine (6-OHDA), or rotenone mimics many aspects of the dopaminergic neuronal death 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 (Kitamura et al., 1998; Sawada et al., 1996). Other less often utilized neurotoxins are rotenone, paraquat, isoquinoline derivatives and methamphetamine (Bezard and Przedborski, 2011).

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. In particular, 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 inflict damage to the catecholaminergic pathways of both the peripheral and the central nervous systems (Jonsson, 1983; Jonsson, 1980). With respect to its mode of action, it is well accepted that 6-OHDA destroys catecholaminergic structures by a combined effect of reactive oxygen species (ROS) and quinones (Cohen, 1984). This view stems primarily from the demonstration that 6-OHDA once 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). However, 6-OHDA lesions do not result in Lewy body formation in the substantia nigra, and can cause nonspecific damage to other neurons (Shimohama et al., 2003).

Another neurotoxin administered in *in vivo* and *in vitro* models for Parkinson's disease 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 the production of reactive oxygen species (ROS) 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 recently 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 *Leguminosa* 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 remembered 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 gains access to all organs including the brain. After a single intravenous and/or intrastriatal injection, rotenone reaches maximal concentration in the central nervous system within 15 min and decays to about half of this level in less than 2h (Talpade et al., 2000). Its brain distribution is heterogeneous (Talpade et al., 2000). Rotenone also freely 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 PSST (20kDA subunit of complex 1) subunit of 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). Interestingly, a protein implicated in some familial forms of PD, parkin, appears to bind to tubulin, thereby enhancing the ubiquitination and degradation of misfolded tubulins, an effect that is lacking with the PD-linked parkin mutants (Ren et al., 2003). 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 potent herbicide paraquat (N,N'-dimethyl-4-4'-bipiridinium) is another prototypic toxin known to exert deleterious effects through oxidative stress. Indeed, paraquat toxicity is mediated by redox cycling with cellular diaphorase such as nitric oxide synthase (Day et al., 1999), yielding ROS. Thus far, there have been several cases of lethal poisoning resulting from ingestion or dermal exposure (Smith, 1988). For many years, experimental studies using paraquat were focused on its effects on lung, liver, and kidney probably because the toxicity induced by this herbicide in these organs is responsible for death after acute exposure. However, significant damage to the brain was seen in individuals who died from paraquat intoxication (Grant and Lantos, 1980; Hughes, 1988) despite the fact that paraquat poorly crosses the BBB spontaneously (Shimizu et al., 2001). Furthermore, epidemiological studies have suggested an increased risk for PD due to paraquat exposure (Liou et al., 1997), raising the possibility that paraquat could be an environmental toxin that can provokes Parkinson's disease. In keeping with this, it is relevant to point out that paraquat exhibits a striking structural similarity to MPTP toxic metabolite MPP^+. Although ROS are incontestably involved in the deleterious mechanism by which paraquat kills dopaminergic neurons, the molecular link between oxidative stress and cell death in this model remains unknown. It appears, however, that paraquat can trigger the sequential activation of c-Jun N-terminal kinase (JNK), and caspase-3 both in vitro and in vivo (Peng et al., 2004), suggesting that JNK signaling pathways could mediate paraquat-induced neurodegeneration.

1.4.2 In vivo models

The brains of PD patients show evidence of impaired mitochondrial complex I function and of the generation of oxidative stress and these might, therefore, be crucial components of 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 evidences. Indeed, 6-OHDA and also other neurotixins poorly crosses the BBB, hence failing to accumulate within the brain parenchyma to meaningful neurotoxic concentrations following systemic injections. To circumvent this problem, 6-OHDA has to be injected directly into the brain either free hand or by stereotaxic means. Several local sites of injection have been used to damage the central dopaminergic pathways including intraventricular, intracisternal, and intracerebral (Jonsson et al., 1983). Discrete intrastriatal administrations of 6-OHDA in the rat can achieve such replication (Kirik et al., 1998). Striking examples of such preferential dorso-ventral 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 are 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).

Recent gene-targeting and transgenic strategies have generated 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) has launched a series of genetic models to study PD. In this sense, a model of PD by 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 occurs. 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 apparent in transgenic flies overexpressing A30P-mutant α-synuclein than in those with A53T-mutant or wild type α-synuclein. Nevertheless, transgenic flies fulfill 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 microinclusions 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 behavior 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).

In the last years, 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 were quickly reported 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 behavior (Yue, 2009). Certain lines exhibiting PD-related pathologies were reportedly used to evaluate LRRK2 kinase inhibitors in neuroprotection, revealing the potential value of the invertebrate LRRK2 models in drug screening (Liu et al., 2011). These models are useful for understanding 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).

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. 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 clinical relevant designs.

2. AIM OF THE STUDY

By means of multidisciplinary approaches, from molecular biology and biochemistry to confocal microscopy and behavior test, the specific aims of the study were: **(1)** To evaluate the expression of the three isoforms of Na^+/Ca^{2+} exchanger (NCX), NCX1, NCX2, NCX3, a plasma membrane protein that plays a prominent role in controlling intracellular homeostasis of Ca^{2+} and Na^+ ions, in animal and cellular models of PD represented by mutant α-synuclein A53T mice and primary neurons obtained from embryos of mutant α-synuclein A53T mice; **(2)** To verify the role of α-synuclein in the modulation of the expression and activity of the three isoforms of the Na^+/Ca^{2+} exchanger in A53T mice and in primary midbrain neurons; **(3)** To investigate whether the modulation of the Na^+/Ca^{2+} exchanger could be correlated with the loss of dopaminergic neurons in striatum and midbrain of transgenic A53T mice and in primary midbrain neurons; **(4)** To verify whether changes in NCX expression could be correlated with an impairment in locomotor activity of mutant α-synuclein A53T mice assessed by means of Pole test and Open Field test; **(5)** To understand the role played by each of these three isoforms in the progression of neuronal damage with particular regard to mitochondrial dysfunction; **(6)** To correlate the deregulation of intraneuronal Ca^{2+} ions homeostasis with the mitochondrial dysfunction in primary midbrain neurons from mutant α-synuclein A53T mice in order to define the temporal and spatial interplay between the two events.

In order to evaluate whether changes in NCX expression could be detected in mutant α-synuclein A53T mice during aging, molecular biology experiments were performed in midbrain and in striatum of mutant α-synuclein A53T and wild type 4, 10 and 16 months old mice. These experiments identified a reduction in protein expression of NCX2 and NCX3 isoforms in midbrain of A53T 4 and 10 months old transgenic mice respectively, in comparison to wild type mice. Conversely, NCX1 protein expression was increased in A53T 10 months old transgenic mice in striatum compared to wild type 10 months old mice. To correlate these results with the deposition of mutated α-synuclein, the expression of this protein was evaluated in WT and transgenic mice during aging. The results of these experiments showed that an accumulation of α-synuclein occurred during aging in both midbrain and striatum of A53T

transgenic mice. Moreover, to demonstrate that the alteration in NCX isoforms expression followed by an accumulation of α-synuclein might be correlated with a loss of dopaminergic neurons observed in PD, tyrosine hydroxylase protein expression was evaluated in midbrain and striatum of WT and transgenic mice during aging. These experiments demonstrated that dopaminergic neuronal loss occurred both in the midbrain of 4 and 10 months old mice and in the striatum of A53T transgenic 4, 10 and 16 months old mice. To verify whether the biochemical events observed correlate with changes in locomotor activity of A53T Tg mice, behavior tests were performed in these mice during aging. Locomotor activity was explored by means of Open Field test to evaluate the spontaneous activity and the Pole test to evaluate balance and bradykinesia. The results obtained by means of the Open Field test showed a decrease in the spontaneous motor activity in 16 months old transgenic mice, since they spent more time on longer rest during their exploration in the arena in comparison to wild type mice at the same age. Pole test was used to evaluate the imbalance and bradykinesia in this mouse model. Accordingly, mice were placed head upward on the top of a pole and the time spent to climb down to the pole until to the floor was recorded. The results obtained showed an impairment in motor activity during aging in transgenic mice. Indeed they took more time to climb down to the pole compared to wild type mice.

Parallel experiments were performed in vitro in primary midbrain neurons obtained from A53T transgenic mice embryos and in the respective wild type in which western blot experiments were performed to evaluate the modulation of NCX isoforms. The results of these experiments demonstrated that among the three isoforms, only NCX3 protein expression was reduced in A53T transgenic neurons. In addition, TH protein expression was also reduced in primary midbrain neuronal culture. Since NCX plays an important role in cellular calcium homeostasis and NCX3 is also localized on the outer mitochondrial membrane (Scorziello et al., 2013) its role in the regulation of in mitochondrial and cytosolic calcium concentrations was explored in primary midbrain neurons from embryos of mutant α-synuclein A53T and wild type mice by means of confocal microscopy techniques. The results obtained showed an increase in cytosolic calcium concentrations of A53T transgenic neurons compared to wild type. This

effect was associated with an increase in mitochondrial calcium concentration and of mitochondrial membrane hyperpolarization.

In conclusion the overall results described let to hypothesize a possible involvement of NCX1 and NCX3 in the degeneration of dopaminergic neurons in A53T transgenic mice probably associated with accumulation of α-synuclein, thus suggesting the identification of new molecular targets in the pathogenesis of PD.

3. EXPERIMENTAL PROCEDURES

3.1 In vivo and in vitro models

3.1.1. Transgenic mice for α-synuclein (A53T)

Mice that express human A53T α-syn under the control of *prion* promoter (Pmp-SNCA*A53T) (Giasson et al., 2002) were obtained from The Jackson Laboratory. Mice hemizygous for the A53T mutation were bred on a mixed C57Bl/6 x C3H background to produce transgenic and non-transgenic littermates. In all cases, 4, 10, 16-months old transgenic mice were directly compared with age-matched wild type littermates. To identify transgenic mice PCR amplifications were performed according to the protocol from The Jackson Laboratory. Mice were group housed (1-5 animals/cage) in temperature and humidity-controlled rooms under a 12-hours light-/dark cycle and fed ad libitum diet of standard mouse chow. Experiments were performed on male and female mice according to the international guidelines for animal research and approved by the Animal Care Committee of "Federico II" University of Naples, Italy.

3.1.2. Primary midbrain neurons from transgenic mice for α-synuclein (A53T)

Primary midbrain cultures were isolated from brains of 15 days-old A53T and wild type mice embryos, were prepared by modifying the previously described method of Fath and collaborators (Fath et al., 2009). The tissue was minced and incubated with a dissection medium containing MEM, $NaHCO_3$ and Dextrose for 30 minutes at 37°C. After incubation, the suspension was centrifuged and subjected to mechanical dissection in order to obtain a cellular suspension. Then the cells were placed on poly-D-lysine-coated (100µg/ml) plastic dishes, in MEM/F12 culture medium containing glucose, 5% deactivated fetal bovine serum, and 5% horse serum, glutamine (2mM), penicillin (50 U/ml), streptomycin (50 µg/ml). For confocal and calcium imaging experiments, cells were plated on glass coverslips coated with poly-D-lysine (Scorziello et al., 2007). The day after plating, cells were treated with Cytosine-β–D-arabino-furanoside in vitro (10 µM) to prevent the non-neuronal cell growth. Neurons

were cultured at 37°C in a humidified 5% CO_2 atmosphere and used after 10 days of the culture for all experiments described.

3.2 Western Blot Analysis

Mice brain tissue and primary neurons were lysed in a buffer containing Tris-HCl (20 mM, pH 7.5); NaF 10 mM; NaCl 150 mM; phenylmethylsulphonyl fluoride (PMSF) 1 mM; NONIDET P-40 1%; Na_3VO_4 1 mM; aprotinin 0.1%; pepstatin 0.7 mg/ml e leupeptin 1 µg/ml. Homogenates were centrifuged at 14.000 rpm for 20 minutes at 4ºC. Supernatant was used to perform western blot analysis. Protein levels were determined using Bradford method. The total protein used for each sample was 50µg and it was separated on 8% sodium dodecyl sulfate polyacrylamide gels with 5% sodium dodecyl sulphate stacking gel (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were blocked in 5% non-fat dry milk in 0,1% Tween 20 (TBS-T; 2mmol/l Tris HCl, 50mmol/l NaCl, pH 7,5) for 1 hour at room temperature and incubated overnight at 4ºC in the blocked buffer with the 1:5000 antibody for NCX1 (polyclonal mouse antibody), 1:1000 antibody for NCX2 (polyclonal rabbit antibody), 1:5000 antibody for NCX3 (polyclonal rabbit antibody), 1:1000 for *α*-synuclein (monoclonal rabbit antibody) and 1:10000 for Tyrosine Hydroxylase (monoclonal mouse 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 hour (1:2000) at room temperature. The immunoreactive bands were visualized by enhance chemiluminescence. The optical density of the bands (normalized with those of tubulin) was determined by *Image J* program.

3.3 Mitochondrial calcium concentrations $[Ca^{2+}]_m$, cytosolic calcium concentrations $[Ca^{2+}]_c$ and mitochondrial membrane potential measurements

To assess the $[Ca^{2+}]_m$, neurons obtained from embryo A53T and wild type mice were loaded with X-Rhod-1 (0.2 µM) for 15 min in a medium containing 156mM NaCl, 3mM KCl, 2mM $MgSO_4$, 1.25mM KH_2PO_4, 2mM $CaCl_2$, 10mM

glucose, and 10mM 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 mitochondria-localized intensity of fluorescence was indicative of mitochondria Ca^{2+} overload (Sisalli et al., 2014).

$[Ca^{2+}]_c$ was measured using the fluorescent dye Fluo-3AM acetixymethyl ester (Fluo-3AM). 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 (Secondo et al., 2007).

Mitochondrial membrane potential was evaluated using the fluorescent dye tetramethyl rhodamine ethyl ester (TMRE) in the “redistribution mode”. Cells were loaded with TMRE (20nM) for 30 minutes in the above described medium. At the end of the incubation, cells were washed in the same medium containing TMRE (20nM) and allowed to equilibrate (Livigni et al., 2006). A decline in mitochondria-localized intensity of fluorescence was indicative of mitochondrial membrane depolarization.

Confocal images were obtained using Zeiss inverted 510 confocal laser scanning microscopy and a 63X oil immersion objective. The illumination intensity of 543 Xenon laser used to excite X-Rhod-1 and TMRE, and of 488 Argon laser used to excite Fluo-3AM fluorescence, was kept to a minimum of 0.5% of laser output to avoid phototoxicity.

3.4 Evaluation of locomotor activity

3.4.1 Open Field Test

Spontaneous exploratory locomotor behavior was evaluated by means of the Open Field test. The Open Field test presents an appropriate paradigm for the phenotyping characterization of transgenic mice (Hinkle et al., 2012). The test has been successfully used with other familial Parkinson disease mouse models, for example mice deficient in parkin gene (Zhu et al., 2007). The behavior of the A53T transgenic mice and their respective wild type were evaluated at ages of 4, 10 and 16 months.

The open field apparatus consisted of a Plexiglas square arena (45 x 45 cm, 40 cm high) that was placed in a homogenously lit experimental room. For

each test, mice were placed individually into the center square and were allowed to explore it for 15 min. Total traveled distance was measured using a video-tracking software.

3.4.2 Pole Test

Pole test was first designed for use with mice by Ogawa and colleagues (Ogawa et al., 1985). It assesses the agility of animals and may be a measure of bradykinesia (Ogawa et al., 1985). This task involves skilled forelimb grasping and maneuvering, which would require an intact basal ganglia and activation of the rubrospinal pathway. This task, therefore, is very sensitive to nigrostriatal dysfunction (Fleming et al., 2004; Ogawa et al., 1985; Hwang et al., 2005; Ogawa et al., 1987; Matsumura et al., 1997).

The test consists by placing the mouse head up-ward on the top of a vertical rough-surfaced pole (diameter 1cm; height 55cm) and recording the time spent by the mouse to reach the floor. The behavior was scored until the mouse reached the floor. For each experimental section, animals received 3 trials and the average scorers were expressed in seconds.

3.4 Statistical analysis

Data were generated from a minimum of 3 independent experimental sessions for in vitro studies, and at least 8 animals for each experimental group for in vivo experiments. Calcium measurements were performed at least in 20 cells for each of the 3 independent experimental sessions. Data were expressed as mean±S.E.M. Statistical comparisons between transgenic and wild type mice and cells and their respective controls were performed using the one-way ANOVA test, followed by Newman Keul's test. P value <0.05 was considered statistically significant.

4. RESULTS

4.1 NCX expression in A53T transgenic mice during aging.

To investigate the expression of the three isoforms of NCX, i.e. NCX1, NCX2, NCX3, during aging, mice expressing human A53T variant α-synuclein were sacrificed at the age of 4, 10 and 16 months old. Brains were removed; midbrain and striatum were isolated and processed by Western blotting. The results obtained showed that NCX1 expression was increased only in the striatum of 10 months old transgenic mice (Fig. 3B) while its expression did not change in the midbrain of the same animals in comparison with 10 months old wild type mice (Fig. 3A).

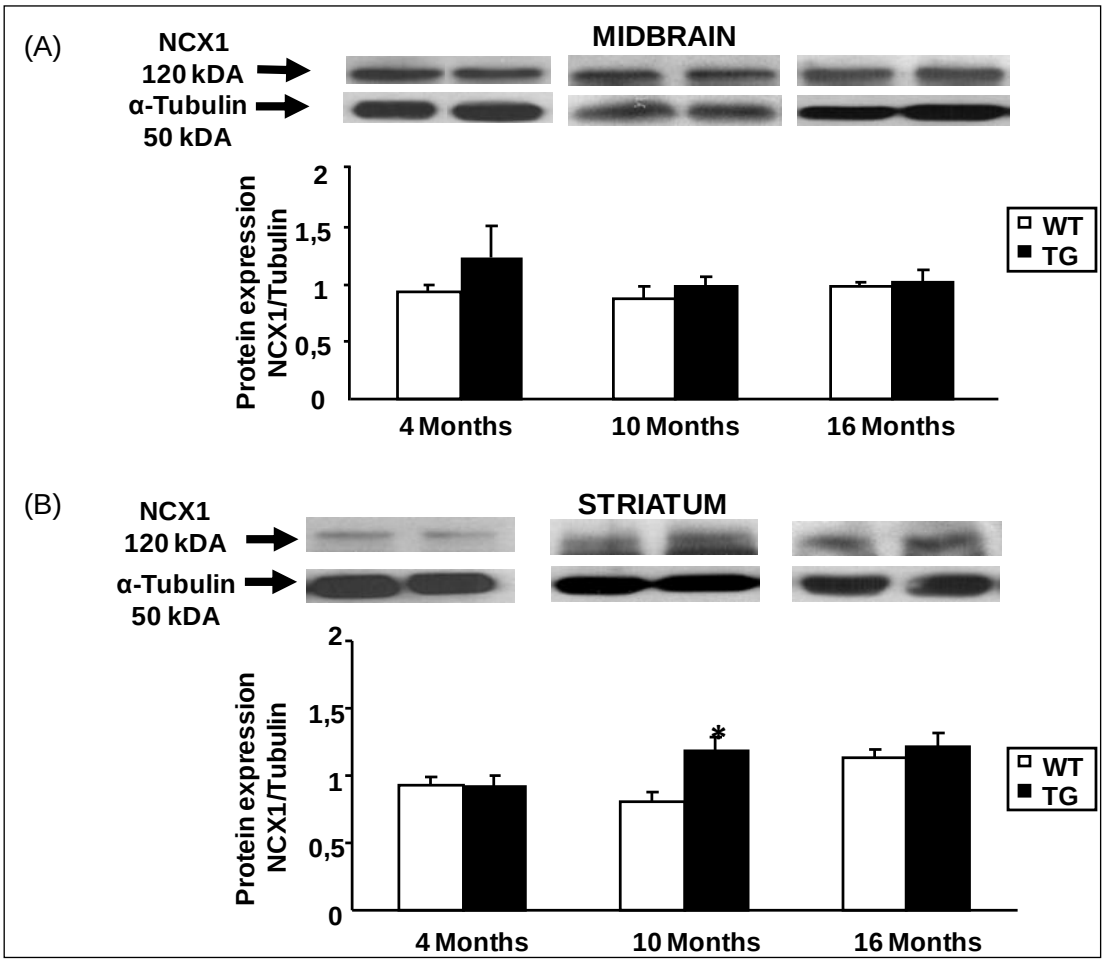

Fig. 3 NCX1 expression in midbrain (A) and in striatum (B) of 4, 10 and 16 months old A53T transgenic and wilde type mice. The bar graphs report the mean ±SEM of the densitometric values of NCX1 band intensity obtained in midbrain and striatum of transgenic and wild type mice and normalized to the respective tubulin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT controls.

Conversely, NCX2 expression did not change during aging in the striatum of transgenic mice compared to the respective Wt controls (Fig. 4B), whereas its expression was reduced in the midbrain of 4 months old mice (Fig. 4A) compared to the respective wild type mice.

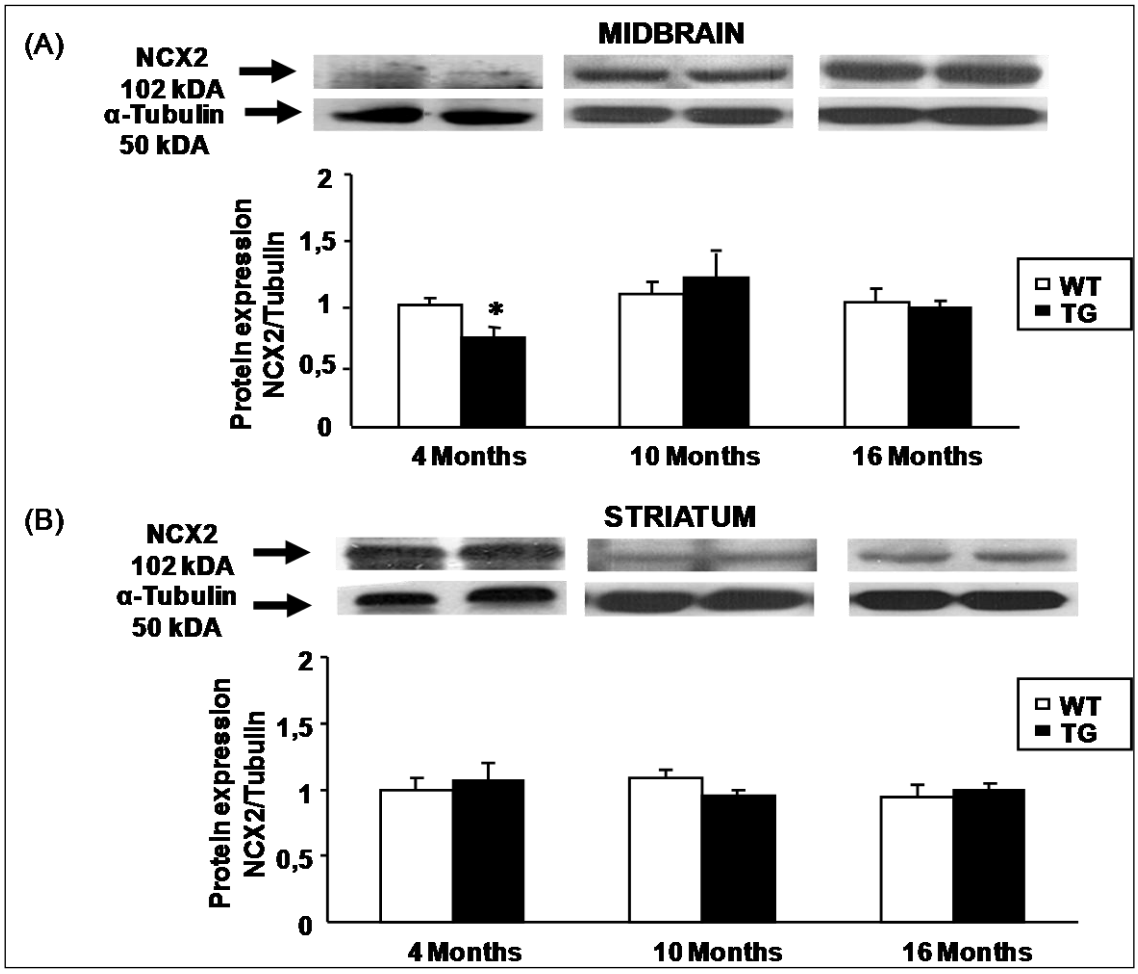

Fig. 4 NCX2 expression in midbrain (A) and in (B) striatum of 4, 10 and 16 months old A53T transgenic and wild type mice. The bar graphs report the mean ±SEM of the densitometric values of NCX2 band intensity obtained in midbrain and striatum of transgenic and wild type mice and normalized to the respective tubulin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT.

On the other hand, NCX3 expression was reduced only in the midbrain of 10 months old A53T transgenic mice respect to wild type (Fig 5A), whereas no change in the striatum was observed in Tg and Wt mice during aging (Fig 5B).

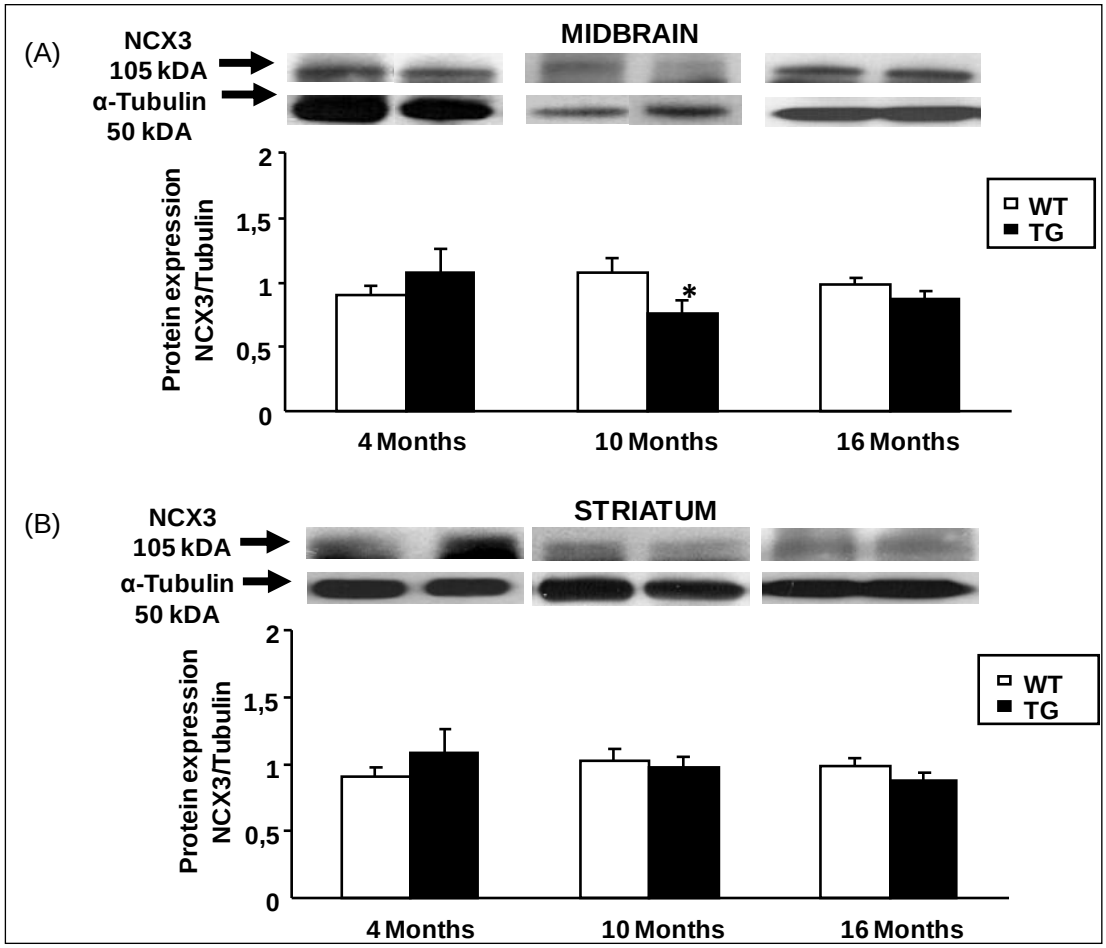

Fig. 5 NCX3 expression in midbrain (A) and (B) striatum of 4, 10 and 16 months old A53T transgenic and wild type mice. The bar graphs report the mean ±SEM of the densitometric values of NCX3 band intensity obtained in midbrain and striatum of transgenic and wild type mice and normalized to the respective tubulin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT.

4.2 α-synuclein expression in midbrain and striatum of A53T transgenic mice during aging

In order to verify whether changes in the expression of the different isoforms of NCX were correlated with an accumulation of α-synuclein in A53T transgenic mice, Western blotting experiments were performed in midbrain and in striatum of 4, 10 and 16 months old A53T transgenic mice to evaluate the

amount of α-synuclein protein expression during aging, since it is well known that α-synuclein deposition occurs in the Lewy bodies found in damaged neurons during PD (Spillantini et al., 1998a,b). The results obtained showed that an increase in α-synuclein expression occurs in both midbrain (Fig. 6A) and striatum (Fig.6B) of A53T transgenic mice during aging, whereas no α-synuclein accumulation was detected in Wt brain at the same times.

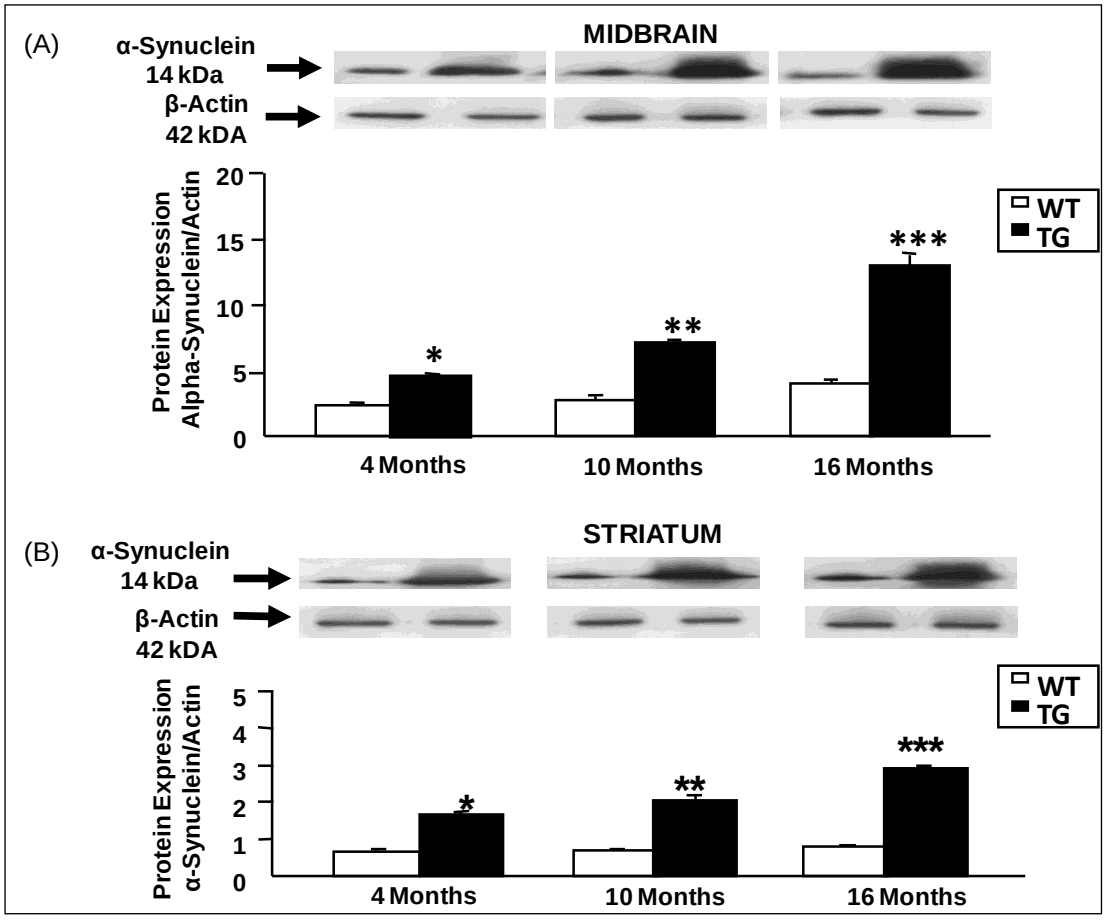

Fig. 6 α-synuclein expression in midbrain (A) and striatum (B) of 4, 10 and 16 months old A53T transgenic and wild type mice. The bar graphs report the mean ±SEM of the densitometric values of α-synuclein band intensity obtained in midbrain (A) and in striatum (B) of transgenic and wild type mice and normalized to the respective actin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT, **p<0.05 vs 4 months TG, ***p<0.05 vs 16 months TG.

4.3 Tirosyne Hydroxylase expression in A53T transgenic mice during aging.

Further experiments were performed to verify whether changes in NCX protein expression and α-synuclein accumulation might be correlated with loss of dopaminergic neurons in A53T transgenic mice in comparison to wild type controls. The dopaminergic neuronal loss was assessed by measuring the expression of the tyrosine hydroxylase (TH) enzyme, which is one of the main markers of dopaminergic neurons. The results obtained showed that in the striatum of A53T transgenic mice the expression of TH was reduced during aging in comparison with wild type controls (Fig.7B), whereas in the midbrain of A53T transgenic mice TH expression was reduced mainly in 4 and 10 months old A53T transgenic mice compared to respective wild type mice (Fig.7A).

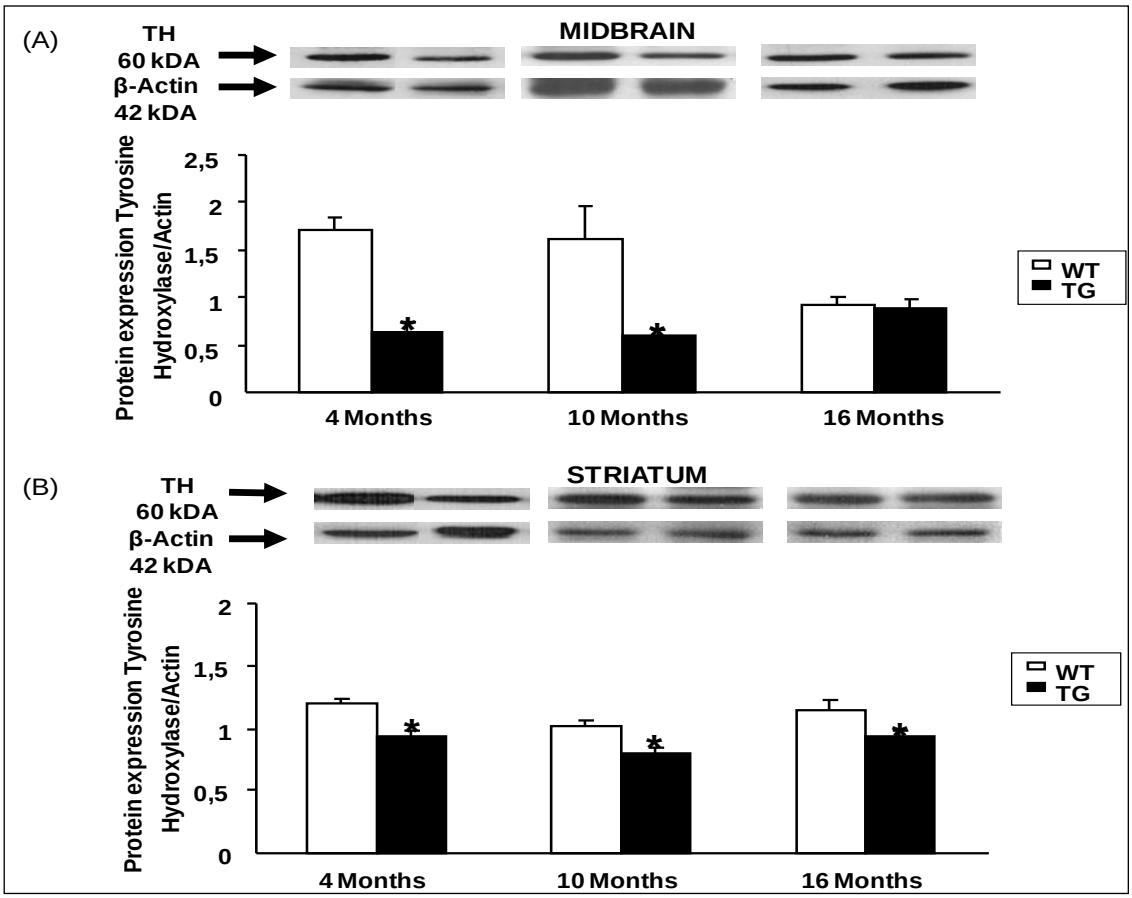

Fig. 7 Tyrosine Hydroxylase (TH) expression in midibrain (A) and in striatum (B) of 4, 10 and 16 months old A53T transgenic and wild type mice. The bar graphs report the mean ±SEM of the densitometric values of TH band intensity obtained in midbrain (A) and in striatum

(B) of transgenic and wild type mice and normalized to the respective actin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT.

4.4 Locomotor activity in A53T transgenic mice during aging.

In order to correlate the alterations in the expression of NCX isoforms, α-synuclein deposition and neuronal cell loss with motor impairment observed in mice expressing human A53T variant of α-synuclein during aging, the locomotor activity was assessed in these mice by means of Open Field and the Pole tests. The results obtained by means of Open Field test showed that in 16 months old transgenic mice a decrease in the spontaneous motor activity occurred. In fact, at this time, the ability of mice to explore the arena was lower compared with that observed in wild type mice at the same age (Fig. 8).

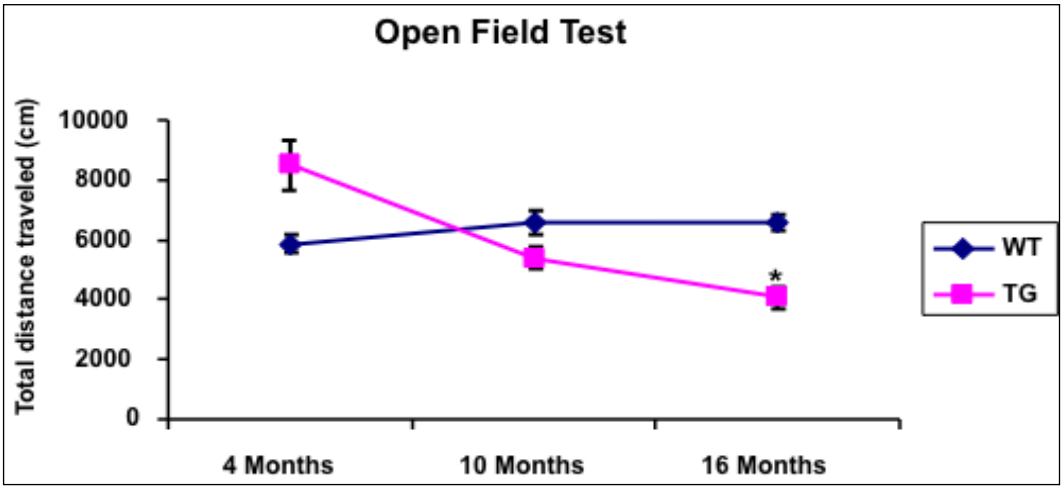

Fig. 8 Spontaneous motor activity in 4, 10, 16 months old Wild type (WT) and A53T transgenic mice (TG) monitored in the Open Field test. Values were reported as mean ±SEM. *p<0.05 Vs WT.

Pole test was used to evaluate the imbalance and the bradykinesia in this mouse model. Accordingly, mice were placed head upward on the top of a pole and the time spent to climb down to the pole until to the floor was recorded. The results obtained showed that an impairment in motor activity occurred in A53T transgenic mice during aging, since they took more time to climb down to the pole compared to wild type mice (Fig. 9).

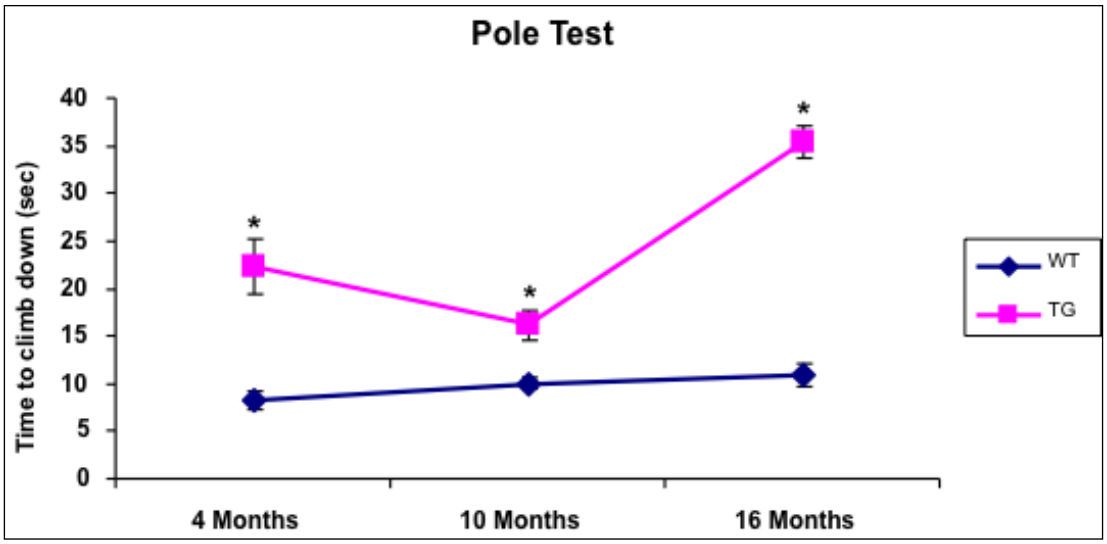

Fig. 9 The difference of the time spent to climb down from the pole in 4,10 and 16 months old wild type (WT) and A53T transgenic mice (TG). Values showed as mean ±SEM.*p<0.05 vs WT.

4.5 NCX and TH expression in midbrain neurons obtained from A53T transgenic mice.

Parallel experiments were performed *in vitro* in primary midbrain neurons obtained from A53T transgenic mice embryos and in the respective wild type neurons in which Western blot experiments were performed to evaluate the modulation of NCX isoforms expression. The results obtained showed that among the three isoforms of NCX, NCX3 is the only one whose expression was reduced in midbrain neurons (Fig.10).

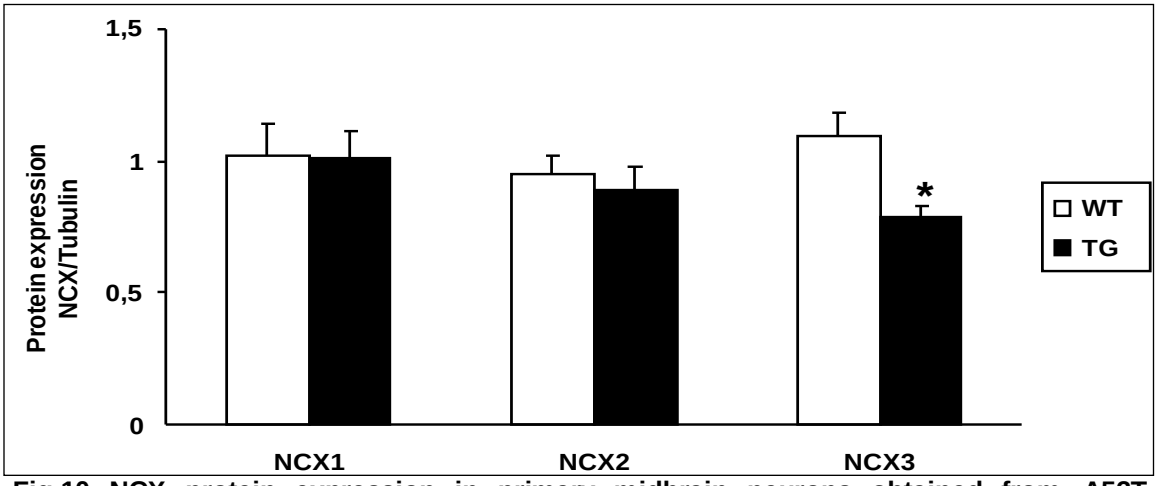

Fig.10 NCX protein expression in primary midbrain neurons obtained from A53T transgenic mice embryos. The bar graphs report the mean ±SEM of the densitometric values of NCX band intensity obtained from primary midbrain neurons of transgenic and wild type mice embryos and normalized to the respective tubulin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT.

To verify whether changes of NCX expression in primary midbrain neurons were correlated with dopaminergic neuronal loss, Western blotting experiments were conducted in these neurons to evaluate the amount of TH protein expression. The results showed that expression levels of TH were significantly reduced in primary midbrain neurons obtained from A53T transgenic mice compared to wild type neurons (Fig.11).

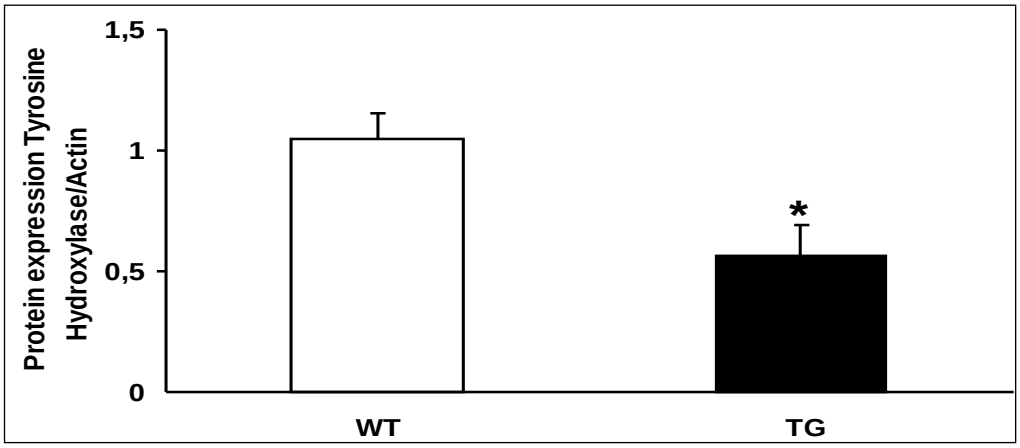

Fig. 11 Tyrosine Hydroxylase (TH) expression in primary midbrain neurons obtained from A53T transgenic mice embryos. The bar graphs report the mean ±SEM of the densitometric values of TH band intensity obtained from midbrain neurons of transgenic and wild type mice and normalized to the respective actin. WT= wild type; TG= transgenic A53T. *p<0.05 vs respective WT.

4.6 Cytosolic and Mitochondrial calcium concentrations and mitochondrial membrane potential in midbrain neurons obtained from A53T transgenic mice

Since it has been recently reported that NCX3 is the only NCX isoform found on the outer mitochondrial membrane where it plays an important role in regulating mitochondrial and cytosolic calcium homeostasis (Scorziello et al., 2013), further experiments have been performed to understand the role played by the impairment of NCX3 protein experession in the progression of neuronal damage. To this aim mitochondrial function was investigated in primary midbrain neurons obtained from A53T transgenic embryos. In particular, mitochondrial membrane potential and mitochondrial calcium concentrations were measured by means of confocal microscopy. Parallel experiments were performed to measure cytosolic calcium concentration. The results obtained showed that both mitochondrial (Fig.12A) and cytosolic (Fig.12B) calcium concentrations were increased in transgenic midbrain neurons compared to wild type neurons.

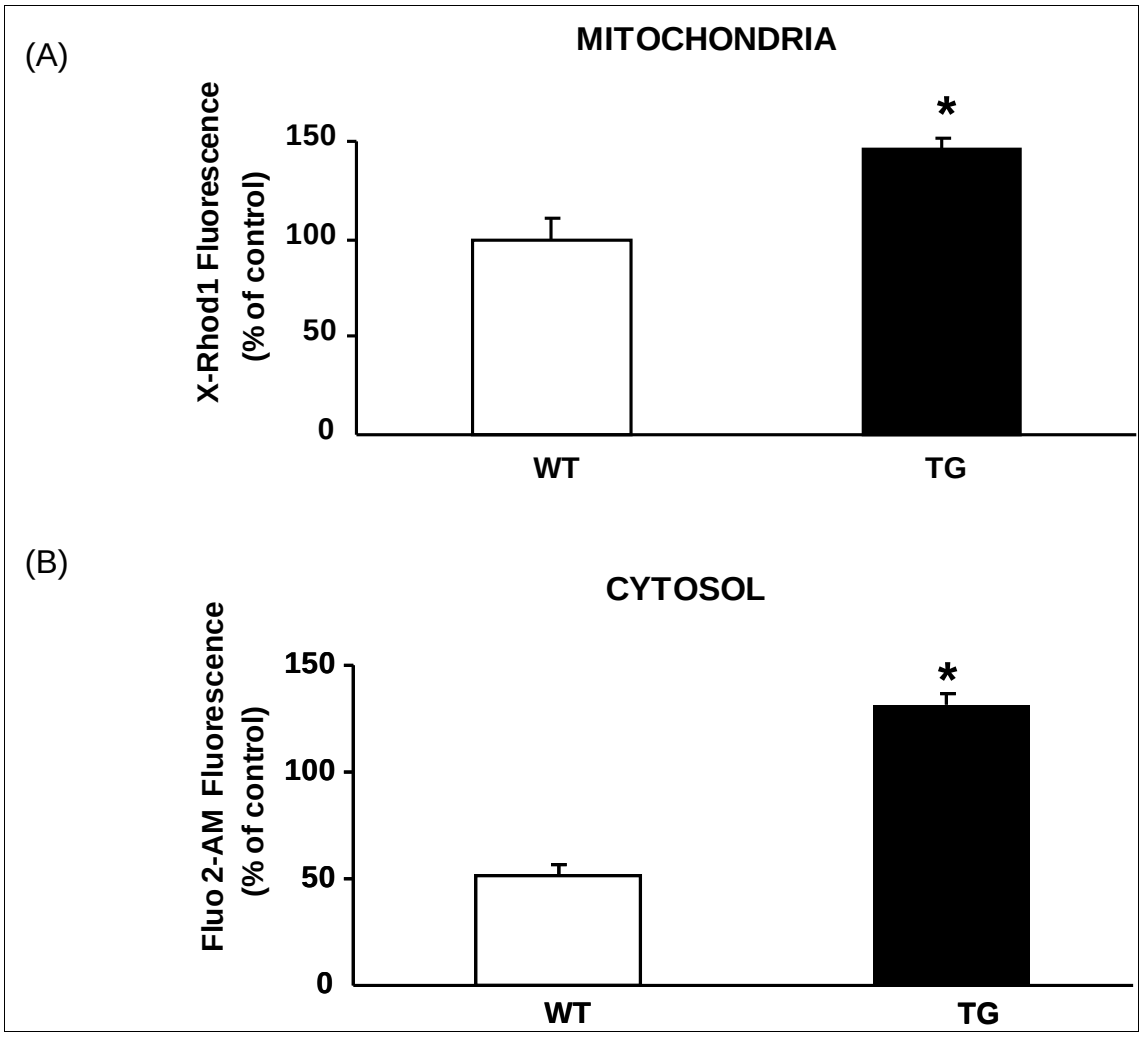

Fig. 12 Mitochondrial (A) and cytosolic (B) calcium concentrations in midbrain neurons obtained from A53T transgenic (TG) and wild type embryos (WT). Values were expressed as mean of the percentage ±SEM. *p<0.05 Vs WT

Interestingly, a significant mitochondrial hyperpolarisation was observed in midbrain neurons obtained from A53T transgenic mice compared to wild type neurons (Fig.13).

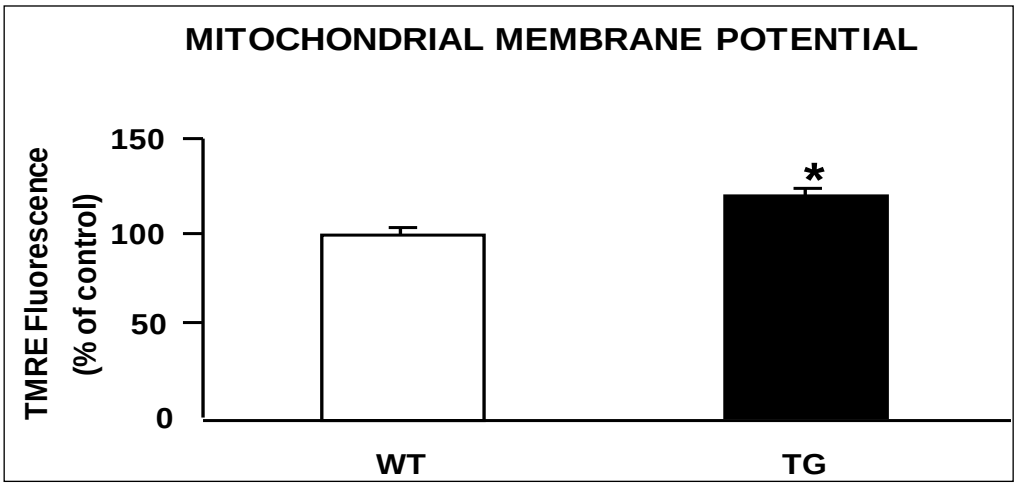

Fig. 13 Mitochondrial membrane potential in midbrain neurons obtained from A53T transgenic (TG) and wild type embryos (WT). Values were expressed as mean of the percentage ±SEM. *p<0.05 Vs WT.

5. DISCUSSION

The results of the present study demonstrate that the three isoforms of the Na^+/Ca^{2+} exchanger are differently modulated in midbrain and in striatum in A53T transgenic mice during aging, a genetic model of PD. In particular, in A53T 4 months old transgenic mice NCX2 is the only NCX isoform whose expression was reduced in midbrain, whereas the expression of NCX1 and NCX3 did not change in comparison to WT mice. Interestingly, at this time the expression of TH protein was reduced, thus suggesting that the decrease in NCX2 expression might be considered as an early maker of dopaminergic neuronal degeneration. This hypothesis is in line with results previously reported by Shin and Colaborators (Jeon et al 2003), who demonstrated that NCX2 works as a temporal regulator of calcium homeostasis and, as such is essential for synaptic plasticity. Therefore, we argued that in our experimental condition, the reduced expression of NCX2 by slowing intracellular calcium clearance, might stimulate DA release and in turn promote oxidative stress and neuronal loss.

Conversely, NCX1 protein expression was found increased in striatum in 10 months A53T transgenic old mice, whereas no changes in NCX1 expression were found in midbrain at this time. Moreover, an impairment in TH protein expression was also detected in striatum of A53T transgenic mice compared with WT 10 months old mice, thus suggesting that the increase in NCX1 might play a role in dopaminergic neuronal death. This hypothesis is in line with the results reported by Ago and colleagues (2011) in a mouse model of PD. In fact, they demonstrated that the inhibition of NCX1 by SEA0400 in mice treated with MPTP ameliorates motor activity and reduces dopaminergic neuronal loss probably by blocking ERK phosphorylation and lipid peroxidation, two effects downstream of NCX-mediated Ca^{2+} influx. These findings support the idea that NCX1 activation might have detrimental role in PD. Moreover, the fact that in our study the increase in NCX1 expression was detected in A53T transgenic mice bearing the human mutation of α-synuclein in that brain area in which an abnormal deposition of α-synuclein occurs, further highlight the involvement of NCX1 in the pathogenesis of PD.

Similarly, in A53T transgenic mice an increase in α-synuclein deposition was observed in midbrain during aging. However, in this brain area a reduction in NCX3 protein expression accompanied with an impairment in the expression of TH was detected. Conversely, in Wt mice no change in α-synuclein deposition was detected and consequently NCX3 protein expression and TH were normally expressed. These findings also suggest that a reduction in NCX3 expression might be correlated with neuronal damage in PD. It is well known that NCX1 and NCX3 are plasmamembrane proteins, which play a crucial role in the regulation of sodium and calcium homeostasis in physiological and pathological conditions (Annunziato et al., 2004; Pignataro et al., 2004; Pannaccione et al., 2012). Therefore, it is possible to speculate that an alteration in NCX1 and NCX3 protein expression in two brain regions, which represent the core of dopaminergic circuits might be associated with a perturbation in intracellular calcium concentration which in turn leads to the selective neuronal degeneration observed in PD. This hypothesis is supported by the finding that in the mouse model used in the present study, an impairment in locomotor activity and balance was observed during aging in comparison to wild type mice.

The hypothesis that the selective degeneration of SNpc neurons might be correlated to elevated intracellular calcium, thus suggesting an alteration of calcium homeostasis in the pathogenesis of PD, has been recently revitalized (Schmitz and Sulzer, 2007; Chan et al., 2007). Indeed, it has been reported that an increased activity of several classes of Ca^{2+} channels including L-type voltage-dependent– and cyclic nucleotide- sensitive channels seem to play a key role in the pathogenesis of PD, leading to an alteration of intracellular Ca^{2+} homeostasis. In this scenario, variation in NCX expression and activity, could assume a relevant role since it works, together with the other plasma membrane and sarco-endoplasmatic pumps, in setting $[Ca^{2+}]_i$ at its resting level (*i.e.*100-200 nM). Accordingly, a deregulation of $[Ca^{2+}]$ in cytosol and in intracellular Ca^{2+} stores has been also considered an important feature of neuronal degeneration in the SNpc a brain region in which a greater level of NCX1 expression has been detected (Papa et al., 2003). Moreover, the observation that cells deficient in complex-I showed an alteration in the cytosolic calcium handling, reduced mitochondrial calcium accumulation and consequent

ATP synthesis (Visch et al., 2004), suggested a possible relationship between mitochondrial dysfunction and perturbation of intracellular calcium homeostasis in the pathogenesis of PD.

It has recently reported that among the three isoforms of NCX, NCX3 is present on the outer mitochondrial membrane where it plays a role in mitochondrial calcium extrusion (Scorziello et al., 2013). In this regard, the results of the present study suggest that the increase in intracellular calcium concentration and mitochondrial calcium content observed in transgenic midbrain neurons is a consequence of NCX3 impairment. This finding strongly supports the hypothesis that in this model a relationship between the perturbation of intracellular calcium homeostasis and mitochondrial dysfunction leads to neuronal degeneration. Accordingly, there is increasing evidence that NCX3, highly expressed in the brain, plays a pivotal role in the maintenance of intracellular Na^+ and Ca^{2+} homeostasis in brain ischemia, thus mediating a neuroprotective effect (Annunziato et al., 2004; Boscia et al., 2006; Molinaro et al.,, 2008; et al., 2013). Moreover, the deletion of the NCX3 gene in mice has detrimental consequences on basal synaptic transmission, LTP regulation, spatial learning, and memory performance (Molinaro et al., 2011). Finally, it has been reported that the absence of NCX3 in the sarcolemma of the muscle fibers was associated with an impairment in neuromuscular transmission (Sokolow et al., 2004).

In conclusion, the results reported in the present study let to hypothesize that the changes in NCXs expression occurring in midbrain and in striatum of A53T mice correlates with loss of dopaminergic neurons probably due to an imbalance of the mechanisms involved in the regulation of intracellular calcium homeostasis. These findings reveal new potential targets useful to develop alternative strategies to treat Parkinson’s disease.

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