Universitat Autònoma de Barcelona

INITIATION, PROGRESSION AND EXTENSION OF PARKINSON'S DISEASE: ROLE OF α-SYNUCLEIN

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Fall seven times and stand up eight

(Japanese proverb)

Per vosaltres Family Trap!

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LIST OF ABREVIATORS

5-HT: serotonin
6-OHDA: 6-hydroxydopamine
AD: Alzheimer's disease
ANS: autonomous nervous system
AP: autophagosome
BP: binding potential
CJD: Creutzfeldt-Jakob disease
CK1: casein kinase 1
CK2: casein kinase 2
CMA: chaperone-mediated autophagy
CNS: central nervous system
CSF: cerebrospinal fluid
CSPα: Cystein string protein
DA: dopamine
DAG: diacylglycerol
DAT: dopamine transporter
DLB: dementia with Lewy bodies
DMV: dorsal motor nucleus of the vagus
ENS: enteric nervous system
ER: endoplasmic reticulum
GFAP: glial fibrillary acidic protein
hGH: human growth hormone
iPSC: induced pluripotent stem cells
IML: intermediolateral nucleus in the spinal cord
LB: Lewy bodies
LBD: Lewy bodies disease
LC: locus coeruleus
LN: Lewy neurites
LP: Lewy-related pathology
LMP: lysosomal membrane permeabilization
MAM: mitochondria-associated endoplasmic reticulum membranes
MCNSC: mouse cortical neuronal stemm cells
MFB: medial forebrain bundle
MPTP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP+: 1-methyl-4-phenylpyridinium
NAC: non-amyloid-β component
NBM: nucleus basalis of Meynert
NE: norepinephrine
NET: norepinephrine transporter
NM: neuromelanin
NSAID: non-steroidal anti-inflammatory drug
OD: optical density
PA: phosphatidic acid
PD: Parkinson's disease
PDGFβ: platelet-derived growth factor-β
PET: positron emission tomography
PFF: preformed fibrils

PLD2: phospholipase D2
PNS: peripheral nervous system
PPN: pedunculopontine nuclei
rAAV: recombinant adeno-associated virus
REM: rapid eye movement
RN: raphe nuclei
RRF: retrorubral field
SERT: serotonin transporter
siRNA: small interference RNA
SNpc: substantia nigra pars compacta
TFEB: transcriptional factor EB
TH: tyrosine-hydroxylase
UCH-L1: ubiquitin C-terminal hydrolase-L1
UPS: ubiquitin-proteasome system
VTA: ventral tegmental area

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown origin mainly characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the presence of intraneuronal proteinaceous cytoplasmic inclusions, called Lewy bodies (LB), in several affected brain areas. Although LB were identified a century ago, their significance to the pathogenic process in PD remains unknown.

Mounting evidence suggest that α-synuclein, a major protein component of LB, may be responsible for initiating and spreading the pathological process in PD. Supporting this concept, intracerebral inoculation of synthetic recombinant α-synuclein fibrils can trigger α-synuclein pathology in mice. However, it remains uncertain whether the observed pathogenic effects of recombinant synthetic α-synuclein can actually apply to PD-linked human α-synuclein and occur in species closer to humans. In this thesis, we addressed this question by assessing the potential pathogenic effect of inoculating α-synuclein-containing nigral LB extracts from PD patients into the brains of wild-type mice and macaque monkeys. Nigral LB containing pathological α-synuclein were purified from postmortem PD brains by sucrose gradient fractionation and subsequently inoculated into the SNpc or striatum of wild-type mice and macaque monkeys. In both mice and monkeys, intranigral or intrastriatal inoculations of PD-derived LB extracts resulted in progressive nigrostriatal neurodegeneration starting at striatal dopaminergic terminals. In LB-injected animals, exogenous human α-synuclein was quickly internalized within host neurons and triggered the pathological conversion of endogenous α-synuclein. At the onset of LB-induced neurodegeneration, host pathological α-synuclein diffusely accumulated within nigral neurons and anatomically interconnected brain regions. LB-induced pathogenic effects required both human α-synuclein present in LB extracts and host expression of α-synuclein. These results indicate that human α-synuclein species contained in PD-derived LB are pathogenic and have the capacity to initiate a PD-like pathological process.

Further supporting a pathogenic role of α-synuclein in PD, increased levels of this protein have been described in PD patients. Therefore, molecular tools able to reverse abnormal α-synuclein expression back to physiological levels might provide therapeutic benefit in PD. Based on this hypothesis, in the second aim of this thesis we assessed the feasibility and safety of downregulating α-synuclein expression *in vivo* specifically in PD-vulnerable neuronal pupulations by intranasal administration of cell-targeted small interfering RNA (siRNA) directed against α-synuclein. To achieve this goal, we performed first an *in vitro* screening of various siRNA sequences to select those able to downregulate basal or overexpressed α-synuclein without decreasing β- or γ-synuclein levels. Once identified, the selected molecule (SNCA499-siRNA) was then confirmed to be able to downregulate nigral α-synuclein mRNA *in vivo* by its local infusion in the SN of mice. This molecule was then chemically modified to enhance its biostability and conjugated to the cell-specific ligand indatraline (IND) to promote its selective delivery into aminergic neurons, the latter being validated in rat ventral midbrain primary cultures. Finally, intranasal administration of IND-SNCA499-siRNA to mice was able to selectively downregulate α-synuclein SNpc expression, both at mRNA and protein levels, without affecting the integrity of the dopaminergic nigrostriatal pathway. These results set the stage for future studies aimed at assessing the disease-modifying potential of intranasally delivered IND-SNCA499-siRNA in experimental PD models associated with increased α-synuclein levels.

INTRODUCTION

1. Parkinson's disease: an overview

Parkinson's disease (PD) is a chronic, progressive neurodegenerative movement disorder with no current treatment; it affects more than six million people worldwide (www.epda.eu.com), making it the most common neurodegenerative disorder after Alzheimer's disease (AD)[1]. The average age at onset of PD is 60, with age being an irrefutable risk factor[2]. In the coming decades, the prevalence of PD is expected to increase markedly due to population aging. Whereas in rare instances PD is inherited (familial PD), most cases are sporadic, and the underlying cause (if any) remains to be determined[3]. Various risk factors have been described for sporadic PD, including pesticide exposure, non-steroidal anti-inflammatory drug (NSAID) use and brain injury, but age remains the most important risk factor documented so far[4].

The diagnosis of PD is largely clinical, although there is no definitive test able to confirm the diagnosis prior to symptom onset, with the exception of gene testing in a reduced number of cases. Clinically, PD is characterized by a syndrome universally known as parkinsonism, which includes four cardinal features: bradykinesia (slowness of movements), resting tremor, rigidity, and postural instability. Although PD has been traditionally considered as a motor disorder, it is now well accepted that PD also presents non-motor symptoms. Some of these non-motor features, which may be present years or even decades before the motor signs[5, 6], include hyposmia (reduced ability to smell), rapid eye movement (REM) disorder, constipation, and depression[5-7,8-10]. It has been suggested that these non-motor symptoms may have a potential diagnostic utility in the early stages of PD. In addition, as PD progresses, frequent motor freezing and falls, treatment-related involuntary movements (dyskinesia), pain and sensory complaints, autonomic dysfunction (urinary incontinence and orthostatic intolerance), and neuropsychiatric manifestations (depression, hallucinations, and dementia) become prominent. These features are probably

due to the spread of the PD pathology to other areas of the brain[11, 12]. Although motor symptoms respond well to dopamine (DA) replacement therapy, most of the non-motor symptoms show little or no response to DA replacement and contribute substantially to overall disability, especially late in the disease.

Neuropathological features of PD. PD is characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) (Fig 1)[13]; these neurons contain the pigment neuromelanin (NM)[14], and project their unmyelinated axons rostrally via the medial forebrain bundle (MFB) to the striatum (composed of the caudate nucleus and putamen), where they release dopamine (DA). It is the loss of striatal DA in PD that results in the characteristic motor signs of this disease. It has been reported that, at

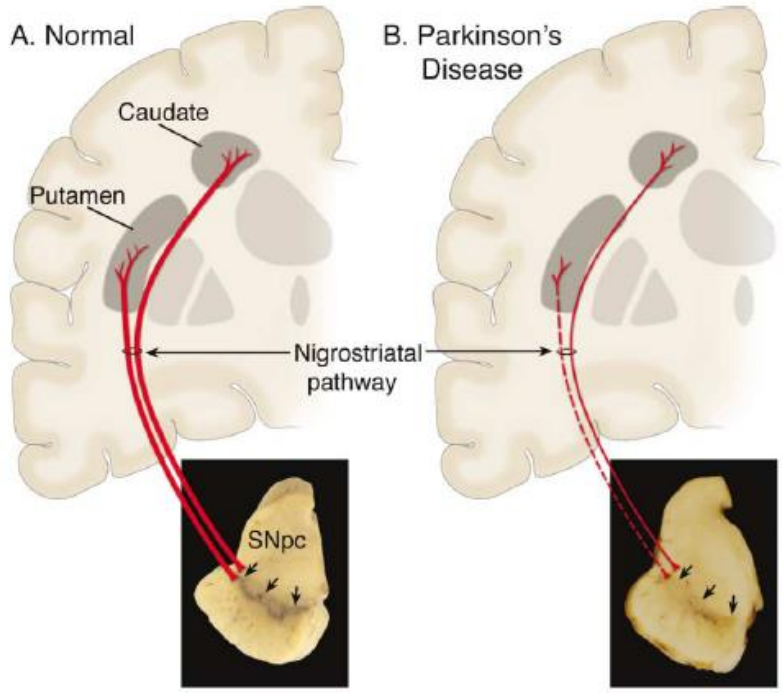


Figure 1. Neuropathology of Parkinson's disease. (A) Schematic representation of the normal nigrostriatal pathway (in red). It is composed of dopaminergic neurons whose cell bodies are located in the substantia nigra pars compacta (SNpc; see arrows). These neurons project (thick solid red lines) to the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

the time of motor symptoms onset, the extent of striatal dopaminergic marker loss (i.e. ~80%) exceeds that of SN DA neurons (i.e. ~30-60%)[15]. In addition, depending on disease duration at the time of death, while 60-80% of SN dopaminergic neurons might have been lost[16,17], there is a much more profound loss of striatal dopaminergic markers[18-20]. In particular, the loss of dopaminergic markers in the striatum (i.e. dorsal putamen) occurs rapidly and is virtually completed by 4 years post-diagnosis[21]. Taken together, these data suggest that the degenerative process in PD may start at the striatal dopaminergic terminals and that neuronal death may result from a "dying back" process[22, 23].

In addition to nigrostriatal degeneration, PD is also characterized by the presence of intraneuronal proteinaceous cytoplasmic inclusions, termed "Lewy Bodies" (LB). Although LB were first described by Friedrich Lewy more than a century ago[24], their significance in the pathogenesis of PD remains unknown[25]. Two morphological types of LB are found in PD: classical (midbrain) and cortical. The aspect of classical LB in hematoxylin and eosine-stained NM-containing SNpc dopaminergic neurons is that of one or more eosinophilic spherical bodies (8-30 μm diameter) with a dense core surrounded by a peripheral halo (Fig. 2A). Ultrastructural examination by electron microscopy revealed a dense granulovesicular core surrounded by a ring of radiating 8-10 nm fibrils[26]. Classical LB are found in several affected regions of the PD brain, including the nucleus basalis of Meynert (NBM), raphe nuclei (RN), locus coeruleus (LC) and SN. Unlike classical LB observed in SNpc dopaminergic neurons, cortical LB are characterized by pale staining and are poorly circumscribed (Fig. 2B). In addition to classical and cortical LB, weakly stained neuronal cytoplasmic inclusions called "pale bodies" are found in pigmented brainstem neurons of the SN and LC[27, 28]. Several pieces of evidence suggest that pale bodies may be early cytologic alterations that precede the classical manifestation of LB[29, 30]. LB are not specific for PD, since they are also found in AD[31] and in "dementia with Lewy bodies" (DLB)[32, 29] in which early cognitive impairment is accompanied by prominent cortical LB

pathology. In addition, nigral LB are also found in people of advanced age without clinical evidence of PD or other neurodegenerative disease[33]; this is known as incidental LB disease (LBD) and could be associated with a pre-symptomatic early stage of PD[34].

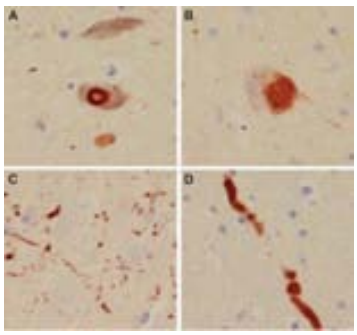


Figure 2. Microscopic findings in PD with α-synuclein immunohistochemistry. A typical brainstem type Lewy body (A) and a pale staining "cortical type" Lewy body (B), Lewy neurites in CA2 sector of hippocampus (C), and intraneuritic Lewy bodies in the medulla (D). From Dickson D.W. (2012) *Cold Spring Harb. Perspect. Med. doi:10.1101/cshperspect.a009258*

Based on immunohistochemistry studies, one of the main components of LB is the protein α-synuclein[35-37]. The presence of α-synuclein in cytoplasmic inclusions represents an aberrant cytological localization of this protein, which is normally located in presynaptic terminals. α-Synuclein is deposited in LB in a β-sheet-rich, fibrillar structure. In addition, several forms of post-translational modified α-synuclein are found in LB, including phosphorylated, nitrated and truncated α-synuclein[38-40], which are believed to promote the abnormal structural conformation of α-synuclein. In addition to α-synuclein, a large number of other constituents have been described in LB, such as ubiquitin, neurofilaments and the ubiquitin binding protein p62 (see Table 1). Accompanying LB (which are located in neuronal perikarya), gross dystrophic neurites, called Lewy neurites (LN), containing α-ynuclein and ubiquitin inclusions are common in PD pathology (Fig. 2C). The combination of LB and LN is sometimes referred to as Lewy-related pathology (LP)[41].

Besides SNpc dopaminergic neurons, a significant number of other central and peripheral neuronal populations exhibit LP, phenotypic dysregulation, or degeneration PD patients (Table 2). While LP has been extensively studied[42], cell loss maps in postmortem PD samples using modern stereological approaches are not common[43]. Within the central

Reported LB components	References
αβ-Crystallin	Lowe, J. et al. (1990) Lancet **336**, 515-516.
α-Synuclein	Spillantini, M. G. et al. (1997)Nature**388**, 839-840.
Calcium-calmodulin-dependent protein kinase II (CaM kinase II)	Iwatsubo, T. et al. (1991) Acta Neuropathol. **82,** 159-163.
Calbindin D_{28K}	Yamada, T. et al. (1990) Brain Res. **526**, 303-307.
Chondroitin sulfate	DeWitt, D. A. et al. (1994) Brain Res. **656,** 205-209.
Chromogranin A	Nishimura, M. et al. (1994) Brain Res. **634,** 339-344.
Clusterin/apolipoprotein J	Sasaki, K. et al. (2002) Acta Neurol. **104,** 225-230.
Cochaperone C terminus of Hsp-70-interacting protein (CHIP)	Shin, Y. et al. Klucken (2005) J. Biol. Chem. **280,** 23727-23734.
Complement proteins (C3d, C4d, C7, and C9)	Yamada, T. et al. (1992) Acta Neuropathol. **84,** 100-104.
Cyclin-dependent kinase 5 (cdk5)	Brion, J.-P. et al. (1995) Am. J. Pathol. **147**, 1465 -1476.
Cytochrome c	Hashimoto, M. et al. (1999) J. Biol. Chem.**274,** 28849-28852.
DJ-1	Jin, J. et al. (2005) Mol. Brain Res. **134,** 119-138.
Dorfin	Hishikawa, N. et al. (2003) Am. J. Pathol.**163,** 609-619.
14-3-3 protein	Kawamoto, Y. et al. (2002) J. Neuropathol. Exp. Neurol.**61,** 245-253.
Gelsolin-related amyloid protein Finnish type	Wisniewski, T. et al. (1991) Am. J. Pathol. **138,** 1077-1083.
Heat-shock proteins 27, 40, 70, 60, 90, and 110	Auluck, P. K. et al. (2002) Science **295,** 865-868. McLean, P. J. et al. (2002) J. Neurochem. **83,** 846-854 .
Phosphorylated IκBα	Noda, K. et al. (2005) Biochem. Biophys. Res. Commun. **331**, 309-317.
Lipids	Gai, W. P. et al. (2000) Exp. Neurol. **166,** 324-333.
Microtubule-associated protein 2 (MAP-2)	D'Andrea, M. et al. (2001) Neurosci. Lett.< /I> **306,** 137-140.
MAP-5/MAP-1b	Jensen, P. H. et al. (2000) J. Biol. Chem. **275,**21500-21507. Gai, W. P, et al. (1996) Acta Neuropathol. **91,** 78-81.
Mitochondria	Roy, S. & Wolman, I. (1969) J. Pathol. **99,** 39-44. Hayashida, K. et al. (1993) Acta Neuropathol. (Berlin) **85,** 445-448. Gai, W. P. et al. (2000) Exp. Neurol. **166,** 324-333.
Multicatalytic proteinase	Masaki, T. et al. (1994) J. Neurol. Sci.< /I> **122,** 127-134.
MxA protein	Yamada, T. (1995) Neurosci. Lett. **195,** 41-44.
NEDD8	Dil Kuazi, A. et al. (2003) J. Pathol.**199,** 259-66.
Neurofilaments	Galloway, P. G. & Mulvihill, P. (1992) Am. J. Pathol. **140,** 809-822. Galvin, J. E. et al. (1999) Adv. Neurol. **80**, 313-324.
NFκB	Noda, K. et al. (2005) Biochem. Biophys. Res. Commun. **331**, 309-317.
Omi/HtrA2	Strauss, K. M. et al. (2005) Hum. Mol. Genet. **14,** 2099-2111.
$P35^{nck5a}$	Nakamura, S. et al. (1997) Acta Neuropathol .**94,** 153-157.
p62/sequestosome 1	Kuusisto, E, et al. (2003) J. Neuropathol. Exp. Neurol. **62,** 1241-1253.
Pael-R	Murakami, T. et al. (2004) Ann. Neurol. **55,** 439-442.
ROC1	Noda, K. et al. (2005) Biochem. Biophys. Res. Commun. **331**, 309-317.
Sphingomyelin	den Hartog Jager, W. A. (1969) Arch. Neurol. **21,** 615-619.
Superoxide dismutase 1 (Cu/Zn superoxide dismutase)	Nishiyama, K. et al. (1995) Acta Neuropathol. **89,** 471-474.
Superoxide dismutase 2 (Mn superoxide dismutase)	Nishiyama, K. et al. (1995) Acta Neuropathol. **89,** 471-474.
Synaptic vesicle-specific protein	Wakabayashi, K. et al. (1992) Neurosci. Lett. **138,** 237-240.
Synphilin-1	Wakabayashi, K. et al. (2000) Ann. Neurol. **47,** 521-523.
Synaptophysin	Nishimura, M. et al. (1994) Brain Res. **634,** 339-344.
Tau	Ishizawa, T. et al. (2003) J. Neuropathol. Exp. Neurol. **62,** 389-397.
TorsinA	McLean, P. J. et al. (2002) J. Neurochem. **83,** 846-854 . Sharma, N. et al. (2001) Am. J. Pathol. **159**, 339 -344.
Tubulin	Galloway, P. G. et al. (1988) J. Neuropathol. Exp. Neurol. **47,** 654-663.
Tyrosine hydroxylase	Nakashima, S. & Ikuta, F. (1984) J. Neurol. Sci. **66,** 91-96.
Ubiquitin	Lowe, J. et al. (1988) J. Pathol. **155**, 9-15.
Ubiquitin C-terminal hydroxylase	Lowe, J. et al. (1990) J. Pathol. **161,** 153-160.

Table 1. Described components of Lewy bodies (LB)

neurons in the SNpc (i.e. DA neurons) and LC [i.e. norepinephrine (NE) neurons] in nearly all PD patients, and of neurons of the dorsal motor nucleus of the vagus (DMV) nerve in most patients. Loss of RN, ventral tegmental area (VTA), retrorubral field (RRF), pedunculopontine nuclei (PPN) and NBM neurons in PD appears common but variable in extent. In the peripheral nervous system (PNS), LP is found in several types of neurons within the autonomous nervous system (ANS) in PD patients, but the only neurons that are known to be lost in this disease are NE neurons innervating the heart and skin, and DA neurons of the enteric nervous system (ENS)[43]. The loss of these PNS neurons might be responsible for orthostatic hypotension, sweating, and constipation that frequently

Brain region	PD pathology
Amygdala	Consistent / severe
Hippocampus	Variable / moderate
Temporal cortex	Variable / moderate
Cingualte cortex	Variable / moderate
Superior frontal gyrus	Uncommon / mild
Motor cortex	Spared
Caudate/putamen	Uncommon / mild
Globus pallidus	Spared
Basal nucleus of Meynert	Consistent / severe
Hypothalamus	Consistent / severe
Thalamus	Spared
Subthalamic nucleus	Spared
Red nucleus	Spared
Substantia nigra	Consistent / severe
Oculomotor complex	Variable / moderate
Midbrain tectum	Spared
Locus coeruleus	Consistent / severe
Pontine tegmentum (including raphe and pedunculopontine nuclei)	Variable / moderate
Pontine nuclein (including pontocerebellar fibers)	Spared
Medullary tegmentum (including dorsal motor nucleus of vagus)	Consistent / severe
Inferior olive (including olivocerebellar fibers)	Spared
Dentate nucleus	Spared
Cerebellar white matter	Spared

Table 2. Described PD pathology. From Dickson DW (2012)Cold Spring Harb. Perspect. Med. doi: 10.1101/cshperspect.a009258

accompany PD. Several risk factors have been proposed to explain the vulnerability of these seemingly diverse sets of neurons within the CNS and PNS in PD, including autonomous pace-making neuronal activity, broad action potentials, low intrinsic calcium buffering capacity, poorly myelinated, long and highly branched axons and terminal fields, the presence of catecholamine-derived NM pigment (e.g. in the SN and LC) and the use of monoamine neurotransmitters [e.g. DA, NE and serotonin (5-HT)][43].

Several reactive changes in astrocytes and microglia have been described to accompany neuronal loss in PD[44], including the expression of activation markers in microglia (e.g. the class II major histocompatibility antigen HLA-DR) and the presence of hypertrophic astrocytes that accumulate the intermediate filament protein, glial fibrillary acidic protein (GFAP). Dying neurons undergo phagocytosis by microglia, a term referred to as neuronophagia. In the SN and LC, NM pigment in the cytoplasm of microglia, which normally do not contain this pigment, serves as evidence of neuronophagia.

The relationship between α-synuclein deposition and neuronal dysfunction remains unclear. However, several observations suggest that α-synuclein deposition itself may not produce dysfunction. These observations include the fact that: (i) LP in the ENS is not always associated with cell loss[45], and that (ii) older individuals (up to 30% of centenarians) exhibit extensive synucleinopathy with no clear neurological symptoms (e.g., ILBD)[46, 47].

2. Pathogenic role of α-synuclein in Parkinson's disease

α-Synuclein is a 14kDa protein consisting of 140 amino acids (pK of 4.7)[48] that is characterized by an amphipathic lysine-rich amino terminus, which has a crucial role in regulating this protein's interaction with membranes, and a disordered acidic carboxy-terminal tail, which is implicated in regulating its nuclear localization, along with its interactions with metals, small molecules and proteins[49, 50]. The central region of α-

synuclein, which is known as the non-amyloid-β component of AD amyloid plaques (NAC), contains a highly hydrophobic motif that comprises amino acid residues 65-90 which has the propensity to adopt the β-sheet structure required for the formation of oligomeric species. α-Synuclein is known as a natively unfolded protein (i.e. in aqueous solution does not have a defined structure) that can adopt α-helical structures when it is bound to negatively charged lipids (such as phospholipids present on cellular membranes) and β-sheet-rich structures during prolonged periods of incubation. Recent findings suggest that α-synuclein may adopt a tetrameric structure under physiological conditions[51, 52], although this tetrameric form remains controversial and requires further investigation[53, 54].

α-Synuclein protein is widely expressed in many neuronal populations within both the CNS and PNS. At a subcellular level, α-synuclein is localized only to presynaptic terminals and portions of the nucleus – hence the name synuclein (Fig. 3)[55]. α-Synuclein is a member of the synuclein family of proteins, which also include β- and γ-synuclein. All three members of this family are predominantly neuronal proteins that, under physiological conditions, localize preferentially to presynaptic terminals[56]. What largely differentiates α-synuclein from the other members is the NAC region, which is only present in α-synuclein. The NAC region is required for the oligomerization and fibrilization of α-synuclein, since it has been reported that deletion or disruption of this NAC domain through the addition

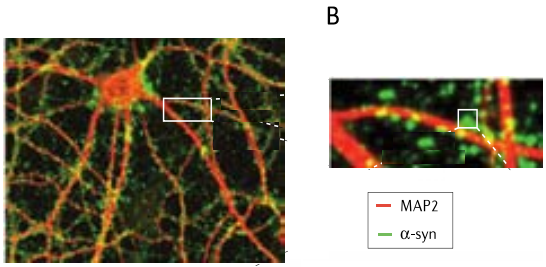


Figure 3. α-Synuclein localization. Wide field (A) and magnified (B) images of cultured cortical neurons form a postnatal day 1 wild-type mouse. A neuronal dendrite (as revealed by MAP2 immunostaining: red) is shown opposed to α-synuclein-positive presynaptic densities (green), indicating that α-synuclein is located in presynaptic terminals. Adapted from Lashuel HA et al., (2013) *Nat. Rev. Neurosci.*

of a charged amino acids abolishes α-synuclein's ability to form the amyloid fibrils [57] that aggregate within LB and LN. Whether α-synuclein is expressed at all under physiological conditions in glia is somewhat controversial; it would appear that, if it exists, such expression is present at very low levels[58].

Although the exact function of α-synuclein remains unknown, substantial evidence suggests that α-synuclein function is related to its capacity to interact directly with membrane phospholipids, particularly highly curved membranes such as vesicles. In fact, α-synuclein seems to play a role in neurotransmission release, vesicle trafficking and mitochondrial function (see "Interaction between membranes and α-synuclein").

2-1 α-Synuclein mutations in PD

Although the vast majority of PD patients are idiopathic, in rare instances PD is inherited. To date, three different missense mutations in the gene encoding for α-synuclein (*SNCA*) have been identified to cause autosomal-dominant forms of PD. These *SNCA* mutations – A53T, A30P and E64K (Fig. 4) – are extremely rare and found in familial PD. Of these, the A53T mutation seems to be by far the most common, having been identified in one Italian, eight Greek, two Korean and one Swedish family[59 60 61 62-64], while the A30P

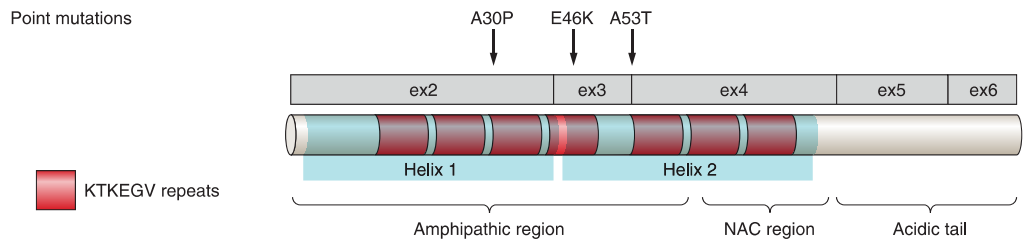


Figure 4. Schematic representation of the α-synuclein protein with the three pathogenic missense mutations above the protein organization. α-Synuclein is a 140-amino acid protein with seven imperfect repeats (KTEGV) in the NH_2 terminus, a central hydrophobic NAC (nonamyloid component) region, and an acidic COOH-terminal region. Adapted from Corti O. (2011) *Physiol. Rev.*

and E64K mutations were identified in one German and one Spanish family, respectively[65, 66].

Clinically, patients with the *SNCA* A53T mutation have a broadly varying phenotype, ranging from typical late-onset PD to atypical PD with more severe features, including an earlier age at onset, more rapid progression, and a heightened prevalence of dementia, psychiatric problems, and autonomic dysfunction[61, 63, 67-69]. The clinical symptoms of patients with the *SNCA* A30P mutation closely resemble those with idiopathic PD, with a late age at onset and a mild phenotype[70]. Carriers of E64K, however, have severe parkinsonism, with an early age at onset and diffuse Lewy body dementia[66]. The brain pathology of patients with *SNCA* mutations is characterized by an abundant α-synuclein pathology involving the brainstem, limbic areas and neocortex.

These PD-associated genetic mutations alter α-synuclein's structure in different ways. The A53T mutation effectively expands the hydrophobic domain from 11 amino acids to ~30 amino acids by destabilizing the α-helical domain between residues 51 and 66[71]. This expanded hydrophobic core confers gain-of-function toxicity[71] by facilitating the protein's ability to adopt the β-sheet structure required for the formation of oligomeric species[72-74]. The A30P mutation disrupts the first α-helical domain of α-synuclein and reduces its affinity for phospholipids[75]. Finally, the E64K mutation is thought to elicit toxicity by altering α-synuclein's interaction with anionic phospholipids, thereby exposing the protein's hydrophobic surfaces for potential intermolecular interactions[76].

2-2 α-Synuclein aggregation

Although α-synuclein is a natively unfolded protein, it can adopt several conformations, characterized as oligomers, protofibrils and fibrils (Figure 5), which have been associated with the pathogenesis of PD. The cascade of events starting from the natively unfolded protein and culminating in mature fibril formation is collectively termed α-synuclein aggregation (Box 1). Fibrils of α-synuclein, which are composed of cross-β-sheet structures, are detected mostly in LB[77, 78]. Indeed, LB and LN contain 5-10 nm filaments that appear to be composed primarily, if not exclusively, of α-synuclein[37]. In classical LB, the pale-staining halo, which contains filaments as identified by Electron microscopy, labels more-strongly for α-synuclein than the acidophilic core[25]. Moreover, dystrophic neurites and cortical LB contain similar α-synuclein filaments[79].

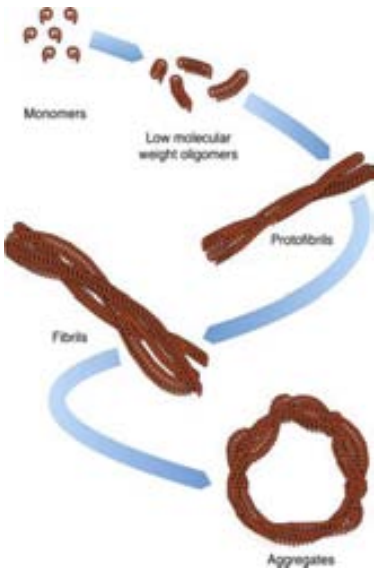


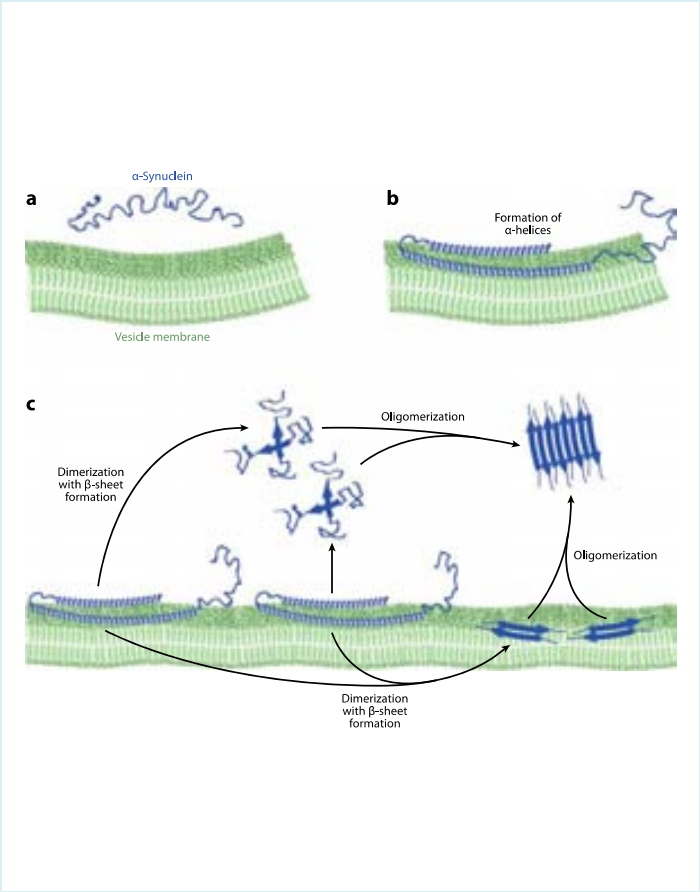
Figure 5. α-Synuclein conformations. From Eisbach S.E. et al. (2013) *J Mol Med.*

It has been proposed that oligomers, as opposed to mature fibrils, may represent the toxic species in PD. This concept first came from observations *in vitro* that acceleration of oligomerization, not fibrillization, is a shared property of both A53T and A30P α-synuclein mutations linked to PD[80]. While the A53T mutation accelerated the process of fibrillization, the A30P mutation fibrillized more slowly than wild-type α-synuclein. However, both mutations accelerated the oligomerization process compared to the wild-type α-synuclein, suggesting that the formation of oligomers, rather than fibrils, is likely to be critical in the pathogenesis of PD. Soon after, *in vitro* studies demonstrated that α-

synuclein oligomers, in contrast to the monomeric and fibrillar forms of this protein, bind synthetic vesicles very tightly via a β-sheet-rich structure, resulting in a permeabilization of these vesicles[81] by a pore-like mechanism[82]. Interestingly, A53T and A30P mutant α-synuclein exhibited higher permeabilizing activities compared to wild-type α-synuclein Inappropriate membrane permeabilization by α-synuclein oligomeric forms could cause degeneration and death of neurons via several mechanisms. These include: (i) up-regulated calcium flux into the cytosol[83], (ii) depolarization of the mitochondrial membrane[84], and (iii) leakage of DA into the cytosol; elevation of cytoplasmic DA leads to cell death[85].

More recently, viral overexpression of α-synuclein variants that form oligomers rather than fibrils (i.e. oligomer-forming) induced a more severe dopaminergic loss in the rat SN compared with fibril-promoting α-synuclein variants[86]. Consistent with the previous

Box 1: α-Synuclein aggregation process



α-Synuclein requires lipid interactions to form dimers, oligomers, and mature amyloid fibrils. (a) In solution, α-synuclein is natively unfolded. (b) In the presence of vesicle membranes containing anionic phospholipids, the N-terminus of α-synuclein forms two α-helices that allow it to associate with the surface of the membrane. (c) Stabilization of α-synuclein's membrane interactions (through α-synuclein mutations) or increased α-synuclein concentration (through genetic multiplication) facilitates the formation of α-synuclein dimers on the membrane surface or in the cytoplasm. Through dimerization, α-synuclein adopts a β-sheet secondary structure that, through association with α-synuclein monomers or other dimers, leads to oligomer formation. These oligomers seed fibril formation and deposit as amyloid within Lewy bodies and Lewy neurites. From Auluck P.K. et al., (2010) *Annu. Rev. Cell Dev. Biol.*

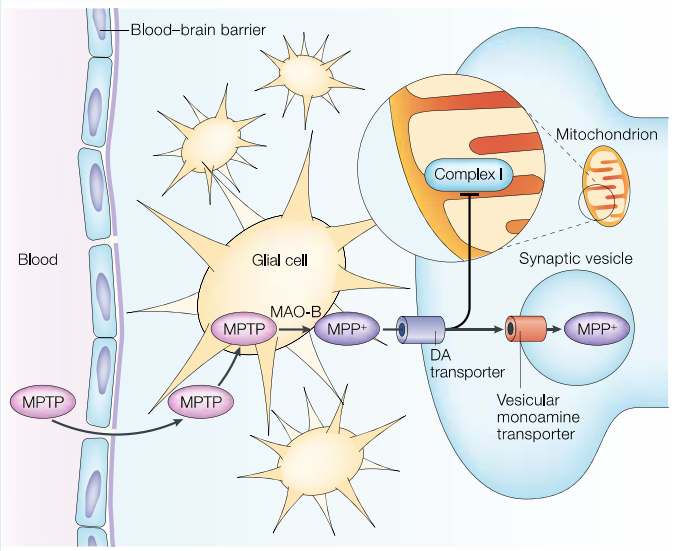
in vitro results, the toxicity observed in this synuclein rat model was associated with alterations in the permeability and integrity of the cell membrane induced by a membrane-associated α-synuclein oligomer. In addition to membrane alterations, α-synuclein oligomers may also cause toxicity by damaging mitochondria[87], triggering lysosomal leakage[88], or disrupting microtubules[89]. In this context, it was demonstrated that α-synuclein oligomers interfered with the axonal transport of synaptic proteins such as synapsin 1, resulting in dysfunctional synapses and eventual neurodegeneration[90]. Although the relationships between the various α-synuclein conformations and the mechanisms of interconversion between these conformations remain poorly understood, the conversion of α-synuclein to a toxic oligomeric form (or forms) might be influenced by interactions with lipids or small molecules and post-translational modifications, including phosphorylation, oxidative stress and truncation (see "α-Synuclein post-translational modifications").

2-3 Increased levels of α-synuclein and PD

Mounting evidence suggests that increased levels of α-synuclein are toxic and may contribute to the neurodegeneration process in PD. One of the first links between PD and α-synuclein up-regulation was reported by Vila et al., in the MPTP mouse model of PD (Box 2). Using a regimen involving chronic exposure to MPTP, α-synuclein protein expression in mouse midbrain extracts, together with the number of α-synuclein-positive neurons within the SNpc, progressively increased from 0 to 4 days after MPTP administration[91]. Interestingly, all α-synuclein-positive neurons in the SNpc were TH-positive, suggesting that α-synuclein up-regulation after exposure to MPTP occurs specifically within dopaminergic neurons. Consistent with protein alterations, α-synuclein mRNA also increased in a time-dependent manner in the midbrain of these mice following chronic MPTP intoxication, peaking at 4 days after intoxication. Importantly, the time-

course of these α-synuclein accumulations paralleled that of MPTP-induced dopaminergic neurodegeneration[92]. Taken together, these results raised the possibility that α-synuclein up-regulation contributed to the cascade of deleterious events that ultimately cause neuronal death. In line with observations in MPTP-treated mice, monkeys injected with a single dose of MPTP exhibited a significant increase in α-synuclein mRNA and protein levels one week and one month after intoxication compared with control monkeys[93]. In addition, MPTP-treated monkeys displayed an increased number of α-synuclein-positive

Box 2. The MPTP model of Parkinson's disease



1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a by-product of the chemical synthesis of a meperidine analogue with potent heroin-like effects that can induce a parkinsonian syndrome in humans almost indistinguishable from PD. Since the discovery that MPTP causes parkinsonism in humans and non-human primates, as well as in various other mammalian species, it has been used extensively as a model of PD. From neuropathological data, MPTP administration causes damage to the nigrostrial dopamine (DA) pathway identical to that seen in PD, with the exception of the intraneuronal inclusions knows as Lewy bodies. It is worthwile to note that postmortem brain samples from patients with PD show a selective defect in the mitochondrial electron transport chain complex that is affected by MPTP. The metabolism of MPTP is a complex, multistep process (see figure). After its systemic administration, MPTP, which is a pro-toxin, rapidly crosses the blood-brain barrier and is metabolized to 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) by the enzyme monoamine oxidase B (MAO-B) in non-DA cells, and then, probably by spontaneous oxidation, to 1-methyl-4-phenylpyridinium (MPP+), the active toxic compound. MPP+ is then taken up by DA transporters, for which it has high affinity. Once inside DA neurons, MPP+ is concentrated by an active process within the mitochondria, where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain. The inhibition of complex I impedes the flow of electrons along the mitochondrial electron transport chain, resulting in an increased production of free radicals, which causes oxidative stress and activation of programmed cell death molecular pathways. From Vila M. & Przedborsky S. (2003) *Nat. Rev. Neurosci.*

cells in the SN one month after MPTP-treatment. The fact that increased α-synuclein levels were seen at the same time that MPTP-induced neurodegeneration commenced in monkeys (i.e. one week post-exposure) further supported the possibility that increased α-synuclein might contribute to neuronal injury. Similarly, enhanced levels of α-synuclein protein were also observed in another mouse model of PD-like nigrostriatal degeneration induced by weekly injections of the toxic herbicide parquet[94].

Although the phenotype of α-synuclein-deficient mice is relatively modest, this animal displays resistance to the MPTP-induced degeneration of DA neurons, suggesting that α-synuclein may contribute to cell death[95]. Moreover, the use of recombinant adeno-associated virus (rAAV) to induce overexpression of human wild-type and A53T mutant α-synuclein in the nigrostriatal DA neurons of adult rats promoted nigrostriatal degeneration (i.e. 30-80% loss of SN cell bodies and 40-50% of striatal fibers 8 weeks after viral injection)[96]. A similar loss of cell bodies and fibers in the nigrostriatal pathway was reported in monkeys injected with rAVV to induce expression of human wild-type and A53T mutant α-synuclein in the SN[97]. These results demonstrated that the mere overexpression of wild-type or mutant α-synuclein is capable of promoting cell death.

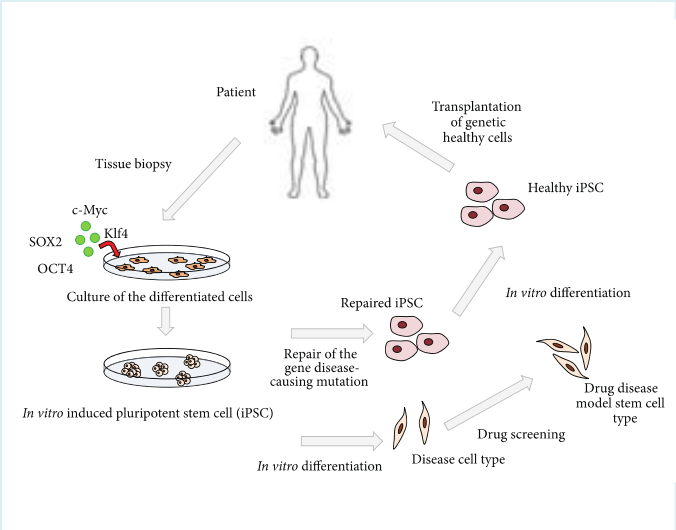
The concept that increased α-synuclein expression is sufficient to produce PD was further corroborated in humans, when duplications and triplications of the chromosomal region surrounding the α-synuclein gene were found to produce dominantly inherited PD[98-100]. While the *SNCA* duplication produces a form of PD similar in onset and symptoms to that of sporadic PD, the triplication causes an exceptionally severe phenotype, with much earlier onset and prominent cognitive as well motor impairment[98, 101, 102]. These findings suggest a dose-dependent correlation of α-synuclein load to the PD phenotype. In addition, polymorphisms in the Rep1 region 10kB upstream of the *SNCA*

promoter are associated with increased risk for PD, likely via a mechanism of α-synuclein overexpression[103-105]. Moreover, UV-laser microdissection and RT-qPCR showed increased α-synuclein mRNA levels in surviving NM- and TH-positive SN dopaminergic neurons from idiopathic PD brains compared to controls[106]. Taken together, these results strengthen the pathophysiologic role of the transcriptional dysregulation of α-synuclein expression in PD.

In addition to the above, α-synuclein accumulation is associated with decreased nigrostriatal activity. Chu and Kordower demonstrated an age-related accumulation of α-synuclein protein within individual nigral neurons both in humans and non-human primates[107]. In particular, increases of 269% and 639% in the number of α-synuclein-positive neurons in middle-aged and elderly human subjects, respectively, were observed relative to a young cohorts[107]. Moreover, the intracellular levels of α-synuclein were increased 56.6% in the aged individuals. These results are of significant relevance for PD, since aging remains the most compelling and prominent risk factor for the development of PD. But the most critical aspect of the Chun and Kordower study was that accumulations of α-synuclein were strongly associated with the loss of DA phenotype, which is one of the earliest cellular manifestations seen within the SN in PD[16, 108-110]. In both humans and monkeys, accumulation of α-synuclein within nigral perikarya is associated with decreased TH expression within those same neurons. Indeed, for both species, regardless of age, decreased TH was only observed within nigral neurons displaying detectable α-synuclein. On the other hand, and also regardless of age, adjacent neurons without detectable synuclein expression displayed stable levels of TH[107]. More recently, it was demonstrated that induced pluripotent stem cells (iPSC) (Box 3) generated from PD patients carrying the G2019S mutation in the PD-associated *LRRK2* gene exhibited α-synuclein accumulation

both at the protein and mRNA levels[111, 112], further supporting the relationship between α-synuclein up-regulation and PD.

Box 3 . Human induced pluripotent stem cells.



Induced pluripotent stem cells (iPSC) are the product of somatic cell reprogramming to an embrionyc-like-state. Differentiated cells are acquired by biopsies from human tissues and *in vitro* cultured in the presence of stem cell transcription factors, such as SOX2, c-Myc, OCT4 and KlF4. After induction of pluripotency phenotype, the cells, known as iPSCs, can be used for redifferentiation into specific disease cell types, for drug screening, or for correction of genomic defects, as well as healthy cells in regenerative theparies. From Fernandez T de S et al. (2013) *Biomed Res Int.*

2.4 α-Synuclein post-translational modifications

α-Synuclein can suffer different post-translational modifications, including phosphorylation, nitration and truncation. Mounting evidence suggests that these modifications could increase the pathogenic role of α-synuclein by promoting the formation of toxic oligomers[80, 113].

Phosphorylation. α-Synuclein phosphorylated at serine129 (Ser129)[39, 114] is present in LB, indicating that α-synuclein phosphorylation may have a role in the pathogenesis of PD. However, α-synuclein phosphorylation is a normal event in the human brain, as demonstrated in brain samples from control subjects and patients with LBD and tauopathies[114]. In that study, all of the human brains examined (including controls) contained phosphorylated α-synuclein at Ser129 in several brain regions, such as the

frontal cortex, putamen, caudate nucleus and cerebellum, with the SN and NBM being the regions with the highest levels of phosphorylated α-synuclein. Consistent with these results, α-synuclein is constitutively phosphorylated in human kidney 293 cells as well as in rat PC12 cells[115]. In particular, α-synuclein can be phosphorylated by a subset of protein kinases, including casein kinase 1 (CK1), CK2 or G protein-coupled receptor kinases[115,116]. Although these results indicate that phosphorylation of α-synuclein is a normal event in human brains, most of the α-synuclein is not phosphorylated under physiological conditions *in vivo*[115], suggesting that the extensive phosphorylation of α-synuclein at Ser129 in PD brains may represent a pathological event. Supporting this idea, phosphorylated α-synuclein at Ser129 induced the formation of a ~4 fold increase in β-sheeted fibrils compared to unphosphorylated α-synuclein as demonstrated by an *in vitro* assay followed by SDS-PAGE analysis[39]. Moreover, the inhibition of phosphorylation at Ser129 in SH-SY5Y cells decreased the formation of inclusions promoted by the overexpression of both human α-synuclein and synphilin-1[117]. These results strongly support the notion that α-synuclein phosphorylation at Ser129 may play a role in PD inclusion formation. The pathological role of α-synuclein phosphorylation at Ser129 has also been reported in transgenic mice expressing human α-synuclein under the control of a Thy-1 promoter. Enhancing α-synuclein phosphatase activity in these transgenic mice, which resulted in a reduced level of α-synuclein phosphorylation, reduced the typical neuropathology and behavioral deficits associated with these transgenic mice (i.e. decreased α-synuclein aggregation, improved neuronal activity and dendritic arborization, reduced astroglial and microglial activation)[118]. Similar results have been reported in *Drosophila* overexpressing human α-synuclein, in which alteration of Ser129 to the phosphorylation-incompetent residue alanine completely attenuated α-synuclein toxicity in *Drosophila*. In contrast,

mutation of Ser129 to aspartate to mimic phosphorylation and overexpression of *Drosophila* G protein-coupled receptor kinase, which increased the phosphorylation of α-synuclein at Ser129, significantly enhanced α-synuclein-induced neurotoxicity[119]. In particular, α-synuclein phosphorylation at Ser129 may induce toxicity in *Drosophila* by promoting the accumulation of toxic α-synuclein oligomeric forms[120] and inhibiting the interaction of α-synuclein with the cell membrane as reported in *in vitro* assays[116].

Although Ser129 phosphorylation is the most studied event of this type, the α-synuclein serine 87 (Ser87) residue can also be phosphorylated[121], and is present in LB[121]. In addition to serine, α-synuclein can also be phosphorylated at tyrosine 125 (Tyr125) in transgenic *Drosophila* expressing wild-type human α-synuclein[120]. Interestingly, Tyr125 phosphorylation is protective against α-synuclein neurotoxicity in the *Drosophila* model of PD. In particular, levels of soluble oligomeric species of α-synuclein in this PD model were decreased by Tyr125 phosphorylation. Strikingly, Tyr125 phosphorylation is diminished during the normal aging process in both humans and flies, and cortical tissue from patients with DLB showed reduced phosphorylation at Tyr125[120]. Taken together, these results suggest that Ser129 may promote the formation of toxic α-synuclein oligomers, while Tyr125 phosphorylation may inhibit its formation.

Nitration. Several studies have demonstrated that nitrated α-synuclein is also present in LB and LN of PD brains[38, 122, 123], and may play a role in the pathogenesis of PD. According to this idea, nitration of human recombinant α-synuclein induced by exposure to nitrating agents (e.g. peroxynitrite/CO_2 or myeloperoxidase/H_2O_2/nitrite) promoted the formation of α-synuclein oligomers[124]. In addition, the nitration of α-synuclein occurs in the mouse striatum and ventral midbrain following MPTP intoxication[125]. Although the significance of α-synuclein tyrosine nitration remains unclear, tyrosine nitration induces

secondary and tertiary structural alterations, which may critically modify protein function[125] and could facilitate interactions with other proteins, thereby promoting protein aggregation. Moreover, nitration decreases the binding capacity of α-synuclein to lipid vesicles and provides α-synuclein with heightened resistance to proteolysis[126]. Because nitration is an oxidative modification promoted by peroxynitrite (i.e. an oxidizing and nitrating agent that results from the combination of superoxide and nitric oxide), impairment of cellular antioxidative mechanisms or overproduction of reactive oxygen species may be a primary event leading to the onset and progression of neurodegenerative synucleinopathies. Further supporting this idea, antioxidant compounds (such as tannic acid, curcumin and rosmarinic) inhibited the formation of α-synuclein fibrils and destabilized preformed α-synuclein fibrils *in vitro*[127].

Truncation. Another modification that may be important in the pathogenesis of PD is the carboxy-terminal (C-terminal) truncation of α-synuclein, which leads to fragments lacking part or all of the acidic tail[40]. Like phosphorylation, α-synuclein truncation is a normal event in the adult human brain[114]. However, C-terminal truncation may have a pathological role, as evidenced by: (i) the enhanced accumulation of C-terminal-truncated α-synuclein in the SH-SY5Y cell line and transgenic mice expressing familial PD-linked mutants[40], (ii) the enrichment of C-terminal-truncated α-synuclein in aggregates from human brains with α-synuclein pathology[40], (iii) a C-terminal-enhanced ability to aggregate in a cell-free *in vitro* self-assembly assay compared to full-length α-synuclein[128], and (iv) enhanced neurotoxicity *in vivo*, since the expression of C-terminal-truncated α-synuclein in *Drosophila* showed increased aggregation into large inclusion bodies, increased accumulation of high molecular weight α-synuclein species, and increased loss of dopaminergic neurons in the dorsomedial cluster compared with flies expressing

wild-type α-synuclein[128]. In particular, lysosomes may have a role in the truncation of α-synuclein at the C-terminal, since lysosomal inhibition in SH-SY5Y cells expressing human α-synuclein blocked the formation of truncated forms of α-synuclein[114].

2.5 Interaction between membranes and α-synuclein

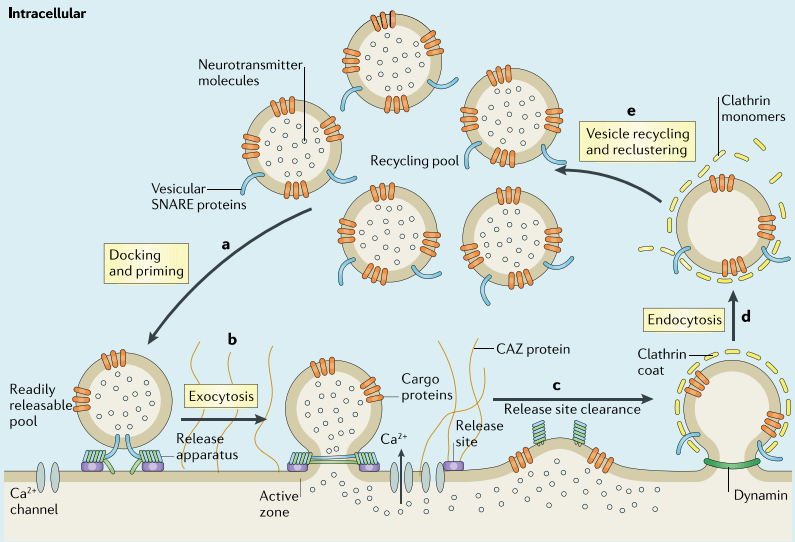
Although the exact function of α-synuclein is still unclear, substantial evidence now exists to suggest that it interacts directly with lipids and membranes, both physiologically as well as pathologically.

α-Synuclein and neurotransmismitter release

The presynaptic location of synuclein in addition to its capacity to interact with membranes, strongly suggests a role of α-synuclein in transmitter release. Indeed, it has been suggested that α-synuclein normally acts to regulate the pool of vesicles available in the synaptic bouton (Box 4). Supporting this idea, in mice lacking α-synuclein, dopaminergic synapses inappropriately release excessive quantities of neurotransmitter in response to multiple stimuli[129]. Moreover, in primary hippocampal neuronal cultures, antisense RNA knockdown of α-synuclein induced a marked reduction (~50%) in the number of vesicles present in the distal pool, while the number of vesicles docked at the synaptic plasma membrane was unchanged[130]. These results suggest that α-synuclein may have a role in regulating the store of vesicles available for transmitter release. Consistent with this idea, hippocampal synapses prepared from mice lacking α-synuclein showed a marked decrease in the pool of undocked synaptic vesicles, whereas the number of docked vesicles was not affected[131]. In particular, it has been suggested that α-synuclein can function as a negative regulator of DA release. Using adrenal chromaffin cells from

transgenic mice and PC12 cell lines overexpressing human wild-type or mutant A30P α-synuclein, Larsen and coworkers[132] hypothesized that α-synuclein inhibits the "priming" step, a reaction that transfers morphologically-docked vesicles to a fusion-competent state (Fig. 6). Both chromaffin cells and PC12 cells exhibited a significant decrease in the amount of stimulation-dependent DA released, which was not attributable to decreased Ca^{2+} entry or decreased sensitivity to Ca^{+}. Moreover, the number of molecules released per fusion event (quantal size) in chromaffin cells was unaffected by α-synuclein expression. In contrast, the number of fusion events in cells from α-synuclein-expressing mice was significantly decreased. Electron microscopy studies revealed that α-synuclein-overexpressing cell lines exhibited a marked accumulation of morphologically-docked

Box 4: Overview of the synaptic vesicle cycle.



Docked and primed synaptic vesicles (a) constitute the readily releasable pool. Following Ca2+ influx they undergo exocytosis (b) and release neurotransmitter into the synaptic cleft where these neurotransmitter molecules can activate postsynaptic receptors. Exocytosis occurs preferentially at release sites within specialized areas of the presynaptic membrane, called active zones, which are defined by their spatial proximity to voltage-activated Ca2+ channels and, presumably, by the presence of cytoplasmic matrix of the active zone (CAZ) scaffolding proteins. To maintain the availability of release sites for subsequent fusion reactions, release sites have to undergo clearance (c), an enigmatic step connecting the exocytic and endocytic limbs of the synaptic vesicle cycle. Synaptic vesicle endocytosis is predominantly mediated by a clathrin- and dynamin-dependent pathway involving endocytic scaffolds and specialized synaptic vesicle sorting adaptors (d). Following clathrin uncoating and concomitant neurotransmitter uptake, synaptic vesicles return to the recycling pool, where they undergo clustering (e). Haucke V. et al., (2011) Nat. Rev. Neurosci.

vesicles. Taken together, these data indicate that α-synuclein overexpression inhibits evoked transmitter release, which is consistent with the previous finding that α-synuclein can inhibit membrane fusion[133, 134], independently of SNARE proteins[134]. In particular, it has been suggested that α-synuclein's membrane association and dissociation cycle is regulated by Rab3a machinery[135]. Rab pull-down assays using brain homogenates from mice overexpressing mutant A30P α-synuclein confirmed that α-synuclein interacts with Rab3a protein[136].

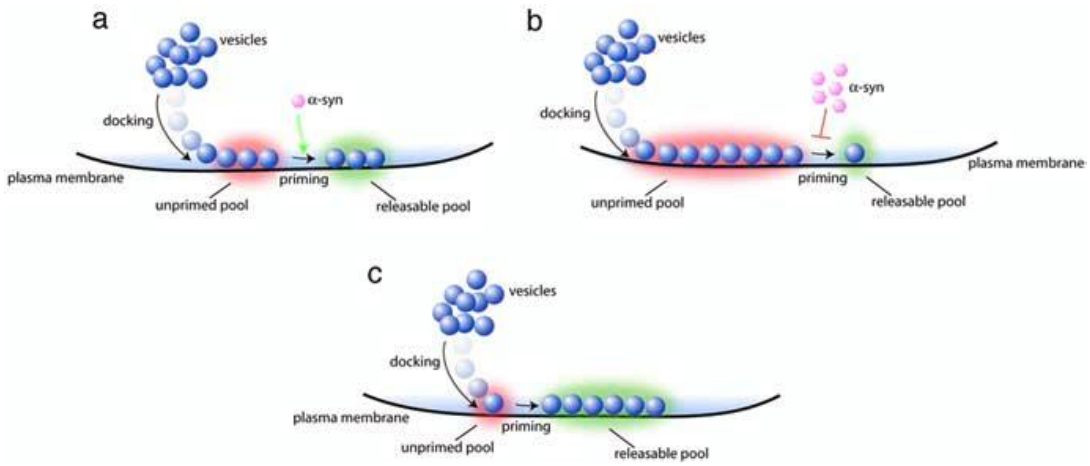


Figure 6: Model for α-synuclein (α-syn) as a regulator of vesicle priming. (***a),*** In the presence of wild-type levels of α-synuclein, vesicles dock with the plasma membrane but are not yet fusion competent (unprimed pool) until primed (releasable pool). α-Synuclein functions as a regulator of this priming step (green arrow). (***b),*** In the presence of high levels of α-synuclein, vesicles continue to dock with the plasma membrane but now linger in a fusion-incompetent state because of an inhibition of priming. Consequently, the unprimed pool of vesicles increases, and the releasable pool of vesicles decreases, leading to an increase in the number of docked vesicles and a decrease in exocytosis. (***c),*** In the absence of α-synuclein (e.g., mouse knock-out), vesicles dock with the plasma membrane, but priming is dysregulated, leading to excessive priming and a build-up in the releasable pool of primed vesicles, potentially explaining the increased evoked neurotransmitter release observed in neurons from α-synuclein -deficient mice. From Gitler AD & Shorter J. (2007) *J. Neurosci.*

The possible role of α-synuclein in regulating synaptic homeostasis is not exclusively related to its direct interaction with synaptic vesicles. For instance, α-synuclein may have a role as a chaperone, very similar to that of the presynaptic chaperone cysteine string protein (CSPα)[137]. Knockown of CSPα in mice does not affect synaptic transmission immediately after birth but eventually results in rapidly progressive synaptic degeneration

and death within 2 months[138]. CSPα thus does not itself appear required for transmitter release, but rather serves to maintain the function of the nerve terminal over a longer time frame. Remarkably, the overexpression of α-synuclein greatly delays degeneration due to the loss of CSPα, while the loss of synuclein exacerbates the CSPα knockout phenotype[137]. This suggests that α-synuclein may have a very similar role to that of CSPα as a chaperone in maintenance of the nerve terminal rather than in transmitter release. Further support for the role of α-synuclein in terminal maintenance was provided by the fact that α-synuclein showed a delayed onset of expression in primary hippocampal neurons compared with other presynaptic proteins[130]. In addition, a lack of gross morphological deficits in the brains of α-synuclein knockout mice[129] strengthens the concept that α-synuclein is not essential for neuronal development and differentiation. Moreover, the role of α-synuclein in the synapse is also associated with phospholipase D2 (PLD2), since α-synuclein is a specific, potent inhibitor of PLD2[139]. PLD2 catalyzes the hydrolysis of phosphatidylcholine to form phosphatidic acid (PA) and diacylglycerol (DAG)[139, 140], both of which are intracellular modulators of neurotransmitter release.

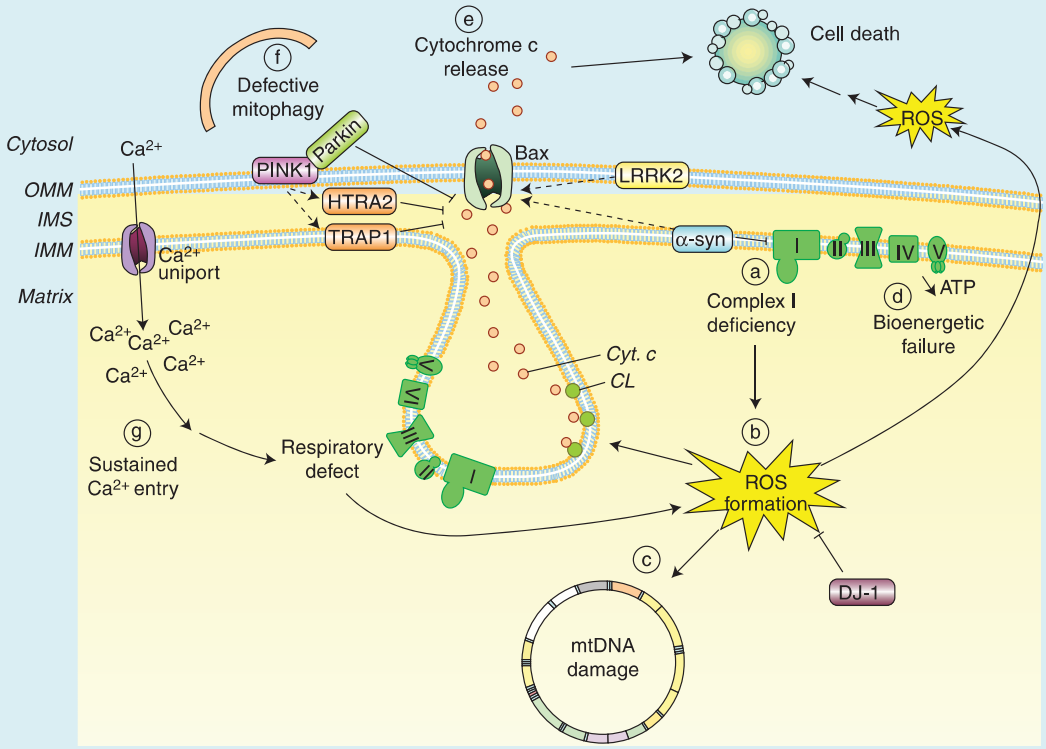
α-Synuclein and mitochondria

The connection between mitochondria and PD stems from the observation that a defect in the activity of respiratory chain protein complex I was identified in the SN[141], the striatum[142], the frontal cortex[143], platelets[144] and leukocytes[145] of PD patients. Mitochondrial alterations are now well recognized as forming part of PD (Box 5) and since α-synuclein and mitochondrial dysfunction are both associated with PD, an interaction between the two may underlie PD pathogenesis. Supporting this idea, overexpression of α-synuclein in a hypothalamic neuronal cell line gave rise to structural mitochondrial alterations, as reflected by the presence of enlarged mitochondria and abnormal cristae, impaired

mitochondrial function and increased oxidative stress[87]. Consistent with these results, mice overexpressing human A53T mutant α-synuclein exhibited (i) mitochondrial structural abnormalities, including shrunken, swollen or vacuolated mitochondria[146], (ii) impaired complex I activity[147] and (iii) mitochondrial DNA damage, frequently in the absence of nuclear DNA damage in brain stem neurons[146], suggesting that α-synuclein-induced mitochondrial DNA damage could precede neuronal cell death[146].

Indeed, α-synuclein is imported into mitochondria via a mechanism dependent on mitochondrial transmembrane potential and mitochondrial ATP[148]. The N-terminal region

Box 5. Mitochondrial dysfunction in PD



Alterations in several aspects of mitochondrial biology have been linked to the pathogenesis of PD, such as: (a) reduced complex I activity, (b) increased production of mitochondria-derived ROS, (c) ROS-mediated mtDNA damage, (d) bioenergetic failure, (e) Bax-mediated cytochrome c release and activation of mitochondria-dependent apoptotic pathways, (f) defective mitophagy, or (g) increased mitochondrial Ca2þ-buffering burden. Many of the mutated nuclear genes linked to familial forms of PD, including PINK1, Parkin, a-synuclein, DJ-1, or LRRK2, have been shown to affect many of these mitochondrial features (see main text for details). CL, cardiolipin; Cyt. c, cytochrome c; HTRA2, high temperature requirement A2; IMM, inner mitochondrial membrane; IMS, intermembrane space; LRRK2, leucine-rich-repeat kinase 2; OMM, outer mitochondrial membrane PINK1, phosphatase, and tensin homolog-induced kinase 1; ROS, reactive oxygen species; TRAP1, tumor necrosis factor receptor-associated protein 1; a-syn, alpha-synuclein. Perier C. and Vila M. (2012) *Cold Spring Harb Perspect Med 2012;4a009332*

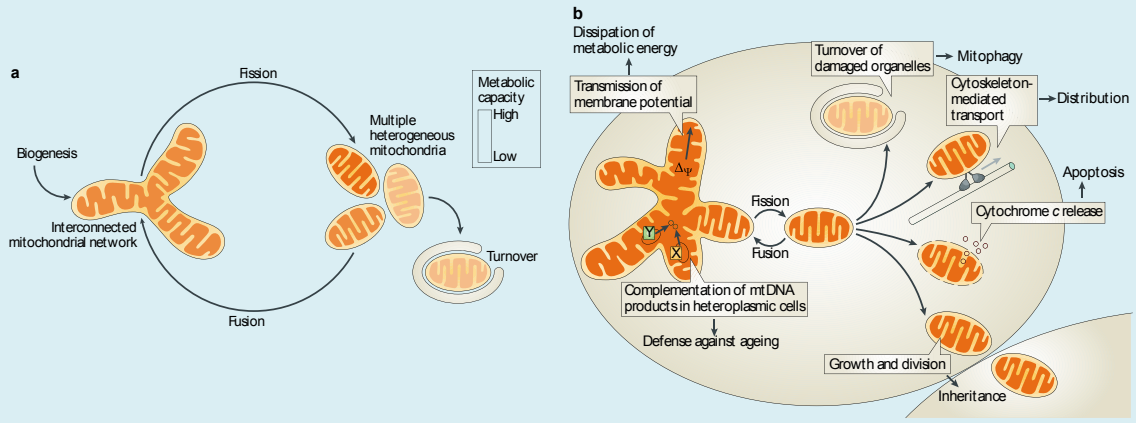
of α-synuclein is critical for its mitochondrial targeting[148]. Moreover, human fetal DA primary neuronal (DAN) cultures overexpressing human wild-type or mutant α-synuclein exhibited a time-dependent accumulation of α-synuclein in the mitochondria, which induced oxidative stress and impaired complex I activity[148].

The relevance of mitochondrial targeting and accumulation of α-synuclein were also studied in mitochondria purified from human fresh postmortem PD and normal brains[148]; compared with normal brains from control subjects, an accumulation of α-synuclein in mitochondria of the SN and striatum was evident in the PD brains. Furthermore, there was a significant correlation between the accumulation of mitochondrial α-synuclein levels and decreased complex I activity in the basal ganglia of PD brains. It is worth noting that mitochondria from the SN, striatum and cerebellum of control subjects exhibited low levels of α-synuclein, suggesting the constitutive presence of α-synuclein in the mitochondria. Consistent with these observations, control DAN cells also exhibited low but detectable levels of mitochondrial α-synuclein. Moreover, the siRNA-mediated knockdown of α-synuclein in DAN cells resulted in decreased complex I activity[148]. Taken together, these results demonstrated that constitutively low levels of mitochondrial α-synuclein may be important for the normal functioning of mitochondrial complexes, whereas mitochondrial accumulation of α-synuclein may contribute to the impairment of complex I function[148]. The inhibitory effect of an increased α-synuclein concentration on complex I activity was also confirmed in rats, which normally express mitochondrial α-synuclein in several brain regions, including the olfactory bulb (OB), hippocampus, striatum and thalamus[149].

Recent studies suggested that α-synuclein might play a role in mitochondrial dynamics (fusion and fission) (Box 6). Overexpression of wild-type or mutant (A30P or

A53T) α-synuclein in neuronal SH-SY5Y cells blocked mitochondrial fusion, which in turn caused increased mitochondrial fragmentation[133]. Conversely, downregulation of α-synuclein with siRNA in SH-SY5Y cells promoted mitochondrial fusion[133]. Mitochondrial fragmentation was also reported in Hela cells overexpressing human wild-type or mutant (A30P or A53T) α-synuclein and in midbrain neurons from transgenic mice overexpressing human α-synuclein[150], although unlike previous work, this mitochondrial fragmentation was associated with a role for α-synuclein in fission rather than fusion. In line with these observations on cultured cells, expression of α-synuclein in *C. elegans* also led to mitochondrial fragmentation and alterations of mitochondrial morphology[133]. In addition, α-synuclein seems to play a role in mitophagy (lysosome-mediated mitochondrial autophagy), since mice overexpressing high levels of mutant A53T α-synuclein exhibited

Box 6: Biological functions of mitochondrial dynamics

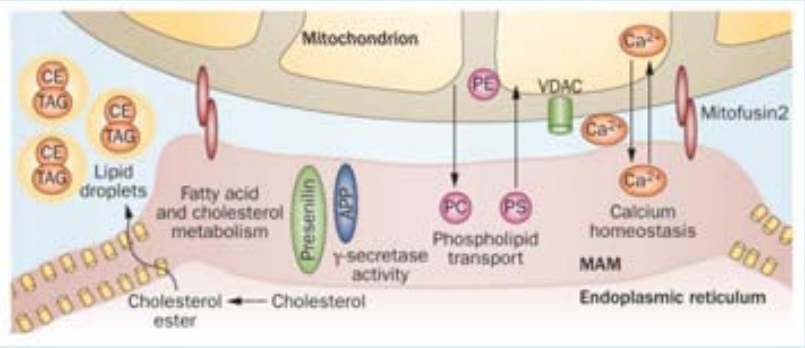


a) The mitochondrial life cycle starts with growth and division of pre-existing organelles (biogenesis) and ends with degradation of impaired or surplus organelles by mitophagy (turnover). In between, mitochondria undergo frequent cycles of fusion and fission that allow the cell to generate multiple heterogeneous mitochondria or interconnected mitochondrial networks, depending on the physiological conditions. **b)** Fusion and fission of mitochondria are important for many biological functions. Division is required for inheritance and partitioning of organelles during cell division, for the release of pro-apoptotic factors from the intermembrane space, for intracellular distribution by cytoskeleton-mediated transport and for turnover of damaged organelles by mitophagy. Fused mitochondrial networks are important for the dissipation of metabolic energy through the transmission of membrane potential along mitochondrial filaments and for the complementation of mitochondrial DNA (mtDNA) gene products in heteroplasmic cells to counteract the decline of respiratory functions in ageing (X and Y depict alleles of different mitochondrial genes). Adapted from Westermann B (2010) *Nat. Rev. Mol. Cell Biol.*

increased mitophagy[151], possibly as a compensatory attempt to remove defective mitochondria.

A recent study demonstrated that α-synuclein is present in mitochondria-associated endoplasmic reticulum (ER) membranes (MAM)[152]. MAM is a subregion of the ER with a unique lipid composition, enriched in cholesterol and anionic phospholipids, and involved in a number of key metabolic functions, including phospholipid and cholesterol metabolism (Box 7)[153]. In addition, MAM is also enriched in proteins related to the control of mitochondrial division[154] and dynamics[155]. Interestingly, different types of cell lines (i.e. M17 and Hela) containing pathogenic mutations in α-synuclein have an altered distribution of this protein between the cytosol and MAM, which was associated with a decreased MAM activity and ER-mitochondria apposition, along with an increase in mitochondrial fragmentation[152]. The localization of α-synuclein at the ER-mitochondrial interface likely contributes to explaining the association between α-synuclein and mitochondria.

Box 7. Structure and function of the mitochondria-associated ER membranes (MAM)

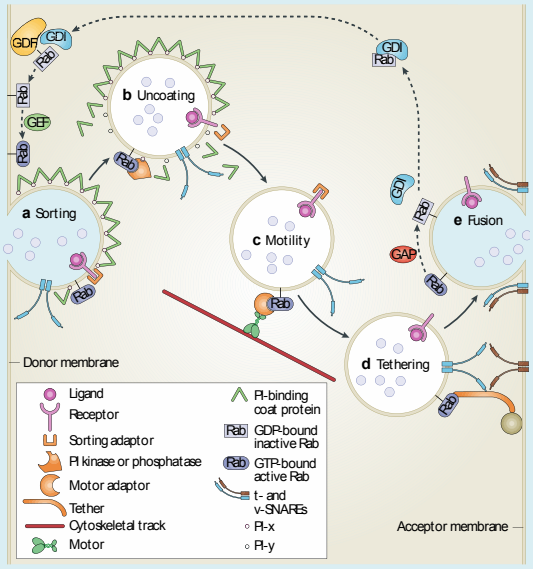


The ER communicates with mitochondria via the MAM, a specialized subregion of the ER with the characteristics of a lipid raft. The MAM is the regulatory site in the cell for phospholipid metabolism, cholesterol ester and fatty acid metabolism, lipid droplet formation, calcium homeostasis, and mitochondrial dynamics. Abbreviations: APP, amyloid precursor protein; CE, cholesteryl ester; ER, endoplasmic reticulum; MAM, mitochondria-associated ER membrane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; TAG, triacylglycerol, VDAC, voltage- dependent anion-selective channel. From Di Mauro et al., (2013) Nat. Rev. Neurosci).

α-Synuclein and Endoplasmic Reticulum (ER) – to – Golgi trafficking.

Using several *in vitro* and *in vivo* models, Lindquist and co-workers identified inhibition of ER–Golgi trafficking as a major component of synuclein-dependent toxicity. Much of Lindquist's work was performed on an α-synuclein yeast model, in which expression of wild-type α-synuclein (αSyn-WT) or disease-associated α-synuclein (αSyn-A53T) induced early increments of ER stress. Cells expressing either αSyn-WT or αSyn-A53T exhibited impaired ER-Golgi vesicular transport, which preceded the onset of ER stress[156]. In particular, in the presence of α-synuclein, vesicles efficiently bud from the ER but fail to dock and fuse with the Golgi membrane (Box 8)[157]. In addition, overexpression of the Rab GTPase Ypt1p protein, which is involved in ER-Golgi trafficking, rescued cells from α-synuclein-induced toxicity[156].

Box 8. ER-to-golgi vesicle trafficking



The ER – Golgi trafficking involves a series of steps consisting of a coat vesicle budding from the ER (a), uncoating of the vesicle (b), vesicle transport from the ER to Golgi (c), tethering and docking to Golgi membrane (d) and finally SNARE protein-dependent vesicle fusion. Adapted from Stenmark H. (2009) Nat. Rev. Mol. Cell Biol.<

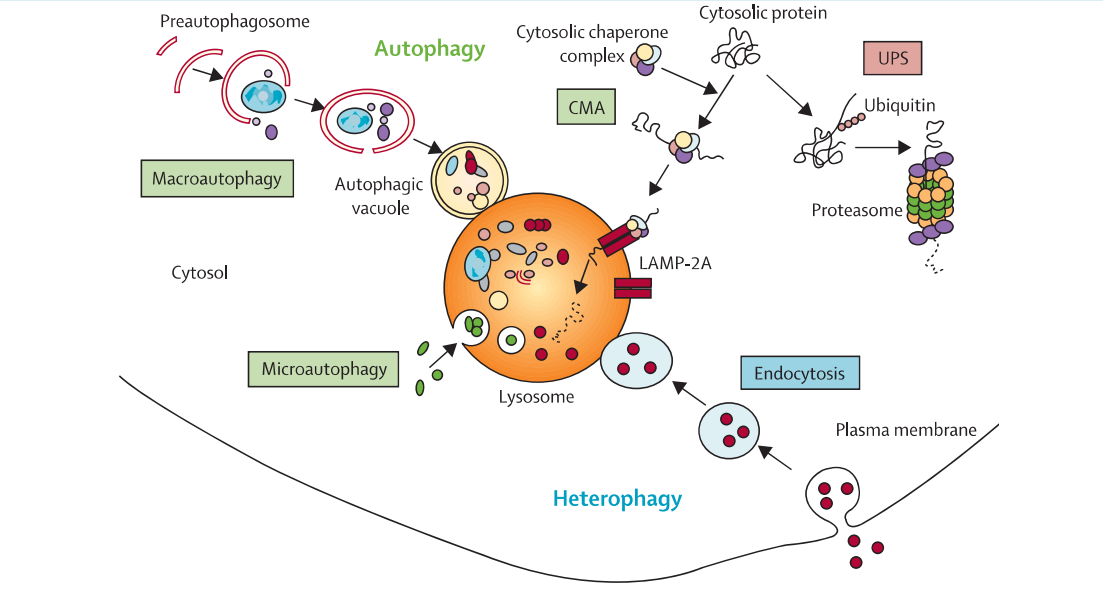
The association between α-synuclein and defects in ER-Golgi trafficking is not unique to yeasts, having also been demonstrated in worms, flies, and mammals. Flies expressing αSyn-WT and αSyn-A53T exhibited DA neuron loss, whereas coexpression of α-synuclein and Rab1 (the murine YPT1 ortholog) fully rescued this loss[156]. In *C. Elegans* expressing α-synuclein under the control of the dopamine transporter (DAT-1) gene promoter, which resulted in 60% of animals exhibiting reduced numbers of DA neurons at

the 7-day stage compared to controls, coexpression of α-synuclein and Rab1 significantly rescued neurodegeneration in three independently established transgenic lines[156]. Furthermore, coexpression of α-synuclein and Rab1 in primary cultures of rat midbrain neurons attenuated the toxicity induced by the overexpression of α-synuclein alone (~50% loss)[156]. Similar results in α-synuclein-mediated disruption of ER–Golgi trafficking were also reported in HEK and PC12 cells overexpressing αSyn-WT and αSyn-A53T[158]; in the presence of α-synuclein, vesicles budded from the ER but failed to fuse with their target Golgi membranes[158]. Further evidences supporting the role of α-synuclein in ER-Golgi trafficking was provided when α-synuclein from mice overexpressing mutant A30P α-synuclein was found to coimmunoprecipitate with Rab8, which is associated with the trans-Golgi network[136].

2.6 α-Synuclein impaired degradation in PD

The presence of LB in PD suggests that defective protein handling, in particular of α-synuclein, may contribute to the pathogenesis of the disease. Under physiological conditions, normal α-synuclein can be selectively degraded by the ubiquitin-proteasome system (UPS) or in lysosomes, where it can be delivered by means of macroautophagy, chaperone-mediated autophagy and endocytosis (Box 9)[159-165]. It has been shown that alteration of these degradation pathways results in the abnormal accumulation of α-synuclein. In addition some pathological α-synuclein species (i.e. PD-related mutant, oligomeric or aggregated) can, in turn, impair the efficiency of cellular proteolytic systems.

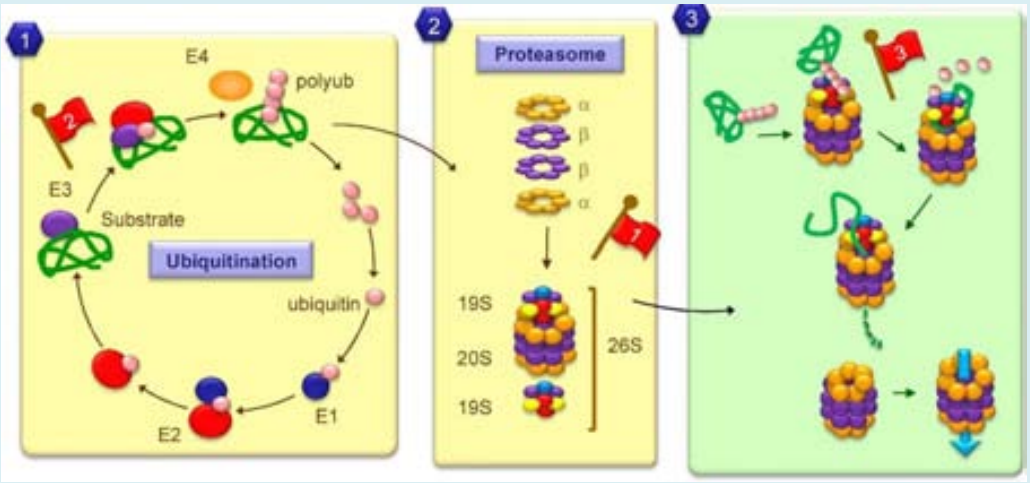
Box 9. Proteolytic systems in mammalian cells



Substrate proteins are delivered to lysosomes from the extracellular media (heterophagy) or from inside the cell (autophagy). The best-described heterophagic pathway is endocytosis. Three different types of autophagy have been described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). In macroautophagy, intracellular components are sequestered by a limiting membrane to form an autophagic vacuole that then fuses with lysosomes. In microautophagy, substrates are directly internalised through invaginations of the lysosomal membrane. In contrast to this "in-bulk" degradation, in CMA, selective substrate proteins are translocated into the lysosomes one by one after binding to a lysosomal receptor (LAMP-2A). The ubiquitin-proteasome system (UPS) is the other major pathway for degradation of intracellular proteins inside cells. Substrates are tagged with a small protein (ubiquitin) that is recognised by the proteasome, the protease of this pathway. Martinez-Vicente M. And Cuervo A.M. (2007) *Lancet Neurol.*

α-Synuclein and the UPS. The UPS is essential for the degradation of the majority of short-lived and misfolded proteins. The proteasome is a multicatalytic enzymatic complex formed by the combination of a barrel-shaped catalytic core (the 20S proteasome) and different regulatory subunits (19S and 11S) that can give rise to various types of intracellular proteasomes, 26S being the best characterized. This degradative pathway is highly regulated (Box 10). An initial link between UPS, α-synuclein and PD came from the observation that inhibition of the UPS could recapitulate some pathogenic features of PD, both *in vitro* and *in vivo*, such as dopaminergic cell death and cytoplasmic α-synuclein -positive inclusions[166-173], although some research groups have failed to reproduce

Box 10. Schematic model of the ubiquitin/proteasome system.



(1) Most proteins are targeted for degradation through the covalent attachment of 4–5 ubiquitins via a lysine residue in their sequence. Ubiquitinization requires the coordinated action of catalytic enzymes (E1, E2 and E3) that act sequentially to activate the ubiquitin and ligate it to the substrate presented by the E3. (2) The proteolytic component, the proteasome or 26S, has a catalytic core (the 20S) formed by four rings containing two types of catalytic subunits (a and b), and a regulatory complex (the 19S). (3) Polyubiquitin chains are recognized by components of the regulatory subunit, where deubiquitinases reverse the covalent conjugation, releasing free ubiquitin for recycling. The substrate is unfolded by unfoldases in the regulatory lid and ATPases in this complex provide the energy required for the injection of the substrate protein into the catalytic barrel or 20S proteasome. Koga H., et al. (2011) *Ageing Res. Rev.*

these results. A more specific connection between these three components came from the identification of familial PD cases caused by mutations in UPS-linked genes, such as parkin and ubiquitin C-terminal hydrolase-L1 (UCH-L1)[174, 175]. Parkin is a component of a multiprotein E3 ubiquitin ligase that catalyzes the addition of ubiquitin chains to target proteins to be degraded by the proteasome while UCH-L1 cleaves polyubiquitin chains into monomeric ubiquitin[176, 177]. Different reports showed that PD-linked mutations in parkin and UCHL1 genes lead to alterations in α-synuclein degradation by the proteasome[174, 175]. It has also been reported that α-synuclein itself, under some conditions, can impair UPS activity. In particular, several studies have shown that the direct interaction of α-synuclein with the proteasome complex can inhibit the activity of the 26S proteasome by occluding this complex and thus blocking the entry of other substrates into the catalytic pore of the proteasome[178]. This α-synuclein-mediated impairment of the proteasome has

been shown to precede the formation of inclusions in different cellular models[179-182]. Although there is some controversy concerning this point, most reports indicate that PD-linked mutant and oligomeric α-synuclein are the main species able to interfere with proteasomal activity[181-184], while other studies indicate that wild-type α-synuclein may also be able to impair UPS[178, 179].

α-Synuclein and the autophagy/lysosomal system. Lysosomes are able to eliminate all types of α-synuclein species through different pathways (Box 9)[163, 164]. Lysosomes are cellular organelles that contain acidic hydrolases able to break up all types of intracellular and extracellular components and macromolecules[185, 186]. In mammalian cells, various types of autophagy exist which differ in their mechanisms, substrate specificities, regulation and functions[159]. These are summarized as follows:

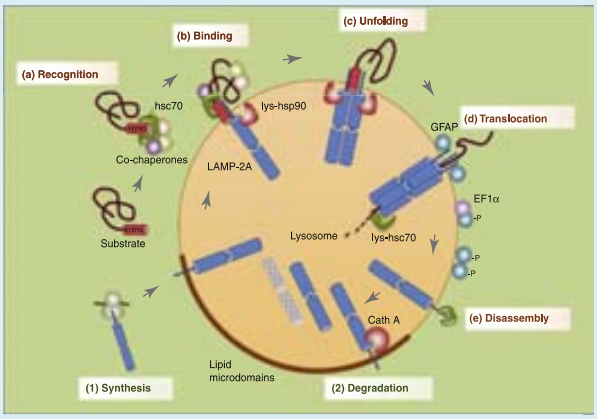
Macroautophagy and α-synuclein: macroautophagy is responsible for the degradation of most intracellular components, including entire organelles, to maintain cellular homeostasis and the appropriate balance between protein synthesis and degradation. During macroautophagy, a portion of the cytosol is surrounded by an intracellular membrane to form a double-membrane-bound structure known as an autophagosome (AP) that contains the sequestered components. The AP later fuses with lysosomes to form single-membrane-bound autophagolysosomes, the contents of which are then degraded by lysosomal hydrolases. Macroautophagy is particularly important for α-synuclein clearance since it is the only intracellular proteolytic system capable of eliminating misfolded α-synuclein once this protein has become insoluble or aggregated. In contrast, other proteolytic systems, like UPS and chaperone-mediated autophagy (CMA; see below) can only handle soluble substrates. The selective degradation of aggregates by macroautophagy (known as aggregophagy or aggrephagy) involves the selective ubiquitination of these aggregates and

subsequent recognition by adapter proteins (e.g. p62, NBR1 among others), which bind both the ubiquinated aggregates and LC3-II, which is the main component of the AP[187-190]. This interaction allows the formation of autophagic membranes around the aggregate until the latter is completely engulfed within an AP structure, which then fuses with lysosomes for its degradation[188-191]. Several groups have shown that activation of macroautophagy can enhance the degradation of different types of pathological α-synuclein species, both *in vitro* and *in vivo*[192, 193]. Conversely, the selective inhibition of macroautophagy increases mutant/pathological α-synuclein levels in different PD models[163, 194]. Recently, it has been reported that α-synuclein, in turn, can directly impair macroautophagy, both *in vitro* and *in vivo*[195]. The inhibitory effect of α-synuclein on macroautophagy seems to be exerted by a loss of function of Rab1, a protein involved in the cellular secretory pathway. As a consequence, AP formation is impaired, thereby resulting in an accumulation of undegraded autophagic substrates[195]. Relevant to PD, a reduced number of intraneuronal lysosomes, decreased levels of lysosomal-associated proteins, and accumulation of undegraded AP are observed in PD patients [i.e. post-mortem brain samples[196, 197], fibroblasts[198, 199] and induced pluripotent stem cell (iPSC)-derived dopaminergic neurons[111]] as well as in toxic and genetic rodent models of PD [i.e. MPTP-treated mice and rats overexpressing mutant α-synuclein[197]]. Mechanistic studies in the MPTP mouse model revealed that PD-linked lysosomal deficiency is secondary to abnormal lysosomal membrane permeabilization (LMP)[196, 200]. In particular, MPTP-induced LMP results in a decreased number of lysosomes and impaired AP-lysosome fusion, leading to a defective clearance and subsequent accumulation of undegraded AP within affected neurons[196, 200]. In addition, LMP can directly participate in MPTP-induced dopaminergic cell death by the leakage of lysosomal proteases into the cytosol, some of which, such as cathepsin B and D, can remain active at neutral cytosolic pH and cause the digestion of vital proteins or the

activation of additional hydrolases, including caspases[196, 200]. Supporting a pathogenic role for PD-related LMP, genetic or pharmacological enhancement of lysosomal biogenesis and function with transcription factor EB (TFEB) or rapamycin, respectively, are able to restore lysosomal-mediated degradation, reverse AP/synuclein accumulation, and attenuate dopaminergic neurodegeneration in toxic and genetic PD models *in vivo*[196,201, 202].

Chaperone-mediated autophagy (CMA) and α-synuclein. In CMA, specific cytosolic proteins are directly translocated into the lysosomes through the lysosomal membrane, without the participation of any vesicular trafficking (Box 11). All CMA substrates contain a CMA-motif with a KFERQ-like sequence[203], which targets the substrate to the lysosome's membrane with the help of cytosolic chaperones by binding the substrate to the membrane receptor protein LAMP-2A. The substrate protein is then translocated into the lysosomal matrix where it is rapidly degraded by the hydrolytic lysosomal enzymes[204]. α-Synuclein contains a KFERQ-like motif needed for CMA-targeting, and is thus recognized by cytosolic-hsc70 chaperone, which delivers α-synuclein to LAMP-2A, resulting in its translocation into the lysosomal lumen for its degradation. Measurement of the half-life for α-synuclein in rat ventral midbrain cultures in the presence of different types of lysosomal inhibition confirmed that CMA was the main lysosomal pathway that degrades soluble

Box 11. Steps and regulation of chaperone-mediated autophagy (CMA)



Steps and regulation of CMA. Steps: (a) Recognition of substrate proteins by hsc70/co-chaperones; (b) binding of substrate–chaperone complex to LAMP-2A; (c) unfolding of the substrate; (d) LAMP-2A multimerization, substrate translocation, and subsequent degradation; (e) disassembly of LAMP-2A multimer/translocon.. Abbreviations: CMA, chaperone-mediated autophagy; EF1alpha, elongation factor 1 a; GFAP, glial fibrillary acidic protein; hsc70, heat shock cognate protein of 70 kDa; LAMP-2A, lysosome-associated membrane protein type 2A; lys-hsc70, lysosome-associated hsc70. From Kaushik and Cuervo 2012 *Trends Cell Biol*

synuclein[205]. Remarkably, the A30P and A53T mutant forms of α-synuclein cannot be degraded by CMA in PC12 cells, although they are still recognized by their CMA-motif and targeted to lysosomes, where the motif binds to the LAMP-2A receptor with an even higher affinity than that of wild-type protein[205]. However, the translocation of mutant forms of α-synuclein across the lysosomal membrane is impaired, resulting in the block of not only their own degradation but also the degradation of other cytosolic CMA[205]. Subsequent studies confirmed that CMA is an important pathway for α-synuclein also in neurons as well, and confirmed the pathogenic effects of mutant α-synuclein on CMA activity on this type of cell[206, 207]. More recently, *in vivo* studies using genetic and toxic PD mouse models provided evidence of lysosomal degradation of α-synuclein by CMA under basal and pathological (i.e. increased α-synuclein levels) conditions, thus confirming the relevance of α-synuclein neuronal degradation by CMA *in vivo*[161]. In addition to PD-linked mutations, various α-synuclein post-translational modifications can also impair the CMA-mediated degradation of this protein and other CMA substrates[208]. For instance, phosphorylated, nitrated and oxidized forms of α-synuclein decreased the capacity of α-synuclein to be translocated and eliminated via the CMA pathway[208]. Similarly, when α-synuclein interacts with oxidized DA it becomes misfolded and can no longer be internalized into the lysosome[208]. Although DA-modified α-synuclein can bind to LAMP-2A, it is retained on the surface of the lysosomal membranes, forming clusters that impair its own lysosomal translocation, as well as the internalization and degradation of other CMA substrates[208]. Consistent with this finding, decreased levels of CMA markers have been reported in post-mortem nigral samples from PD patients[209].

Endocytosis and α-synuclein: endocytosis is a process by which cells internalize extracellular components with a coat assembly, by invagination of the membrane and

subsequent formation of a vesicle called endosome. There are many types of endosomes and the final destiny of these vesicles and their contents can vary depending on the receptors and proteins present in the endosomal membrane, the type of molecules trapped inside the endosome, the cell type and many other factors. One possible destination of endosomes is the lysosome, in which all the intraendosomal material can be degraded[210]. It has been recently reported that α-synuclein associated with lipid membranes can be degraded by the endosomal-lysosomal pathway[162]. In particular, it was shown that Nedd4, a ubiquitin ligase enzyme involved in the ubiquitination of membrane-associated proteins, can ubiquitinate α-synuclein for its specific degradation in lysosomes via a process involving endocytosis[162]. In the mentioned study, it was postulated that the endosomal-lysosomal pathway may specifically catalyze the degradation of a membrane-associated pool of α-synuclein, whereas the proteasome and autophagy pathways may degrade the soluble and aggregated forms of α-synuclein, respectively[162].

2-7 Extension of α-Synuclein pathology in PD

The notion that α-synuclein lesions in PD may self-propagate and spread progressively between interconnected brain regions via a cell-to-cell transmission mechanism, which has recently been strongly promoted, is based on the followings: (i) the discovery that PD patients exhibit accumulations of pathological α-synuclein in different brain regions, which has been hypothesized to follow a caudo-rostral progression pattern[41, 211, 212] and, (ii) the finding that embryonic mesencephalic neurons grafted into the striatum of PD patients develop LB many years after grafting, suggesting a host-to-graft transmission of the LB pathology in the human brain[213, 214].

Widespread α-synuclein pathology in PD.

In 2003, Braak et al. suggested the possibility that sporadic PD might progress in six stages that follow a caudo-rostral pattern based on the presence of α-synuclein accumulations in different brain regions (Fig. 7)[211]. In the first stage, α-synuclein lesions are found in the OB and/or the dorsal motor nucleus of the glossopharyngeal and vagal nerves, suggesting that PD begins in the gastrointestinal and olfactory systems. In stage 2, α-synuclein lesions are found in the pontine tegmentum, especially in the caudal RN, gigantocellular reticular nucleus, and coeruleus-subcoeruleus complex. In stages 1 and 2, affected subjects are not usually diagnosed with PD, but exhibit some of the typical PD non-motor symptoms, such as hyposmia and constipation. It is unknown whether these individuals will develop PD later on. It is not until stage 3 that the midbrain is affected, and subjects are clinically diagnosed with PD. The principal characteristic of stage 3 is the

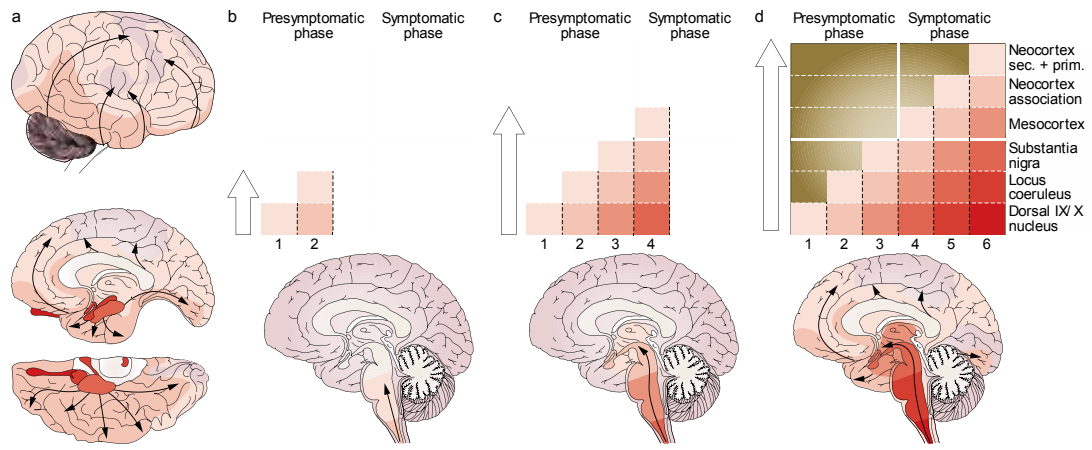


Figure 7. Six stages of PD pathology. Cases with α-synuclein inclusions fall into one of six stages according to the brain regions involved. Progression between stages involves additional brain areas and worsening of pathology in previously affected brain regions. A) Rostrocaudal progression of the pathology process (arrows). Variable red shading reflects the ascending disease process and increasing severity of pathology. B) Stage 1: lesions occur in the olfactory bulb, the anterior olfactory nucleus and/or the dorsal motor nuclei of the vagal and glossopharyngeal nerves in the brainstem. Stage 2: lesions are observed in the pontine tegmentum (locus coeruleus, magnocellular nucleus of the reticual formation, and lower raphe nuceli). C) Stages 3 and 4: lesions reach the pedunculopontine nucleus, the cholinergic magnocellular nuclei of the basal forebrain, the pars compacta of the subtantia nigra (stage 3), the hypothalamus, portions of the thalamus and, as the first cortical region, the anteromedial temporal cortex (stage 4). First clinical symptoms of PD appear during stage 3 or early stage 4. D) Stages 5 and 6: lesions reach neocortical high-order association areas (stage 5), followed by first-order association areas and primary fields (stage 6). Abbreviation: PD, Parkinsons' disease. From Goedert et al., (2013*) Nat. Rev. Neurol.*

involvement of the SN, although macroscopically there is no detectable depigmentation of the SN. At stage 4, marked degeneration of the NM-containing neurons of the SN is found, and mesocortical areas begin to be affected. During stages 5 and 6, α-synuclein lesions appear in the neocortical areas.

The Braak et al. study[211] supported the idea that the key lesions in PD begin developing a considerable time prior to the appearance of motor dysfunction. It is now well known that non-motor symptoms usually pre-date the PD-associated motor dysfunctions by many years. Some of these non-motor symptoms include an impaired sense of smell years prior to motor manifestations of PD, as well as several autonomic dysfunctions. The presence of α-synuclein lesions in the areas affected during stages 1 and 2 may be related to the appearance of these non-motor symptoms. In contrast, motor symptoms appear around stages 3 and 4, when the midbrain, particularly the SN, is affected. Finally, during stages 5 and 6 important limbic structures, including the amygdala, hippocampal formation and anteromedial temporal mesocortex, are affected. The presence of α-synuclein lesions in these areas may be related to the declining intellectual faculties that are frequently seen in advanced PD. Although other groups have confirmed some of these PD stages[215-217] not all sporadic PD cases follow this theoretical caudo-rostral pattern of progression. Moreover, this staging does not explain the absence of clinical symptoms in subjects who on autopsy have widespread α-synuclein pathology. Regardless of the validity of Braak staging, this model has the merit of showing that α-synuclein lesions in PD are not only present in the SN, but in several other brain areas, and in both the PNS and CNS, indicating a widespread accumulation of α-synuclein lesions. Another important observation of Braak's work is that the majority of α-synuclein lesions are found in neuritic processes rather than in the cell body.

According to the Braak staging hypothesis, PD might originate outside of the CNS by a causative pathogen capable of entering the CNS by way of retrograde axonal and transneuronal transport, with misfolded α-synuclein being a possible candidate for such a pathogen. In fact, α-synuclein pathology is abundant in the peripheral ANS (pANS) of patients with LB diseases[218]. In particular, the more-affected pANS regions in PD patients are the stellate and sympathetic ganglia, the vagus nerve, gastrointestinal tract, adrenal gland and/or surrounding fat and heart. Interestingly, epicardial fat tissue obtained during cardiac surgery from patients without parkinsonism but with some premotor symptoms such as constipation and acting dreams, exhibited α-synuclein pathology[219]. These results strengthen the concept that pANS involvement is an intrinsic component of PD and suggest that the presence of α-synuclein pathology in the pANS of patients without PD could represent a pre-clinical stage of the disease

Host-to-graft α-synuclein transmission.

Embryonic mesencephalic neurons grafted into the striatum of PD patients can develop LB many years after grafting, suggesting a host-to-graft transmission of the LB pathology in the human brain. In particular, 3 out of 4 patients who died a decade after transplantation (11-16 years after) exhibited LB and LN pathology in the grafted neurons. In contrast, patients who died 4-9 years after transplantation did not exhibit LB pathology, suggesting that the development of LB pathology is a slow process, which may require more than a decade to manifest.

The mechanism by which α-synuclein lesions spread in grafted cells is currently unknown. One hypothesis is that grafted neurons are exposed to an unfavorable microenvironment, where inflammation, oxidative stress, excitotoxicty and/or failure of neurotrophic factors may induce protein misfolding within grafted neurons (Fig. 8)[220]. Host-to-graft transmission of α-synuclein might also explain the presence of LB in recipient cells. In this scenario, α-synuclein could be released by living cells (via an active process such as exocytosis), or by dying cells into the surrounding extracellular milieu. Thereafter, grafted

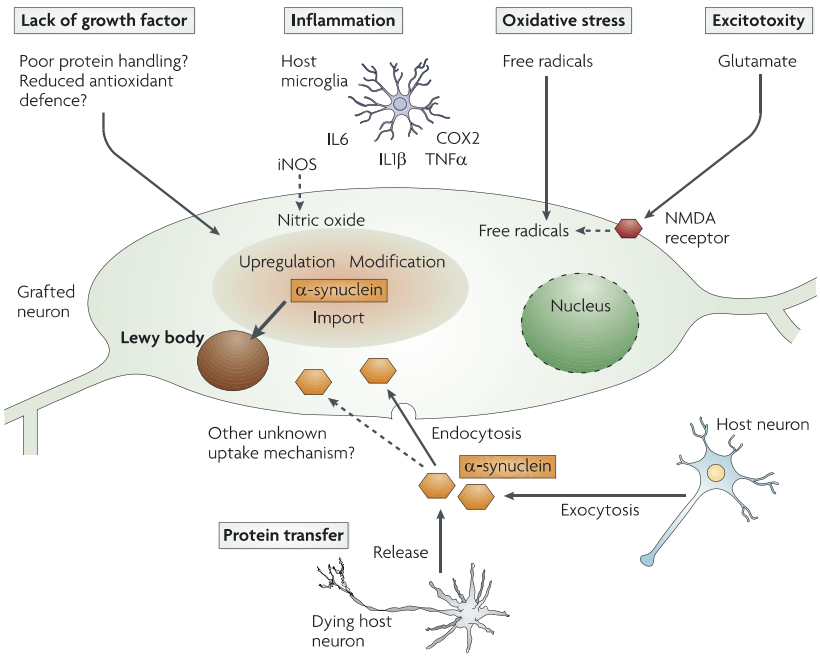


Figure 8. Potential mechanisms of α-synuclein host-to-graft transmission. This diagram illustrates cellular mecanisms that might underlie the spread of Parkinson's disease (PD) pathology from the host brain to grafted neurons. Neurotrophic factors have been reported to be deficient in the striatum of PD patients. Certain neurotrophic factors are known to counteract oxidative stress, and one could speculate that they normally also promote the molecular defence against misfolded proteins. A relative lack of these growth factors could therefore lead to the accumulation and aggregation of misfolded α-synuclein in the grafted cells. Inflammatory mediators (including tumour necrosis factor-α (TNFα), interleukin-1β (IL1β), IL6, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2) are upregulated in the parkinsonian brain. Some of them have been shown experimentally to promote upregulation of α-synuclein and α-synuclein nitrosylation, and could thereby promote the generation of Lewy bodies (LBs) in grafted neurons. There is ample evidence for oxidative stress in the PD brain. Free radicals from the host brain migh attack grafted neurons, leading to modifications of α-synuclein and increasing its propensity to misfold. Excitotoxicity, potentially triggered by excessive glutamate in the PD striatum, could lead to increased free-radical stress inside grafted neurons. Host cells adjacent to the grafted neurons might also be a source of misfolded, LB-generating α-synuclein. α-Synuclein can be released from living cells into the surrounding extracellular milieu, and dying neurons in the PD brain could conceivably also release α-synuclein. Recent studies have shown that neurons take up different molecular forms of α-synuclein through different pathways, one of which is endocytosis. Once inside the grafted neurons, the exogenous α-synuclein could act as a template that promotes misfolding of endogenously produced α-synuclein, ultimately leading to LB formation. NMDA, N-methyl-D-aspartate. From Brundin P. et al. (2008) *Nat. Rev. Neurosci.*

neurons could take up this released α-synuclein through different pathways, including endocytosis. Once inside the grafted neurons, the exogenous α-synuclein could act as a template that promotes misfolding of endogenously-produced α-synuclein, ultimately leading to the formation of LB.

Cell-to-cell transmission of α-synuclein

The potential cell-to-cell transmission of α-synuclein implies that α-synuclein can be secreted and internalized by cells (Fig. 9). Since α-synuclein lacks an ER signaling sequence that would direct it to secretory pathways, it was initially though that α-synuclein was exclusively an intracellular protein. However, the finding that α-synuclein species can be detected in human plasma and cerebrospinal fluid (CSF)[221, 222] suggests that α-synuclein can be secreted by cells. Indeed, it has been demonstrated that α-synuclein can actually be secreted into the culture medium by several types of cultured neuronal cells[221, 223-226]. Although the mechanism of α-synuclein release has not been fully elucidated, recent results point towards a non-classic secretory pathway. In particular, it seems that α-synuclein may

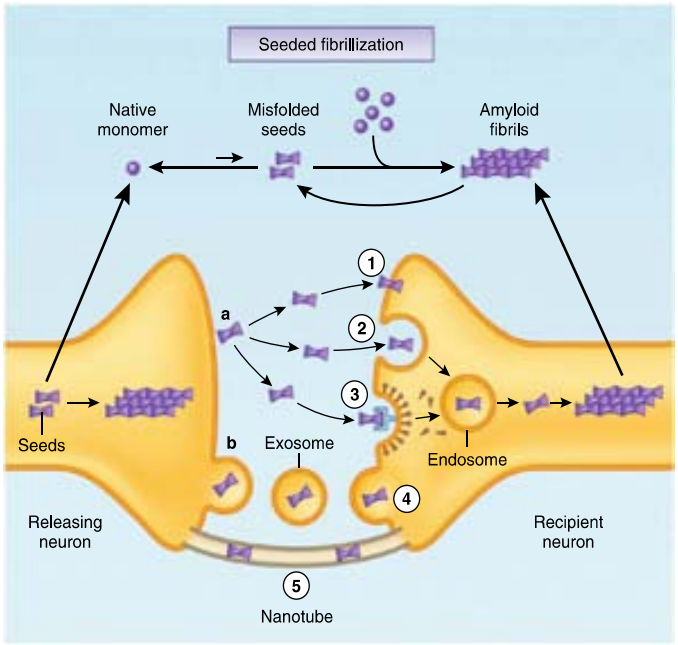


Figure 9. Potential mechanisms mediating cell-to-cell transmission of α-synuclein. (a,b) Misfolded α-synuclein seeds first in the cytoplasm of the releasing neuron (left), where soluble native monomers are recruited into large intracellular aggregates and a positive feedback loop can be initiated by generation of more seed through fragmentantion or secondary nucleation. A small amount of protein aggregates can be released into the extracellular space in the "naked" form (a) or via membrane-bound vesicles such as exosomes (b). Free-floating seed may directly penetrate the plasma membrane of the recipient neurons (1) or enter by fluid-phase endocytosis (2) or receptor-mediated endocytosis (3), whereas exosomes containing seeds may fuse with the membrane of the receipient neuron (4). Intercellular transfer of seeds may also occur by nanotubes that directly connect the cytoplasm of two cells (5). Internalized seeds then nucleate the fibrilliation of native monomers in the cytoplasm of the recipient neuron. From Guo J.L & Lee V.M. (2014) *Nat. Med. Rev.*

be released by exosomes in a calcium-dependent manner[223, 226]. Interestingly, exosomes have been previously shown to play a role in the secretion of prion proteins and β-amyloid peptides[227-229].

In addition, several studies demonstrated that α-synuclein can be internalized by cells when this protein is added to the culture medium[230-236]; this probably occurs via a classical endocytic mechanism[235, 237-239] that could include dynamin-dependent receptor-mediated endocytosis[238, 240]. However, considering the size of α-synuclein fibrillar aggregates, receptor-mediated endocytosis, which requires specific interactions between ligands and cell-surface receptors, seems unlikely to be the principal mode of fibril internalization. Other mechanisms could potentially mediate the transcellular movement of cytosolic α-synuclein aggregates (Fig. 9), although these have not been fully demonstrated. Such mechanisms include nanotubes (tunnel-like structures connecting two cells), which have been shown to be involved in the spreading of prionic proteins[241]. Finally, α-synuclein monomers could potentially enter cells via passive diffusion by interacting with membranes and lipids[237, 242, 243]

Recently, *in vitro* studies demonstrated that synthetic recombinant preformed α-synuclein fibrils (PFFs) could be internalized by cells (overexpressing α-synuclein, or not) and act as a seed to induce the recruitment of endogenous soluble α-synuclein into insoluble pathologic aggregates[232, 238, 239]. The formation of these α-synuclein aggregates within recipient cells leads to alterations in synaptic functions, compromises neuronal excitability and connectivity, and culminates in neuronal death.

One of the first *in vivo* studies demonstrating that α-synuclein can be spread via a cell-to-cell transmission mechanism was by Desplats et al.[240]. GFP-labeled mouse cortical neuronal stem cells (MCNSCs) were injected into the hippocampus of transgenic mice

expressing human α-synuclein under the control of the Thy-promoter. Four weeks after transplantation, 15% of the grafted GFP-labeled MCNSc exhibited human α-synuclein immunoreactivity. Interestingly, few of these cells exhibited inclusion bodies within the cytoplasm. Furthermore, 4 weeks after transplantation, about 20% of the transplanted MCNSC exhibited caspase-3 activation. In a separate study, 5% of fetal post-mytotic dopaminergic neurons grafted into the striatum of mice overexpressing human α-synuclein, exhibited human α-synuclein immunoreactivity six months after transplantation[238], thus confirming the transfer of human α-synuclein from host-to-graft *in vivo*. In addition, this study also demonstrated that different forms of human α-synuclein, including monomers, oligomers and fibrils, could be taken up by neurons *in vivo* by endocytosis[238]. Host-to-graft transmission of human α-synuclein has also been reported in rats[244], although in a less-physiological situation. In this case, embryonic ventral mesencephalic rat neurons were grafted into the dopamine-depleted striatum of rats that had previously received 6-OHDA. One month after the transplant, human α-synuclein was virally overexpressed in a striatal region distal to the graft. Five weeks after viral infusion, grafted cells exhibited human α-synuclein, supporting the concept of a cell-to-cell α-synuclein transmission from host-to-graft in rats.

Once demonstrated that α-synuclein could be transmitted *in vivo* between cells, the next step was to explore the potential pathogenic effect of α-synuclein transmission *in vivo*. In this context, it was reported that both synthetic and murine disease-associated forms of α-synuclein are able to induce a PD-like α-synuclein pathology *in vivo*[245]. Indeed, the intracerebral injection of brain homogenates derived from old α-synuclein transgenic mice (which exhibited α-synuclein pathology) into the neocortex and striatum of young asymptomatic transgenic mice induced a widespread accumulation of pathological α-

synuclein throughout the anterior/posterior extent of the neural axis spanning the CNS, from OB to the spinal cord. These effects were mostly observed by 90 days post-injection (dpi), although at 30 dpi some α-synuclein pathology was already evident. Similar results were obtained after the injection of synthetic recombinant α-synuclein PFFs (Fig. 10), providing the first evidence that PFFs alone are sufficient to initiate and propagate the α-synuclein pathology *in vivo*. Surprisingly, although inoculations were performed unilaterally, α-synuclein lesions were observed bilaterally. Furthermore, the inoculation of either symptomatic brain lysates or α-synuclein PFFs accelerated and increased the accumulation of α-synuclein in these transgenic mice and reduced their lifespan. Some of these results were reproduced by Mougenot et al.,[246]. The injection of brain homogenates from symptomatic α-synuclein transgenic mice into the brains of healthy transgenic mice accelerated the characteristic clinical signs of paralysis observed in this mouse model and reduced the lifespan of injected animals. Insoluble phosphorylated α-synuclein at Ser129 was also found in the brains of inoculated mice.

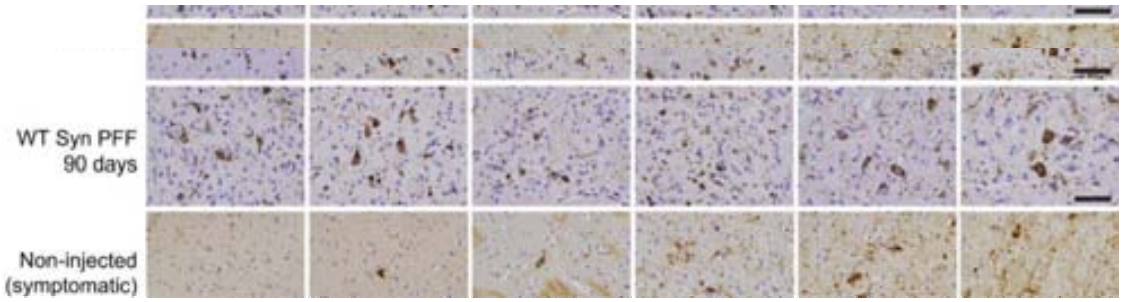


Figure 10. Recombinant α-Syn PFFs induce α-synuclein pathology in the CNS. (A) pSyn immunohistochemistry of various CNS regions in young α-synuclein transgenic mice 90 d after intracerebral injection with WT α-Syn PFFs. Asterisks indicate level of injection sites. Bottom panels show phosphorylated α-synulcein staining in aged symptomatic transgenic mice. Immunohistochemial analyses were performed on multiple transgenic mice after injection with PFFs assembled from α-Syn$^{1-120}$Myc (30d, n=3; 90 d, n=10) or from WT α-Syn (n=4). Luk KC. et al., (2012) *J. Exp. Med*

Pathological spreading of α-synuclein was also reported in wild-type mice[247]. Indeed, the injection of synthetic recombinant α-synuclein PFFs into the striatum of wild-type mice induced a pathological time-dependent accumulation of endogenous α-synuclein

(Fig. 11) that was associated with cell loss in the SN and impaired motor coordination. The formation of an LB/LN-like pathology in PPF-inoculated mice occurred upstream of SNpc DA neuron loss, indicating that the α-synuclein pathology is sufficient to induce the cardinal behavioral and pathological features of sporadic PD. The injection of PFFs directly into the SN of wild-type mice also induced a time-dependent widespread accumulation of α-synuclein pathology[248], although no neuronal loss in the SN or motor impairment were found in this case.

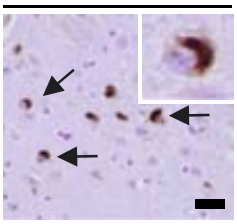


Figure 11. Dense LB-like inclusions (black arrows) in the SNpc, 90 days after the injection of α-synuclein PFFs into the striatum of wild-type mice .Adapted from Luk KC. et al (2012) *Science*

Peripheral transmission of α-synuclein pathology to the brain

While the studies mentioned above involved a direct intracerebral inoculation of pathological α-synuclein, other studies have addressed the possible transmission of α-synuclein pathology from the periphery to the brain. For example, rAAV expressing human α-synuclein were injected into the left vagus nerve in the neck of rats[212]. This induced a strong expression of human α-synuclein expression in the medulla oblongata (MO), leading to a caudo-rostral spreading of the α-synuclein pathology into other interconnected brain regions, such as the pontine coeruleus-subcoeruleus complex, the dorsal raphe, the hypothalamus and the amygdala (Fig. 12). In addition, α-synuclein accumulation present in the aforementioned areas was accompanied by morphological evidence of neuronal abnormalities (i.e. thread-like axons with irregularly spaced, densely labeled varicosities). Surprisingly, the transmission of α-synuclein did not reach the SN, and neuronal damage was not induced in this brain region at least 18 weeks after the injection.

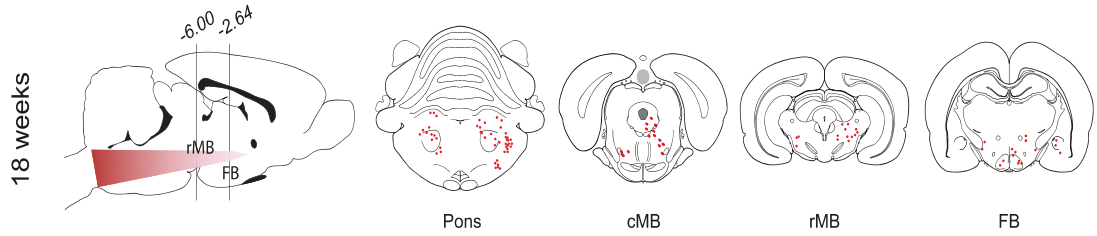


Figure 12. Human α-synuclein (hα-syn) progressively spreads from the medulla oblongata to more rostral brain regions. Topographical plot of the distribution and spreading of hα-syn-labeled neurites. Neuronal fibers from representative brain sections are shown as red dtos. Bregma values indicate the caudo-rostral value. cMB: caudal midbrain; rMB: rostral midbrain; FB: forebrain. From Ulusoy A. et al. (2013) *EMBO Mol. Med.*

In another study, Brundin et al. examined if α-synuclein can transfer from the OB to other brain structures through neuronal connections[249]. In this way, different molecular species (monomers, oligomers composed of soluble high molecular weight species, and fibrils) of recombinant human α-synuclein were injected into the OB of normal mice. The authors reported that cells in different layers of the OB (i.e. the glomerular layer, mitral cell layer and granule cell layer) readily take up recombinant monomeric and oligomeric α-synuclein. Fibrillar α-synuclein was also taken up, but to a much lesser extent within the time frame of the experiments. Soon after the injection (1.5h and 3h), soluble and oligomer, but not fibrillar, α-synuclein species were detected in several interconnected brain regions, including the anterior olfactory nucleus, the frontal cortex, the tenia tecta, the olfactory tubercle, the periform cortex, the striatum and the amygdala. At these time points, few microglial cells in the OB, anterior olfactory nucleus and frontal cortex were positive for human α-synuclein. More than 12h after injection into the OB, α-synuclein in microglial cells was present only locally, and not in other brain regions. In contrast, at later time points (12h and 72h post injection), α-synuclein was extensively detected in microglial cells, suggesting that microglia might clear the human α-synuclein released into the extracellular space by the neurons.

In this context, Pan-Montojo and colleagues reported that intragastric administration of the environmental toxin rotenone induced α-synuclein accumulation in both the ENS and CNS following the same pattern of progression as hypothesized by Braak[250]. Firstly, they reported α-synuclein accumulations in ENS neurons as soon as 1-5 months after rotenone treatment. Next, they determined whether the local effect of rotenone on the ENS could lead to alterations in the synaptically connected ANS centers in the spinal cord and brainstem (i.e. in the intermediolateral nucleus in the spinal cord (IML) and DMV). Both the IML and DMV exhibited accumulation and aggregation of α-synuclein 1.5 and 3 months after rotenone treatment, although α-synuclein pathology in these areas was not associated with neuronal death. Interestingly, the SN also exhibited α-synuclein accumulation, phosphorylation and inflammatory signs 3 months after rotenone treatment. Unlike the DMV and IML, α-synuclein increments in the SN were associated with neuronal loss. After intragastric rotenone administration, pesticide was not detected in the blood or brain, and no inhibition of complex I activity in muscle or brain was found, suggesting that the reported alterations in the mentioned brain regions were not due to a systemic effect of rotenone. Remarkably, the rotenone-induced α-synuclein pathology was specific, as only neuronal subpopulations with direct connections to the ENS showed alterations, while nearby areas (such as the striatum, cerebellum and cortex) remained unaffected. This specificity together with the fact that the appearance of α-synuclein accumulations in the SN were only detected at the last treatment time-point (i.e. 3 months), raised the possibility of a direct mechanism between cells being responsible for this pattern of progression of the α-synuclein pathology. To confirm this hypothesis, Pan-Montojo and colleagues severed some of the connecting nerves between the CNS and the gut (i.e. the sympathetic and parasympathetic nerves)[251], which delayed the appearance of motor symptoms after oral rotenone treatment. This treatment also stopped the progression of α-

synuclein pathology into the IML and DMV, and prevented cell death in the SN[251]. These finding thus supported the idea that the α-synuclein pathology can be transmitted via a cell-to-cell mechanisms.

α-Synuclein transmission and neuroinflammation

The secretion of α-synuclein by neurons may not only induce toxicity once inside the cytoplasm of neighboring cells, but also in the extracellular space; this may activate glial cells and induce chronic inflammation (i.e. a common pathological feature of PD), thereby contributing to the progression of the pathology throughout the brain. Supporting this idea, glial cells (i.e. astrocytes and microglia) are able to take up and degrade synthetic recombinant α-synuclein aggregates even more efficiently than neurons[252]. Indeed, α-synuclein can be transmitted between neurons and glial cells *in vitro*[253, 254]. Interestingly, the exposure of neuron-derived α-synuclein induced an inflammatory reaction in rat primary astrocytes[254] and microglia[236, 253, 255], suggesting that accumulation of the neuron-derived α-synuclein within astrocytes and microglia leads to an inflammatory response. Moreover, α-synuclein-activated microglia promoted neurotoxicity both in rat mesencephalyc primary cultures and in a MES23 dopaminergic cell line. The direct transfer of α-synuclein from neurons to astrocytes was demonstrated *in vivo* using transgenic mice overexpressing human α-synuclein under the neuronal promotor (PDGFβ). In these transgenic mice, abundant human α-synuclein accumulation was observed not only in neurons but also in glial cells[254]. Consistent with these results, recombinant α-synuclein oligomers and monomers injected into the neocortex of wild-type mice were taken up by oligodendrocytes[256]. Similarly, in rAAV-treated rats overexpressing human α-synuclein, embryonic oligodendrocytes grafted into the striatum were found to contain this human α-

synuclein, thus further demonstrating the neuron-to-astrocyte transmission of α-synuclein[256].

Cell-to-cell transmission of proteinophaties in other neurodegenerative diseases.

At almost the same time that a self-propagating pathology was demonstrated for α-synuclein, a collection of recent studies has provided convincing evidence that this same self-propagating mechanism may be applicable to a wide range of disease-associated proteins, including Aβ, tau, huntingtin with polyQ repeats, superoxide dismutase 1 (SOD1) and TDP-43 (Table 3). For each of these proteins, aggregate-containing lysates and/or synthetic fibrils assembled from recombinant proteins were shown to act as a template or seed that could efficiently recruit their soluble counterparts into elongating fibrils in cultured cells and/or living animals.

		Seeded aggregation in different model systems			
Protein	**Type of seed**	**Non-neuronal cells**	**Neuronal cells**	**Mice**	**References**
Aβ	Synthetic fibrils	Not tested	Not tested	Yes	Stöhr, J. et al. *Proc Natl. Acad Sci USA* (2012)
	Mouse brain lysates	Not tested	Not tested	Yes	Stöhr, J. et al. *Proc Natl. Acad Sci USA* (2012) Meyer-Luehmann, M. et al. *Science* (2006) Eisele, Y.S. et al. *Science* (2010)
	Human brain lysates	Not tested	Not tested	Yes	Meyer-Luehmann, M. et al. *Science* (2006)
Tau	Synthetic fibrils	Yes	Yes	Yes	Guo, J. L & Lee, V. M. *J Bid. Chem* (2011) Frost, B. et al. *J Bid. Chem* (2009) Guo, J. L & Lee, V. M. *FEBS Lett* (2013) Nonaka, T. et al. *J Bid. Chem* (2010) Iba, M. et al. *J Neurosci* (2013)
	Mouse brain lysates	Not tested	Not tested	Yes	Clavaguera, F. et al. *J Bid. Chem* (2009)
	Human brain lysates	Not tested	Not tested	Yes	Lasagna-Reeves, C.A. et al. *Sci. Rep.* (2012) Clavaguera, F. et al. *Proc Natl. Acad Sci USA* (2013)
TDP-43	Synthetic fibrils	Yes	Not tested	Not tested	Chen, A.K. et *al. J Am Chem Soc* (2010)
	Human brain lysates	Not tested	Yes	Not tested	Nonaka, T. et al. *Cell Rep.* (2013)
SOD1	Synthetic fibrils	Not tested	Yes	Not tested	Münch, C. et al. Proc. Natl. Acad. Sci USA (2011)
PolyQ	Synthetic fibrils	Yes	Not tested	Not tested	Ren, P.H. et al. *Nat. Cell. Bid.* (2009)

Table 3. Summary of studies demonstrating the transmissibility of protein aggregates in other neurodegenerative diseases. From Guo J.L & Lee V.M. (2014) *Nat. Med. Rev.*

2.8 Summary of α-synuclein pathogenic role in PD

Although the exact mechanisms by which α-synuclein induces cell death remains unknown, it is likely that α-synuclein toxicity involves several α-synuclein forms and affects several cellular process (Fig. 13):

- α-Synuclein mutations cause autosomal-dominant forms of PD, most likely by disturbing the normal structure of this protein (thus resulting in abnormal α-synuclein function) and promoting the formation of toxic oligomers.

- α-Synuclein can adopt several conformations, including oligomers and fibrils, which are associated with the pathogenesis of PD. In particular, oligomer toxicity is associated with an abnormal permeabilization of membranes. On the other hand, LB and LN may disrupt the normal cellular architecture to the extent that cell function is compromised, eventually leading to cell death.

- Duplications and triplications in the *SNCA* gene cause familial PD, indicating that increased levels of α-synuclein can cause PD. Therefore, alterations in the machinery underlying α-synuclein synthesis and clearance could be toxic.

- Post-translational α-synuclein modifications could increase the pathogenic role of α-synuclein by promoting the formation of toxic oligomers and disrupting the normal function of this protein.

- α-Synuclein toxicity is highly associated with this protein's capacity to bind to membranes. In particular, the pathological α-synuclein species (i.e. post-translationally modified, mutant or oligomeric α-synuclein forms), may induce toxicity by: (i) disrupting the normal function of α-synuclein in neurotransmission

release, where it probably acts as a negative regulator of DA release, (ii) impairing mitochondrial structure and complex I activity, as well as mitochondrial dynamics and mitophagy and (iii) disrupting ER-Golgi vesicular transport, which results in toxic ER stress.

- The pathological α-synuclein species impair the efficiency of some protein-degradation mechanisms, thereby interfering with the normal physiology of the cell, and eventually leading to cell injury and death. In addition, the α-synuclein-

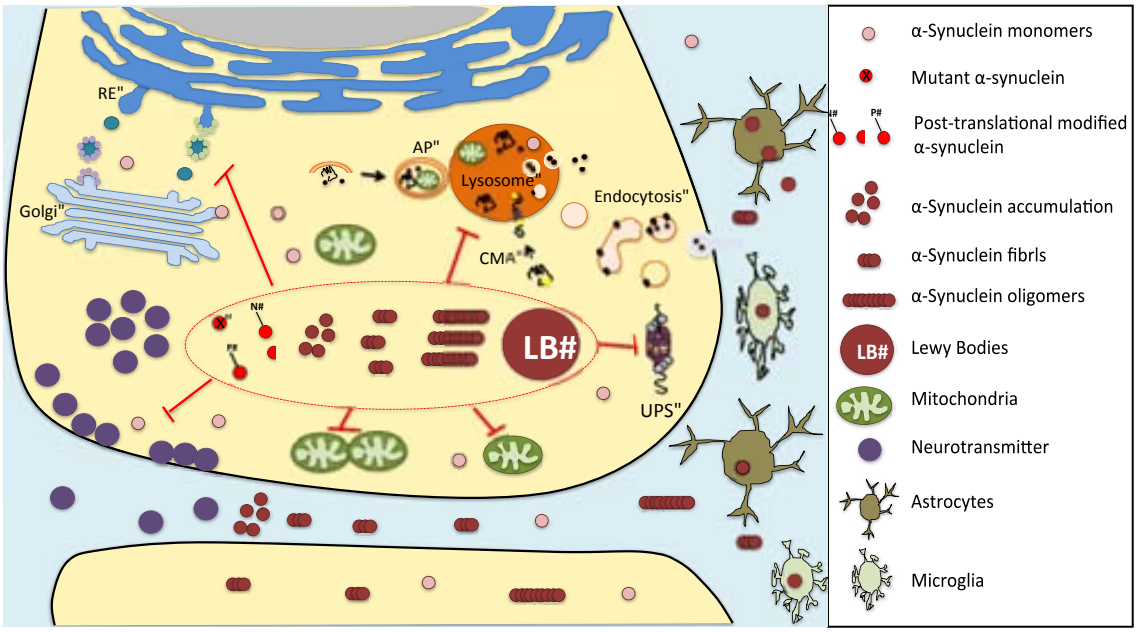


Figure 13. **Initiation, progression and extension of PD: role of α-synuclein**. Although the exact mechanism by which α-synuclein causes cell death remains unknown, mounting evidence suggest that α-synuclein toxicity involves several toxic species (i.e. post-translationally modified, mutant and oligomeric forms) and impaired cellular functions. In particular, toxic α-synuclein species can (i) disturb the normal function of α-synuclein in neurotransmission release, where the protein probably acts as a negative regulator of DA release, (ii) impair mitochondrial structure and complex I activity, as well as mitochondrial dynamics and mitophagy, (iii) disrupt ER-Golgi vesicular transport, resulting in toxic ER stress, and (iv) impair the efficiency of some protein-degradation mechanisms, which in turn lead to toxic accumulations of α-synuclein. All these α-synuclein pathological functions interfere with the normal physiology of the cell, and can eventually lead to cell injury and death. In addition, α-synuclein pathology can be propagated between neurons by a cell-to-cell transmission mechanism, thus contributing to the progresion of PD. The presence of extracellular α-synuclein can also activate an inflammatory reaction, which might play a role in the progression of PD. AP: Autophagosome; CMA: chaperone-mediated autophagy; RE: endoplasmic reticulum; UPS: Ubiquitin-proteasome system.

induced impairment of proteolytic systems in turn leads to toxic increments of α-synuclein.

- Finally, α-synuclein is secreted and internalized by cells, suggesting that α-synuclein pathology can be transmitted between cells. The cell-to-cell transmission of α-synuclein pathology may contribute to the spread of the PD pathology throughout the brain. In addition, the presence of extracellular α-synuclein can activate an inflammatory reaction, which in turn may play a role in the progression of the disease.

HYPOTHESES AND AIMS

Hypothesis 1: Mounting evidence suggest that α-synuclein, a major protein component of LB, may be responsible for initiating and spreading the pathological process in PD. Supporting this concept, intracerebral inoculation of synthetic recombinant α-synuclein fibrils can trigger α-synuclein pathology in mice. However, it remains uncertain whether the observed pathogenic effects of recombinant synthetic α-synuclein can actually apply to PD-linked human α-synuclein and occur in species closer to human. Furthermore, it is not known whether LB material eventually released from affected neurons can propagate to intact neighboring neurons and thus contribute to the spreading of the disease process in PD. Here we hypothesize that LB-linked pathological α-synuclein derived from PD patients is pathogenic and can participate to initiation and extension of the pathological process in PD.

Objective 1: Assess the potential pathogenic effects of inoculating α-synuclein-containing nigral LB extracts from postmortem PD brains into the brains of wild-type mice and macaque monkeys. To achieve this goal we will perform the following steps:

1. Purification and characterization of LB-enriched extracts obtained from freshly frozen postmortem SNpc tissue derived from patients with sporadic PD.

2. Intracerebral injection of LB extracts into either the SN or striatum of wild-type mice and monkeys.

3. Evaluation of the integrity of the dopaminergic nigrostriatal pathway, both at the level of SNpc cell bodies and striatal axon terminals, at different time-points following LB inoculations.

4. Assessment of α-synuclein pathology (i.e. aggregation/accumulation, hyperphosphorylation) induced by the intracerebral injection of LB.

5. Evaluation of the spread of α-synuclein pathology into other brain regions induced by the intracerebral injection of LB.

6. Determine the requirement of (i) human α-synuclein present in LB extracts and (ii) host (endogenous) expression of α-synuclein for the pathogenic effect of inoculating LB.

Hypothesis 2: Mounting evidence indicates that α-synuclein levels are increased in PD. Here we hypothesize that molecular tools able to reverse abnormal α-synuclein expression back to basal physiological levels might provide therapeutic benefit in PD.

Objective 2: develop a novel molecular approach to specifically decreases α-synuclein levels in PD-vulnerable neuronal populations. If successful, this approach will be tested in future studies as a potential disease-modifying strategy in experimental animal models of PD. To achieve this goal we will perform the following steps:

1. Perform an *in vitro* screening of several α-synuclein-directed siRNA sequences to assess their potential at knocking-down endogenous or overexpressed α-synuclein levels. Based on this assessment, the best candidate sequence will be selected for further studies *in vivo*.

2. Validate *in vivo* the level of α-synuclein downregulation after local infusion of the selected siRNA sequence into the SN of wild-type mice.

3. The selected siRNA sequence will be chemically modified by nLife Therapeutics to enhance its biostability (to be able to be delivered intranasally) and conjugated to the cell-specific ligand indatraline (IND-siRNA), to promote its selective delivery into aminergic neurons.

4. Validate *in vitro* and *in vivo* the specific delivery of IND-siRNA into aminergic neurons.

5. Assess the degree of α-synuclein downregulation in the SN obtained by the intranasal administration of IND-siRNA to mice.

6. Determine whether downregulation of endogenous α-synuclein levels in the SNpc of mice by intranasal administration of IND-siRNA is safe and does not induce nigrostriatal degeneration.

MATERIALS AND METHODS

1. HYPOTHESIS 1: PATHOGENIC POTENTIAL OF PD-DERIVED LB EXTRACTS

1.1 Human samples.

Postmortem human brain samples and sucrose gradient purification of LB-enriched extracts. Human NM-containing SNpc tissue was dissected from fresh frozen postmortem midbrain samples from three patients with sporadic PD exhibiting abundant nigral LB pathology on neuropathological examination (mean age at death = 72 ± 4.3 years, cold postmortem interval = 6.47 ± 2.72 hours, frozen postmortem interval = 10.63 ± 2.82 hours, see Table 4). The samples were obtained from the New York Brain Bank at Columbia University and the University of Barcelona Brain Bank.

	Gender	Mean age at death	Frozen postmortem interval
PD #1	male	76 years old	13.58h
PD #2	male	75 years old	13.33h
PD #3	male	65 years old	5h

Table 4. Postmortem human brain samples used in our studies. PD: Parkinson's disease patient.

Tissue was homogenized in 9 vol (w/v) ice-cold MSE buffer (10 mM MOPS/KOH, pH 7.4, 1 mM EGTA, 1 mM EDTA and 1 M sucrose) with protease inhibitor cocktail (Complete Mini; Boehringer Mannheim) with 12 strokes of a motor-driven glass/Teflon homogenizer. For LB purification, a sucrose step gradient was prepared by overlaying 2.2 M with 1.4 M and finally with 1.2 M sucrose in volume ratios of 3.5:8:8 (v/v)[257]. The homogenate was carefully layered on the gradient and then centrifuged at 160,000 x g for 3h using a SW32.1 rotor (Beckman). Twenty fractions of 500 μl were collected from each gradient from top (fraction 1) to bottom (fraction 20), which were further analyzed.

Filter retardation assay (FRA). All the fractions were analyzed by FRA as previously described[258]. Briefly, after heating at 100ºC for 5min, samples (60 μg) were diluted in 200 μl of migration buffer (25 mM Tris-HClBase, 200 mM glycine, SDS 1%) and filtered through a

cellulose acetate membrane (Schleicher & Schuell; 0.2 μm pore size) using a Minifold-1 Dot-Blot System (Schleicher & Schuell). Membranes were saturated in 5% dried skimmed milk in PBS and probed with antibodies against α-synuclein, phosphorylated α-synuclein, ubiquitin or p62 (see Table 6 in page 82). Appropriated secondary antibodies coupled to peroxidase were revealed using a Super Signal West Pico Chemiluminescent kit (Pierce). Chemiluminescence images were acquired using the ImageQuant RT ECL Imager (GE Healthcare). This assay permits the detection and quantification of detergent- or urea-insoluble amyloid-like fibrils and high molecular weight insoluble protein aggregates based on their ability to be retained on cellulose acetate membranes with a 0.2 μm pore size[258-260] (Fig. 14).

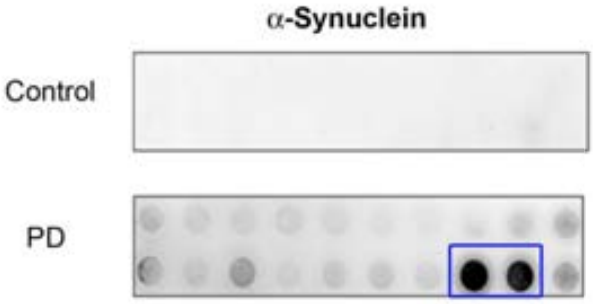


Figure 14. Filter retardation assay probed with a human α-synuclein antibody to assess the presence of insoluble α-synuclein aggregates in the different fractions obtained by sucrose gradient fractionation from freshly frozen post-mortem nigral brain tissue from one control subject without α-synuclein pathology at neuropathological examination (82 years old) and one PD patient exhibiting abundant α-synuclein pathology (76years old).

Immunofluorescence analysis of LB fractions. Indicated fractions from the sucrose gradient were spread over slides coated with poly-D lysine and fixed with 4% paraformaldehyde (PFA) in PBS for 30min. Fixed slides were stained with 0.05% thioflavin S for 8min and then washed 3 times with 80% EtOH for 5min, followed by 2 washes in PBS for 5min. Finally, all samples were washed 3 times with PBS and blocked with 2% casein and 2% normal goat serum (NGS) for 30min. For immunofluorescence analyses, samples were incubated with human α-synuclein antibody Ab-2 (Table 6) for 30min, washed 3 times with PBS, incubated with goat-anti-mouse-TRITC (Jackson, 1:500), before being coverslipped for microscopic visualization using fluorescence mounting medium.

Immunogold electron microscopy. Carbon-coated nickel grids were covered for 1min with corresponding fractions of interest prior to and 5min after bath-sonication and then washed 3 times with distilled water. Grids were transferred for 30min to 4% normal goat serum (NGS) in PBS and then incubated for 2h at room temperature (RT) in a solution of PBS containing anti-α-synuclein primary antibody supplemented with 1% NGS (Table 6). Grids were subsequently washed in PBS and incubated for 1h in PBS containing anti-mouse IgG secondary antibody conjugated to 15 nm gold particles (1:100; Aurion), washed in PBS and fixed with 2% glutaraldehyde in distilled water. They were then washed again in distilled water and stained for 5min with 2% uranyl acetate, before being air-dried. Digital images were obtained with a computer linked directly to a CCD camera (Gatan) on a Hitachi-H-7650 electron microscope.

Human brain sample preparation for animal inoculations. For stereotactic inoculations of mice and monkeys, LB-containing fractions from three sporadic PD patients were mixed together in the same proportion (PD #1, fractions 18 and 19; PD #2, fractions 15; PD #3, fractions 16; see Fig. 21 in page 86). Control-injected animals were inoculated with a mixture of non-LB fractions (Fractions 6) derived from the same three PD patients (which contain soluble α-synuclein) or the corresponding buffer (vehicle) obtained from a sucrose gradient purification performed without the addition of any brain sample. In all cases, samples were bath-sonicated for 5 min prior to the in vivo inoculations.

1.2. Mouse experiments.

Animals and stereotactic inoculations. Male C57BL/6 mice (4-7 months old; Charles River, Lyon, France) were housed under controlled conditions (22ºC ± 1ºC; 12h light/dark cycle) with food and water available *ad libitum*. Animal procedures were conducted in accordance with standard ethical guidelines (EU regulations L35/118/12/1986) and approved by the local ethical committee. Mice received 2 µl of either LB fractions, non-LB fractions or appropriated buffer (vehicle) by stereotactic delivery to the region immediately above the SN (-2.9 AP, 1.3 L and -4.5

DV) at a flow rate of 0.4 µl/min. After each injection, the syringe was left in place for 10 min to prevent leakage along the needle track. Animals were euthanized at 24h, 4 weeks, 4 months and 17 months post-inoculation and the position of the 30 G needle was determined histologically. Only animals with a correct placement of the needle were processed for further analyses.

Pole test. This behavioral test was performed by the group of Isabel Fariñas (University of Valencia, Spain). The pole test has been previously validated in mice to assess motor alterations linked to striatal dopamine depletion or basal ganglia abnormalities[261-265]. This test was performed as previously described[264] with minor modifications. Briefly, each mouse was placed head-upward on the top of a vertical rough-surfaced wooden pole (1 cm diameter and 45 cm long) with a cardboard barrier to prevent upward traversal. When placed on the pole, mice oriented downward and descended the length of the pole. The time between the placement on the pole and the moment when the four paws touched the floor was measured, with a limit of up to 90s[266]. A Motor Ability Scale (M.A.S) was defined to score the behavior of the animals in three degrees: 0 points for draggins or falling form the pole, 1 point for T-down between 7s and 49s and 2 points for T-down < 7s (which is the mean time in the control group to complete the test with a correct motor strategy). Therefore, the higher the score in the motor ability scale, the better motor performance in the pole test. Mice were subjected to the test on three consecutive trails. The average of those three trials was calculated and considered for statiscal analyses.

Immunohistochemistry. Twenty-four hour to 17 months after LB inoculations, mice were euthanized by perfusion with 4% PFA and their brains were processed for immunohistochemical analyses. Immunostaining was performed on 20 µm-thick free-floating sections. Sections were incubated with different primary antibodies (Table 6) for 24h at 4ºC. Biotinylated secondary antibodies, followed by signal amplification using the avidin-biotin complex (ABC) method, were used. Immunostaining was revealed using 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Aldrich). For Iba1 immunostaining, the Vector SG Peroxidase Substrate Kit (Vector

Laboratories) was used to reveal the staining. For immunofluorescence, sections were incubated with primary antibodies (Table 6) for 24h at 4ºC, washed in PBS incubated for 1h at RT with fluorescent secondary antibodies, incubated with Hoechst (1:2000; Invitrogen) for 10min at RT, washed in PBS again and finally mounted using DAKO Fluorescent Mounting Medium (DAKO). When mouse secondary antibodies were required, a Vector M.O.M Immunodetection kit (Vector Laboratories) was used, according to manufacturer's instructions.

Proteinase K digestion. 20 μm-thick sections were washed with TBS and incubated in proteinase K (PK, Invitrogen; 1 μg/ml in TBS) at RT for 10min. The sections were then washed in TBS and immunostained for α-synuclein as indicated above.

Quantitative morphology. To assess the integrity of the nigrostriatal pathway, TH immunohistochemistry was performed on SNpc and striatal sections.

TH-positive SNpc neurons: The total number of TH-positive SNpc neurons was assessed by stereology in 12 regularly-spaced 20 μm-thick sections spanning the entire SNpc using StereoInvestigator software (MBF Bioscience). The SNpc was delineated for each slide (Fig. 15) and probes for stereological counting were applied to the map obtained (size of counting frame was 50 x 50 μm spaced by 250 x 250 μm).

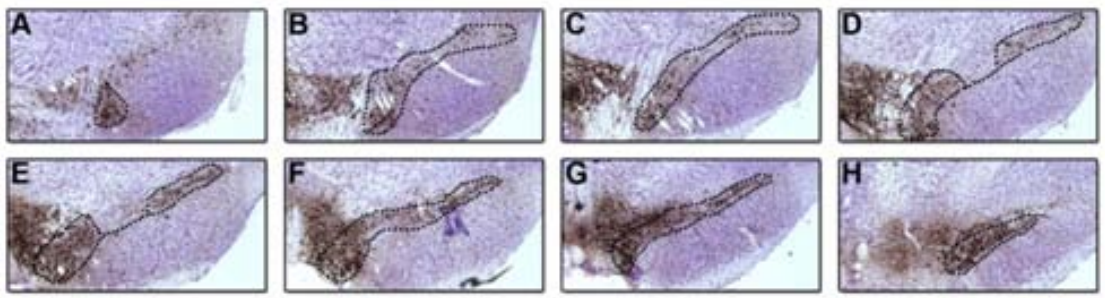


Figure 15. Coronal sections across the entire SNpc from caudal (A) to rostral (H). Photomicrograph in (A) corresponds to the first caudal section to consider for stereology. The brown color represents TH-positive neurons and the purple color, the Nissl staining.

Each TH-positive cell with its nucleus included within the counting frame was counted (Fig. 16). The optical fractionator method was finally used to estimate the total number of TH-positive cells in the SNpc of each hemisphere.

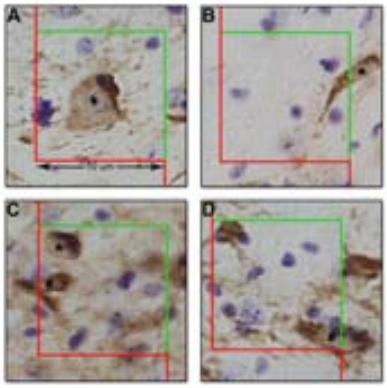


Figure 16. Stereological counting using optical fractionator. (A-D) illustrate the way we TH-positive cells with a bicolor 50 μm-squared counting frame. Every nucleus of TH-positive cells inside the counting frame (asterisk in A and C) or touching the green line (asterisk in B) has to be count. Every nucleus of TH-positive cells touching the red line (pound sign in C) has to be excluded from the counting. (D) If the nucleus of TH-positive cell crosses both, green and red lines, the cell has to be excluded (pound sign in D).

TH-positive striatal fibers: Striatal TH innervation was assessed by optical densitometry (OD) in 4 regularly spaced 20 μm-thick sections corresponding to different striatal anatomical levels (Fig. 17). Sections were scanned in an Epson Perfection V750 PRO scanner and Sigma Scan software was used to analyze the grey intensity in the region of interest: striatum (Str) and cortex (Ctx). Intensity in Ctx was used as the blank. OD was assessed with the formula $OD=-\log_{10}(I_{str}/I_{ctx})$.

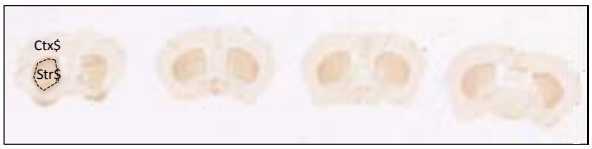


Figure 17. TH-immunohistochemistry in 4-regularly spaced 20 μm-thick section corresponding to different striatal anatomical levels. Ctx: cortex; Str: Striatum

Alpha-synuclein: Alpha-synuclein expression levels were estimated after immunohistochemistry (Table 6), as follows: (i) OD at regional levels: 20 μm sections mounted on slides were scanned with an Epson Perfection V750 PRO scanner, after which Image J software was used to quantify the grey level in each region of interest; (ii) intracellular densitometry: the intracellular OD of transmitted-light microscopy images of α-synuclein-positive cells from the different brain regions of interest were analyzed using Sigma Scan. An average of ~500 neurons per group, randomly selected among the differet animals, was analyzed as follows: each neuron was

delineated and the intensity of its area was measured by SigmaScan (I_{neuron}). An area without staining was used as a blank (I_{blank}). OD was assessed by the formula: $OD=-\log_{10}(I_{neuron}/I_{blank})$

Inflammatory reaction: For inflammatory reaction assessment, the total number of Iba1-positive cells in the SNpc was measured stereologically using StereoInvestigator software (MBF Bioscience). Astrocyte density was measured by OD at regional levels in GFAP-immunostained sections using Sigma Scan software.

Alpha-synuclein enzyme-linked immunosorbent assay (ELISA). Total protein concentrations in the different sucrose extracts was determined using the bicinchoninic acid (BCA) assay (Thermo Scientific). Samples were diluted to 20 μg/ml and analyzed in duplicate for total α-synuclein protein level with a specific ELISA Kit against human α-synuclein (Invitrogen) according to the manufacturer's instructions.

Immunodepletion of α-synuclein. LB fractions (prepared as described above) were diluted 1:10 in RIPA buffer supplemented with protease inhibitors (complete MP Inhibitor cocktail, Roche), and bath-sonicated for 5min. Diluted extracts were incubated overnight at 4ºC with a mouse monoclonal anti-α-synuclein antibody (Table 6). Immune complexes were then incubated with 40 μl of protein G-agarose beads (Sigma), previously washed with PBS for 3h at 4ºC. Samples were centrifuged at 1000 x g for 1min at 4ºC and the resultant pellets (P1) were washed 3 times in PBS by sequential sedimentation and resuspension. Finally, immunoprecipitated α-synuclein was eluted in 25 μl of 2x Laemmli buffer by heating to 95ºC for 5min and the supernatant (S1) was subjected to a second immunoprecipitation. The final supernatant (S2) was used for inoculations whereas eluted material and supernatants from each step were collected for western blotting. Proteins were resolved by SDS-PAGE on 12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes (GE Healthcare), which were blocked in 5% non-fat milk powder in PBS for 1h at RT and incubated overnight at 4ºC with the α-synuclein

antibody (Table 6). Incubation with the anti-mouse secondary antibody coupled to horseradish peroxidase (dilution 1:5000; Amersham Biosciences) was performed at RT for 1h, followed by repeated washing with PBS. Immunoreactive bands were visualized using SuperSignal Femto Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions on an ImageQuant RT ECL imaging system (GE Healthcare).

1.3. Monkey experiments.

All the experiments in monkeys were perfomed in the laboratory of Erwan Bezard at the University of Bordeaux (Bordeaux, France)

Animals. Four female rhesus monkeys (*Macaca fascicularis*; mean weight, 5.1 kg; mean age 7 years) were used, two of which had been treated three years earlier with MPTP hydrochloride (0.2 mg/kg i.v.; Sigma-Aldrich) dissolved in saline according to a previously described protocol[267]. Animals were housed in individual primate cages under controlled conditions of humidity (50 ± 5%), temperature (24 ± 1ºC), and light (12h light / 12h dark cycle, lights on at 8:00 A.M.); food and water were available *ad libitum*. Tissues from four additional untreated control animals and three additional MPTP-treated only monkeys were used for post-mortem experiments when needed. All experiments were carried out in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC) revised in 2010 (2010/63/EU) for the care of laboratory animals in government-approved facilities. The study design was approved by the Ethics Committee of the Centre National de la Recherche Scientifique, Region Aquitaine, for primate experiments, and by the Ethics Committee for Animal Testing of the University of Navarra. Veterinarians skilled in the care and maintenance of non-human primates supervised their overall well-being.

Stereotactic inoculations. For stereotactic delivery of LB fractions, the standard Horsley-Clarke technique was improved by using sagittal and frontal ventriculography to locate the

borders of the third ventricle and the edges of the anterior and posterior commissures as previously described[268-270]. The intrastriatal injection of LB was performed at 2 rastrocaudal levels of the motor striatum (anterior commissure, -1 mm and -5 mm). The total injected volume per hemisphere was 100 μl. Intranigral LB inoculations of 10 μl were performed with electrophysiologial guidance considering the AC-posterior commissure line as a reference: -7 mm anterior; -4 depth, and ± 3 lateral. After each injection, the syringe was left in place for 10min to prevent leakage along the needle track. The Hamilton syringe was refilled between each track. Monkeys recovered from surgery for 6 weeks before the imaging experiments began. At the end of the experiment (14 months postinjection), all monkeys were euthanized by sodium pentobarbital overdose (150 mg/kg i.v.), followed by perfusion with saline solution (containing 1% heparin) and 4% PFA, and brains were quickly removed and processed for histological studies.

Positron emission tomography (PET) Scans. ^{11}C-DTBZ PET studies were performed every 3 months by the group of Jose Obeso at the Center for Applied Medical Research of the University Clinic of Navarra (Pamplona, Spain). The synthesis of radioligands was performed at the Cyclotron Unit of the Nuclear Medicine Department of the University Clinic of Navarra following standard protocols. On each study day, anesthesia was initially induced by intramuscular injections of ketamine (10 mg/kg) and midazolam (1 mg/kg) to facilitate the preparation and handling of the monkeys. Anesthesia was maintained during the scans with a mixture of ketamine (5 mg/kg) and midazolam (0.5 mg/kg). All PET studies were conducted during lighting hours and animals were fasted overnight prior to the PET scan. PET imaging was performed in a dedicated small animal Philips Mosaic tomography (Cleveland), with 2 mm resolution, 11.9 cm axial field of view (FOV) and 12.8 cm transaxial FOV. The standard acquisition protocol used has been previously described in detail[271]. In brief, anesthetized animals were placed on the bed in the prone position with the head centered in the FOV. A transmission

study prior to the emission scan was carried out with an external 137Cs source (370 MBq). Radiotracers were injected intravenously via the saphenous vein simultaneously with the beginning of a 40min list mode study for ^{11}C-DTBZ. The mean (SD) injected activity was 2.15 (0.46) mCi for ^{11}C-DTBZ. For each study, a summed sonogram of the whole emission study and dynamic sonograms were created. From these sonograms, images were reconstructed in a 128 x 128 matrix with a 1 x 1 x 1 mm^3 voxel size using the 3D Ramla algorithm with two iterations and a relaxation parameter of 0.024. Dead time, decay, attenuation, random and scattering corrections were applied. To obtain parametric images, PET studies were analyzed by suitable tracer kinetic models using PMOD software (v.2.5; PMOD Technologies Ltd). The computed parameter was the binding potential (BD) of VMAT2 transporter for ^{11}C-DTBZ. The Ichise Multilinear Reference Tissue Model[272] was used for ^{11}C-DTBZ quantification, using the striatum as a VMAT2 transporter-rich region and the occipital cortex as a transporter-poor region. The parametric images were transformed into standard stereotaxic space using a species-specific template for each radioligand[271]. After this, a ROI analysis was conducted using a volume of interest (VOI)-map template that delineated the striatum (i.e. caudate and putamen), caudate nucleus, and pre-commissural and post-commissural putamen defined in a single-subject MRI in the same sterotaxic space[273]. Values for specific ^{11}C-DTBZ BP were obtained for each region. Imaging data were analyzed using PMOD software.

Postmortem processing. At the end of the experiment (14 months post-injection), all animals were euthanized by sodium pentobarbital overdose (150 mg/kg i.v.), followed by perfusion with saline solution (containing 1% heparin) and 4% PFA performed in accordance with accepted European Veterinary Medical Association guidelines. Brains were removed quickly after death. Each brain was bisected along the midline and each hemisphere was divided into three parts. The two hemispheres were post-fixed overnight in the same fixative, cryoprotected in PBS containing 20% sucrose before being frozen by immersion in a cold isopentane bath (-45ºC) and

stored at -80ºC until sectioning. Medial regions containing the basal ganglia of each hemisphere were cut on a cryostat into 50 μm-thick free-floating serial sections and stored in PBS containing 0.2% sodium azide at 4ºC until use.

Quantitative morphology. To assess the integrity of the nigrostriatal pathway, TH immunohistochemistry was performed on SNpc and striatal sections. Briefly, sections from three representative levels of the striatum (anterior, medial and posterior) and serial sections (1/12) corresponding to the whole SNpc were incubated with a mouse monoclonal antibody raised against human TH (Table 6) overnight at RT and revealed by an anti-mouse peroxidase EnVision™ system (DAKO) followed by DAB visualization. Free-floating SNpc sections were mounted on gelatinized slides, counterstained with 0.1% cresyl violet solution, dehydrated and coverslipped, while striatal sections were mounted on gelatinized slides and coverslipped.

TH-positive SNpc neurons: TH-positive SNpc cells were counted by stereology using a Leica DM6000B motorized microscope coupled with Mercator software. The SNpc was delineated for each slide and probes for stereological counting were applied to the map obtained (size of counting frame was 100 x 80 μm spaced by 600 x 400 μm). Each TH-positive cell with its nucleus included within the counting frame was counted. The optical fractionator method was finally used to estimate the total number of TH-positive cells in the SNpc of each monkey hemisphere.

TH-positive striatal fibers: The extent of the lesion in the striatum was quantified by OD. Sections were scanned in an Epson expression 10000XL high resolution scanner and images were used in ImageJ software to compare the grey level in each region of interest: i.e. caudate nucleus and putamen.

Alpha-synuclein: Alpha-synuclein expression levels were estimated for different regions after immunohistochemistry. Sections of the striatum (anterior, median and posterior part), superior

frontal gyrus, prefrontal gyrus, entorhinal cortex, pallidum (external and internal part), subthalamic nucleus, and SN were incubated with a mouse monoclonal antibody raised against human α-synuclein (Table 6)[274-276] overnight at RT and revealed by an anti-mouse peroxidase EnVision™ system (DAKO) followed by Vector SG Peroxydase incubation. Free-floating sections were mounted on gelatinized slides, and scanned using an Epson expression 10000XL high resolution scanner. ImageJ software was used to quantify the grey levels across the regions of interest.

Satistical analysis. All values are expressed as the mean ± standard error of mean (S.E.M). For experiments in mice, differences among means were analyzed by using one- or two-way ANOVA. When ANOVA showed significant differences, pair-wise comparisions between means were subjected to Student-Newman-Keuls post-hoc testing. For experiments on monkeys, statistical analyses were carried out with Sigma Stat software (version 11.0). For normally distributed data, means between groups were compared with one-sample Students' t-test and Chi-squared analysis. In all analyses, the null hypothesis was rejected at the 0.05 level. All analyses were performed blinded to the researcher.

2. HYPOTHESIS 2: FEASIBILITY AND SAFETY OF INTRANASAL siRNA-MEDIATED α-SYNUCLEIN DOWNREGULATION.

2-1 Conjugated siRNA.

The synthesis and purification of Indatraline-conjugated siRNA molecules (Fig. 18) directed against α-synuclein (SNCA2-siRNA, MAYO2-siRNA, SNCA499-siRNA and IND-SNCA499-siRNA) and nonsense (NS-siRNA and IND-NS-siRNA) were performed by nLife Therapeutics S.L. (Granada, Spain) as reported in patent application numbers EP12382414.6 and US 61/719,284 (see Table 5 for sequences).

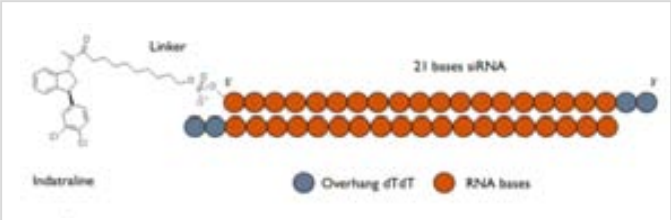


Figure 18. Schematic representation of the siRNA conjugated to the cell-specific ligand Indatraline to promote their selective delivery into aminergic neurons used in our experiments.

siRNA	Sequence	References
SNCA 499	AS: gcucccuccacugucuucuTT	Han Y. et al., (2011) *Brain Res*
	SS: agaagacaguggagggagcTT	Khodr C.E. et al. (2011) *Brain Res.*
SNCA 2	AS: gccuacauagagaacaccTT	Fountaine T.M. et al. (2008) *Eur J Neurosci.*
	SS: gguguucucuauguaggcTT	Gorbatyuk O.S. et al. (2009) *Md Ther .*
MAYO 2	AS: uuggucuucucagccacuguuTT	Lewis, J. et al. (2008) *Md Neurodegener.*
	SS: aacaguggcugagaagaccaaTT	

Table 5. Sequences of the siRNA used in our study

In brief, siRNA (sense and antisense strands) synthesis was performed using ultramild-protected phosphoramidites (glen Research, Sterling) and H-B DNA/RNA Automatic synthesizer (K&A Laborgeraete GbR). Sense strand was amino-modified by performing a 5'-C6 amino modification and condensation with a succinimide active ester of Indatraline. Conjugated single strand oligonucleotides were purified by high performance liquid chromatography using a RP-C18 column (4.6 x 150 mm, 5 μm) under a linear gradient condition of acetonitrile shifting the concentration from 5% to 35% for 30min in 100 mM TEAA (pH 7.0). The molecular weights of

the siRNA strands and the conjugate were confirmed by MALDI-TOF mass spectrometry (Ultraflex, Bruker Daltonics) as predicted. The yield of the conjugates was spectrophotometrically calculated on the basis of absorbance at 260 nm wavelength. Complementary strands were annealed in an isotonic RNA-annealing buffer (100 mM potassium acetate, 30 mM HEPES pH 7.4, 2 mM magnesium acetate), pre-incubated by 1min at 90ºC, centrifuged for 15s and incubated 1h at 37ºC. Duplex RNA formation was confirmed using 20% polyacrylamide gel electrophoresis (PAGE, 30 mA, 60min) and visualized by silver staining (DNA silver stain kit, GE Healthcare).

To study *in vitro* and *in vivo* intracellular distribution of conjugated siRNA into aminergic neurons, conjugated NS-siRNA molecule was additionally bound to the fluorocrom dye Cy3 in the antisense strand. Stock solution of all siRNA molecules were prepared in RNAse-free water and stored at -20ºC until use.

2.2 *In vitro* experiments.

Cell culture. BE(2)-M17 cells stably expressing α-synuclein or the corresponding empty vector were grown in Optimem (Gibco) medium supplemented with 10% FCS and 0.5 mg/ml active Geneticin (Gibco). Transfection with 200 nM of the corresponding siRNAs was done in 24-well plates using lipofectamine RNAiMAX (Invitrogen). Equal transfection efficiency in independent experiments was controlled by using BLOCK-IG Fluorescent Oligo (Invitrogen).

Primary culture. Postnatal (P1-P3) ventral midbrain neurons were cultured on rat astrocyte monolayers following the Sulzer Lab protocol (http://sulzerlab.org/). Briefly, to obtain the astrocyte monolayer, cortex of postnatal P1-P3 rat pups was mechanically disrupted in warmed M10C-G medium with a 10 ml syringe, and after diluting the homogenate in M10C-G medium it was plated on laminin-coating 24-well-plates. When glial culture was 70% confluent, it was maintained with MEM containing FDU. P1-P3 ventral midbrain was enzymatically digested with

papain and then mechanically tritured in SF1C-medium using different size tips. After centrifugation at 1000 x g for 5min, cells were resuspended in SF1C medium and plated above the astrocyte monolayer. To validate the targeting of the siRNA, primary culture was treated with non-sense siRNA conjugated to Cy3 with or without the cell-specific ligand indatraline, so-called IND-siRNA Cy3 and siRNA-Cy3 respectively, in a final concentration of 100 nM. Fluorescent-inverted microscope was used to visualize the uptake of the siRNA 3h after transfection. At the selected time-point, the transfection medium was removed and substituted by PBS.

mRNA extraction and reverse transcription PCR (RT-PCR). Isolation of mRNA was performed 24h post-transfection by the TRIZOL™ (Invitrogen) method following the manufacturer's protocol. Briefly, cell pellets were lysed in 1 ml TRIZOL™ Reagent (Invitrogen) and frozen overnight to help homogenization. Then 0.2 ml chloroform was added and samples were centrifuged for 15min at 10000 rpm at 4ºC. The aqueous supernatant was mixed with 0.5 ml isopropanol and centrifuged again for 20min at 15000 rpm at 4ºC. Finally, isopropanol was replaced by 0.9 ml of cold 75% ethanol and the samples were centrifuged for 5min at 10000 rpm at 4ºC. Air-dried samples were ressuspended in RNAse-free water and genomic DNA was removed by digestion with DNase I (Qiagen). Total mRNA concentration was measured on a Nanodrop 200 (Thermo Fischer Scientific). One μg of total mRNA was reverse-transcribed with Oligo dT by using SuperScript III™ first-strand synthesis system for RT-PCR (Invitrogen). After RNAse treatement, cDNA was diluted to 8 ng/ul in 10 mM Tris-HCl (pH = 8.0).

Quantitative Real-Time PCR (RT-qPCR). RT-qPCR was performed on a 7900HT SDS (Applied Biosystems) with TaqMan Universal Master Mix II with UNG (Roche Applied Biosystems) for detection using 20 ng of cDNA per reaction in a total volume of 10 μl. The cycling conditions were as follows: 2min at 50ºC and 10min at 95ºC, followed by 40 cycles, each consisting of 15s at 95ºC and 1min at 60ºC. Fluorescence-labelled specific probes were used to detect the expression levels of the target genes alpha- (SNCA-FAM, #Hs00240907-m1), beta-

(SNCB-FAM, #Hs00608185-m1) or gamma-synuclein (SNCG-FAM, #Hs00268306-m1), normalized against the housekeeping genes beta-actin (FAM, #4333762F), RPLPO (VIC, #4326314E) and GADPH (FAM, #4333764F), all from Applied Biosystems. The threshold cycles (Ct) were calculated using the software ABI PRISM 7900HT SDS version 2.2 (Applied Biosystems). Relative quantification using the comparative Ct method was used to analyze the data output. Values were expressed as the fold-change over corresponding values for the control by the 2-ΔΔCt method.

Immunoblot. Cells were recollected and centrifuged at 1500 rpm for 5min. Pellets were washed with PBS three times. Lysis buffer (Tris-HCl 50 mM pH=7.4, NaCl 150 mM, EDTA 1 mM, Triton X-100 1%) was added to the pellet and then incubated on ice for 30min. Total protein concentration was determined using the BCA assay (Thermo Scientific). Thirty μg of protein were resolved by SDS-PAGE on 12% polyacrylamide gels and electrotransferred onto nitrocellulose membranes (GE Healthcare), which were blocked in 5% non-fat milk powder in PBS for 1h at RT and incubated overnight at 4ºC with the human α-synuclein primary antibody (Table 6). Incubation with the anti-mouse secondary antibody coupled to horseradish peroxidase (dilution 1:5000; Amersham Biosciences) was performed at RT for 1h, followed by repeated washing with PBS. Immunoreactive bands were visualized using SuperSignal Femto Chemiluminescent Substrate (Pierce) according to the manufacturer's instructions on an ImageQuant RT ECL imaging system (GE Healthcare).

2.3. *In vivo* experiments.

Animals. Male C57BL/6NCrl mice (10-14 weeks; Charles River, Lyon, France) were housed under controlled conditions (22ºC ± 1ºC; 12h light/dark cycle) with food and water available *ad libitum*. Animal procedures were conducted in accordance with standard ethical guidelines (EU regulations L35/118/12/1986) and approved by the local ethical committee.

Intracerebral siRNA infusion. Wild-type C57BL/6 mice were anesthetized (pentobarbital 40 mg/kg, i.p.) and silica capillary microcannulae (110 mm-OD, 400 mm-ID; Polymicro Technologies, Madrid, Spain) were stereotaxically implanted into the SNpc (coordinates in mm: anteroposterior –AP, -2.9; mediolateral-ML, -1.3; dorsoventral-DV, -4.2). siRNA microinfusion was performed with a perfusion pump at 0.5 μl/min flow rate 20-24h after surgery in awake mice. siRNA targeting α-synuclein or nonsense were prepared in artificial cerebrospinal fluid (aCSF, 125 mM NaCl, 2.5 mM KCl, 1.26 mM $CaCl_2$ and 1.18 mM $MgCl_2$ with 5% glucose) and infused at dose of 10 μg or 20 μg of siRNA per mouse. Intra-SNpc siRNA infusion was repeated 24h later (1 μl aliquots; 2 administrations in total). Control mice received aCSF[277-279].

Intranasal siRNA administration. Wild-type C57BL/6 mice were anesthetized by 2% isofluorane inhalation and placed in a supine position, with the head supported at a 45 degree angle to the body[277]. A 5 μl drop of PBS or conjugated siRNA (IND-NS-siRNA and IND-SNCA499-siRNA prepared in PBS) was applied alternatively to each nostril once daily. A total of 10 μl of solution containing 30 μg (2.1 nmol/day) of conjugated siRNA was delivered for 4 days and, mice were euthanized at 1, 3 or 7 days after the last administration.

In situ hybridization. In situ hybridization experiments were performed and analyzed at the laboratory of Analia Bortolozzi (IDIBAPS, Barcelona, Spain). At selected time points, mice were killed by pentobarbital overdose and brains rapidly removed, frozen on dry ice and stored at -80ºC. Coronal tissue sections (14 μm thick-coronal) were cut using a microtome-cryostate, thaw-mounted onto 3-aminopropyltriethoxysilane (Sigma-Aldrich)-coated slides and kept at -20ºC until use. Antisense oligoprobes were complementary to bases: α-synuclein/441-447 (GenBank accession NM-001042451) and γ-synuclein/336-416 (NM-011430). The oligonulceotides were individually labeled (2 pmol) at the 3'-end with [^{33}P]-Datp (<2500 Ci.mmol^{-1}; DuPont-NEN) using terminal deoxynucleotidyltransferase (TdT, Calbiochem). The labeled oligoprobes were purified using QIAquick Nucleotide Removal Kit (QIAGEN GmbH). Sections were hybridized

as previously described[277-279]. Briefly, frozen tissue sections were first brought to RT, fixed for 20min at 4ºC in 4% PAF in PBS, washed 3 times in PBS at RT, and incubated for 2min at 21ºC in a solution of predigested pronase (Calbiochem) at a final concentration of 24 UmL^{-1} in 50 mM Tris-HCl, pH 7.5, and 5 mM EDTA. The enzymatic activity was stopped by immersion for 30s in 2 mg/ml glycine in PBS. Tissues were finally rinsed in PBS and dehydrated through a graded series of ethanol. For hybridization, the radioactively labeled probes were diluted in a solution containing 50% formamide, 4x standard saline citrate, 1x Denhardt's solution, 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer, pH 7.0, 250 μg/ml yeast tRNA, and 500 μg/ml salmon sperm DNA. The final concentrations of radioactive probes in the hybridization buffer were in the same range (~1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probes, overlaid with Nescofilm coverslips (Bando Chemical Ind.), and incubated overnight at 42ºC in humid boxes. Sections were then washed 4 times (45min each) in a buffer containing 0.6 M NaCl and 10 mM Tris-Hcl (pH 7.5) at 60ºC. Hybridized sections were exposed to Biomax-MR film (Kodak, Sigma-Aldrich) for 1-4 weeks with intensifying screens. For specificity control, adjacent sections were incubated with an excess (50x) of unlabelled probes. The cytoarchitecture of different mouse brain areas were analyzed in an adjacent series of cresyl-violet stained frozen section. Autoradiograms were analyzed and the relative optical densites (ROD) were obtained using a computer assisted image analyzer (MCID). The slide background was substracted. ROD were evaluated in SNpc/VTA, DR and LC at the three antero-posterior coordinates by duplicate and averaged to obtain individual values. MCID system was also used to acquire pseudocolor images. Black and white photographs were taken from autoradiograms using a Wild 420 macroscope (Leica) equipped with Nikon KXM1200F digital camera and ACT-1 Nikon software (Soft Imaging System Gmbh). Images were processed with Photoshop (Adobe Systems, Mountain View) by using identical values for contrast and brightness.

Immunohistochemistry. Immunohistochemistry for α-synuclein and TH were performed as previously described in pages 66-67.

Quantitative morphology. Analysis of α-synuclein levels and nigrostriatal integrity were performed as previously described in pages 67-69.

Statistical analyses. In vitro: data are presented as mean ± S.E.M. Statistical analyses were performed by one-way analysis of variance (ANOVA) followed by a post-hoc Turkey test to determine the differences between the groups. Data were analyzed using SigmaStat software (version 3.0; Jandel Scientific Software). In vivo: all results are presented as the mean ± S.E.M. Data were analyzed using GraphPad Prism 6.0 (San diego, CA). For all experiments, one-way or two-way ANOVA with repeated-measure were applied to the data as appropriate. Significant main effects were followed by Turkey's post-hoc test. In all analyses, the null hypothesis was rejected at the 0.05 level. All analyses were performed blinded to the researcher.

Antibody	Source	Host	Type	Dilution	
Human Samples					
hSyn Ab-2 (clone syn211)	Thermo Scientific, #MS-1572	Mouse	Monoclonal	1/1000 (WB)	
pSyn (phospho 129)	WAKO, #014-20281	Mouse	Monoclonal	1/1000 (WB)	
Ubiquitn	Sigma-Aldrich U5379	Rabbit	Polyclonal	1/1000 (WB)	
p62	Progen GP62-C	Guinea Pig	Polyclonal	1/1000 (WB)	
α-Synuclein	BD Transduction Laboratories, #610786	Mouse	Monoclonal	1/1000 (WB)	1/20 (IP)
M17 Cell-line					
hSyn Ab-2 (clone syn211)	Thermo Scientific, #MS-1572	Mouse	Monoclonal	1/1000 (WB)	
Mice					
α-Synuclein	BD Transduction Laboratories, #610786	Mouse	Monoclonal	1/1000 (DAB)	1/1000 (IFX)
hSyn Ab-2 (clone syn211)	Thermo Scientific, #MS-1572	Mouse	Monoclonal	1/250 (DAB)	1/50 (IFX)
pSyn (phospho S129)	Abcam, #2014-1	Rabbit	Monoclonal	1/750 (DAB)	1/500 (IFX)
Tyrosine Hydroxylase	Milipore #MAB5280	Mouse	Monoclonal	-	1/1000 (IFX)
	Calbiochem #657012	Rabbit	Polyclonal	1/2000 (DAB-SNpc), 1/5000 (DAB-Str)	1/1000 (IFX)
Iba1	Wako Pure Chemical Industries #019-19741	Rabbit	Polyclonal	1/1000 (DAB)	-
Glial Fibrillary Acid Protein (GFAP)	Sigma-Aldrich #G3893	Mouse	Monoclonal	1/1000 (DAB)	-
Monkey					
hSyn Ab-2 (clone syn211)	Thermo Scientific, #MS-1572	Mouse	Monoclonal	1/1000 (DAB)	-
pSyn (phospho S129)	WAKO, #014-20281	Mouse	Monoclonal	1/1000 (DAB)	-
Tyrosine Hydroxylase	Milipore #MAB318	Mouse	Monoclonal	1/10000 (DAB-SNpc), 1/1000 (DAB-Str)	-

Table 6: list of primary antibodies used in our study. DAB: 3,3'-diaminobenzidine tetrahydrochloride; IFX: immunofluorescence; IP: immunoprecipitation; WB: Western blot

RESULTS

1 LEWY BODY EXTRACTS FROM PARKINSON'S DISEASE BRAINS TRIGGER α–SYNUCLEIN PATHOLOGY AND NEURODEGENERATION IN MICE AND MONKEYS

1.1 Purification of Nigral LB Extracts From PD Patients.

LB-enriched fractions were obtained from freshly frozen postmortem SNpc tissue derived from 3 patients with sporadic PD exhibiting abundant LB pathology at neuropathological examination (Fig. 19). LB purification from these samples was achieved by sucrose gradient fractionation, as previously validated[256,280], with LB-containing fractions being recovered at the 1.4/2.2M interface (Fig. 20).

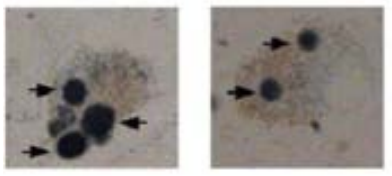


Figure 19. Immunohistochemistry images of α-synuclein-positive LB (arrows, in black) in nigral post-mortem brain sample of the PD #3 patient before sucrose gradient purification; brown pigments correspond to neuromelanin.

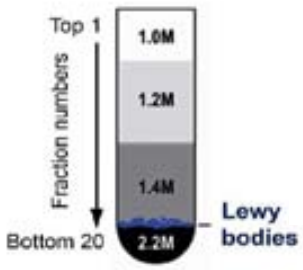


Figure 20. Schematic representation of the sucrose gradient fractionation procedure used to purify LB-containing fractions from freshly frozen post-mortem nigral brain tissue of three sporadic PD patients.

To identify LB fractions, all recovered sucrose gradient fractions (~19-20) were screened by FRA for the presence of insoluble α-synuclein aggregates. This assay permits the detection and quantification of detergent- or urea-insoluble amyloid-like fibrils and high molecular weight insoluble protein aggregates based on their ability to be retained on cellulose acetate membranes with a 0.2 μm pore size[258-260]. Using this biochemical assay, LB fractions containing insoluble aggregated α-synuclein are mostly sedimented within fractions ~15 to 19 (Fig. 21).

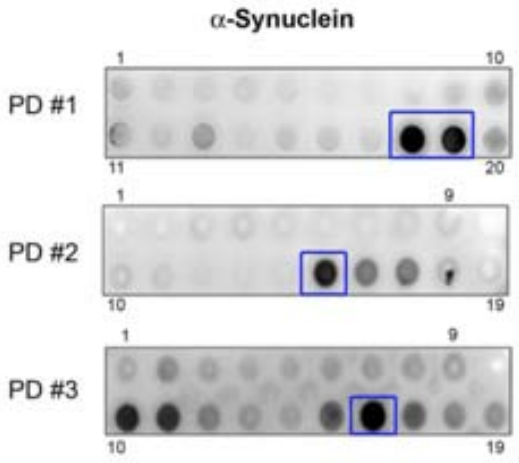


Figure 21. Filter retardation assay probed with a human α-synuclein antibody to assess the presence of insoluble α-synuclein aggregates in the different fractions obtained by sucrose gradient fractionation from freshly frozen post-mortem nigral brain tissue from three sporadic PD patients. Blue rectangles indicate the LB-containing fractions selected to prepare the mixtures used for inoculations.

The presence of LB in these fractions was further confirmed by the immunodetection of hyperphosphorylated α-synuclein (pSyn), ubiquitin and p62 (Fig. 22), all of which are known components of LB (see Table 1 page 5).

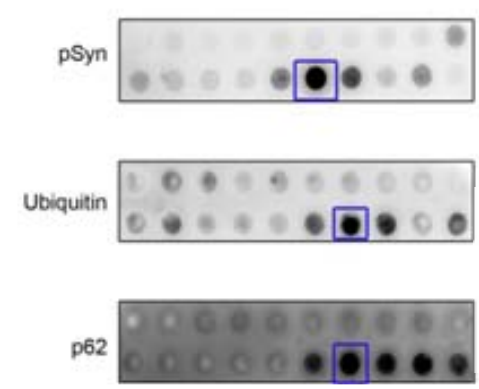


Figure 22. Filter retardation assay probed with antibodies against phosphorylated α-synuclein (pSyn), ubiquitin and p62 in the different fractions obtained by sucrose gradient fractionation from freshly frozen post-mortem nigral brain tissue from PD #3. Blue rectangles indicate the LB-containing fraction selected to prepare the mixture used for inoculations.

In addition, examination of LB fractions by fluorescence microscopy confirmed the presence of immunolabeled α-synuclein, which colocalize with the amyloid-binding dye thioflavine S (Fig. 23A). Ultrastructural examination of LB fractions by electron microscopy revealed the presence of spherical or amorphous aggregates with an α-synuclein immunoreactive filamentous structure as determined by immunogold labeling (Fig. 23B).

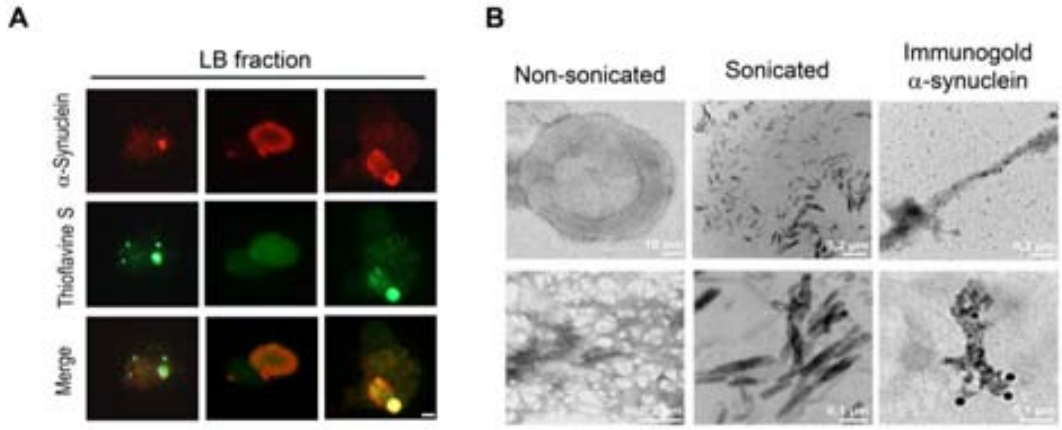


Figure 23. (A) Confocal examination of purified LB fractions with α-synuclein immunofluorescence (red) and thioflavin S staining (green). Scale bar, 10 μm. (B) Ultrastructural examination of LB fractions by electron microscopy (before and after sonication) and immunogold labeling

For stereotactic inoculations in mice and monkeys, LB-containing fractions from the three PD patients were mixed together in the same proportion (PD #1, fractions 18 and 19; PD #2, fraction 15; PD #3, fraction 16; see Fig. 21). Prior to inoculation, this mixture was sonicated for 5 min, resulting in the disruption of the aggregates into fibrillar fragments of different sizes (Fig. 23B)[245, 281, 282]. Indeed, the signal of α-synuclein after FRA was decreased in the samples sonicated compared to non-sonicated samples, confirming that the levels of aggregated α-synuclein were diminished after sonication (Fig. 24).

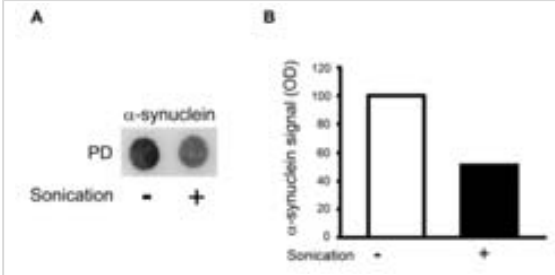


Figure 24. (A) Filter retardation assay (FRA) probed with a human α-synuclein antibody to assess the presence of insoluble α-synuclein aggregates in the LB-fractions derived from PD #1 before and after sonication. (B) Bar graphs showing the densitometric quantification of the FRA.

Quantification by ELISA indicated that this mix contained ~15ng of α-synuclein per milligram of total protein (corresponding to ~6pg of α-synuclein per microliter of injected sample, see Table 7). Control-injected animals were inoculated with a mixture of non-LB fractions (i.e. fraction 6, at the beginning of the 1.2M interface) derived from the same three PD patients, which contains soluble or finely granular α-synuclein but lacks large LB-linked α-synuclein aggregates[257, 280, 283]. The amount of α-synuclein in the non-LB

mixture, as quantified by ELISA, was ~28ng per milligram of total protein (corresponding to ~10pg of α-synuclein per microliter of injected sample, Table 7). Additional control animals were injected with the corresponding buffers (i.e. vehicle) obtained from a sucrose gradient purification performed without the addition of any brain sample.

Fractions	BCA results (μg/μl)	ng of α-synuclein per mg of total protein	pg of α-synuclein per μl of injected sample
LB-fraction	0,4	15	6
Non-LB fraction	0,36	28	10

Table 7. Concentration of total protein and soluble α-synuclein protein in the LB- and Non-LB fraction measured by BCA and ELISA respectively.

1.2. Intracerebral Inoculation of Human LB Extracts Initiates Nigrostriatal Dopaminergic Neurodegeneration in Mice

For the experiments on rodents, adult wild-type C57BL6 mice received a single unilateral stereotaxic inoculation of either LB fractions, non-LB fractions or vehicle immediately above the right SNpc (Fig. 25). Following inoculations, the animals were euthanized at different time-points (i.e., 24hrs, 4 weeks, 4 months and 17 months) after LB-inoculation and their brains were processed for histochemical examination.

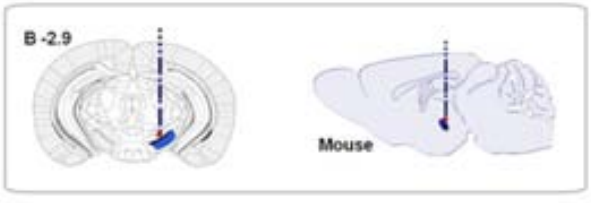


Figure 25. Schematic diagram indicating the site of nigral stereotactic LB inoculations in mice.

The integrity of the dopaminergic nigrostriatal system, at the level of both SNpc cell bodies and striatal axon terminals, was assessed by stereological cell counts of dopaminergic TH-positive SNpc neurons and by optical densitometry (OD) of striatal

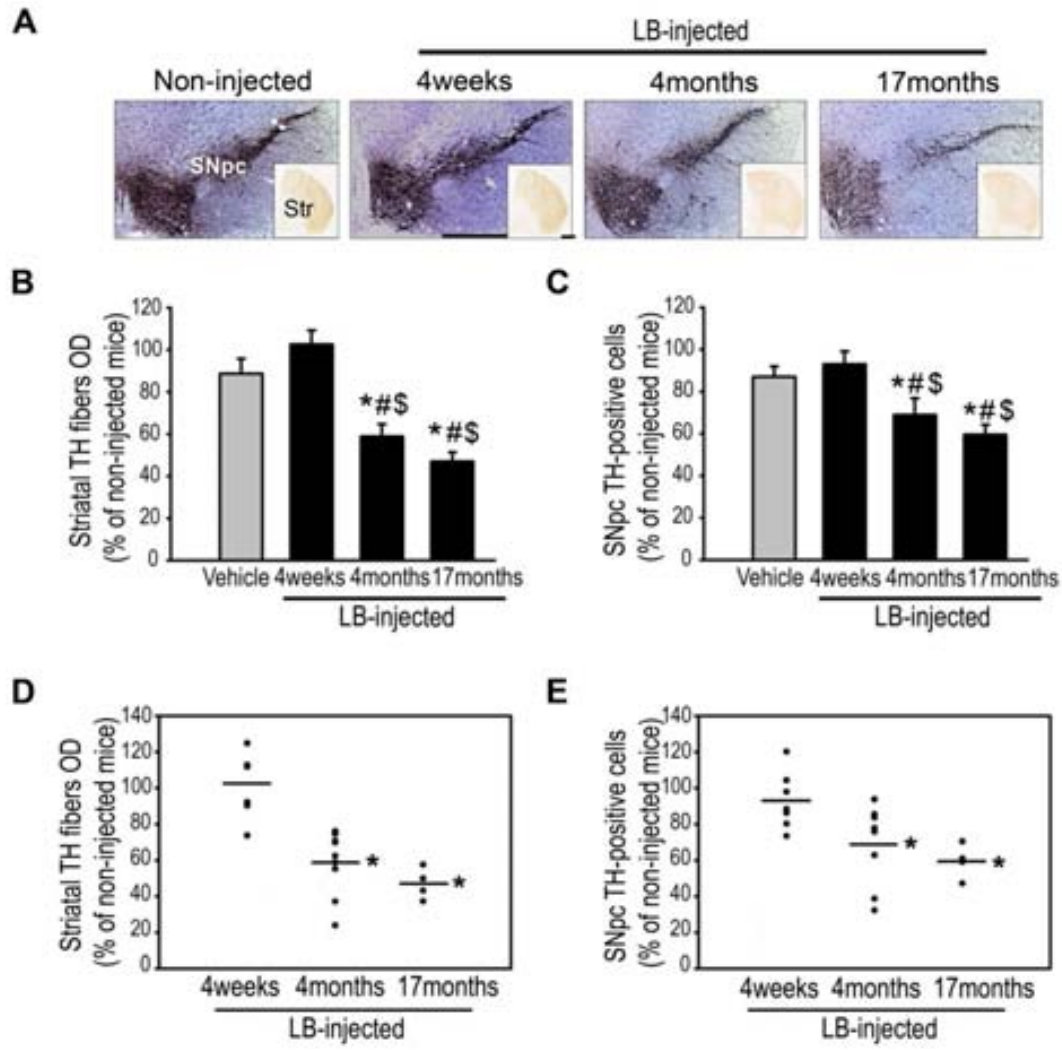


Figure 26. (A) Representative photomicrographs of TH-positive SNpc neurons (Brown; thionin staining in purple) and TH-positive striatal terminals (Str, inset) in non-injected and LB-injected mice at different time-points after LB inoculations. Scale bars, 500μm. (B-C) Quantification of TH-positive striatal terminals by optical densitometry (B) and the number of TH-positive SNpc neurons by stereology (C) in vehicle- and LB-injected mice, relative to non-injected animals. Histograms represent average ± S.E.M. Non-injected mice, N=23-26; vehicle-injected mice, N = 13; LB-injected mice at 4 weeks, N = 7; LB-injected mice at 4 months, N = 8-9; LB-injected mice at 17 months, N = 4. * $p< 0.05$ compared with non-injected mice; # $p<0..05$ compared with vehicle-injected mice; $ $p<0.05$ compared with LB-injected mice at 4 weeks post-inoculations. (D-E) Point plot representation of the same quantifications shown in (B-C), in which the individual values per LB-injected animal are represented. Each point represents an individual animal with the horizontal line indicating the average value per group. Quantifications correspond to either striatal TH terminals by optical densitometry (D) or number of SNpc TH-positive neurons by stereology (E) in the different groups of LB-injected mice, relative to non-injected animals. *$p<0.05$ compared with either non-injected animals and to LB-injected animals at 4 weeks post-injections.

dopaminergic TH-positive fibers, respectively (Fig. 26A). No evidence of nigrostriatal degeneration was observed in these animals in the first 4 weeks after LB inoculation. However, by 4 months, LB-injected mice exhibited a progressive loss of striatal TH-positive fibers up to 17 months after LB inoculation (Fig. 26B, D). Concomitant with striatal dopaminergic denervation, some LB-injected mice began to exhibit SNpc

dopaminergic cell loss by 4 months, which increased and extended to all LB-injected animas by 17 months (Fig. 26C, E). LB-induced decreases in the number of SNpc TH-positive neurons corresponded to an actual cell death and not to a mere downregulation of TH expression, as similar decreases were also observed in the number of thionin-stained SNpc neurons at 17 months after LB injection [12,500 ± 538.75 SNpc Nissl-positive neurons in non-injected mice vs 7,837.5 ± 259.9 in LB-injected mice; $p<0.001$, Student-t test]. In contrast, no nigrostriatal degeneration was observed in animals injected with either vehicle or non-LB fraction obtained from the same PD patients (Fig. 27).

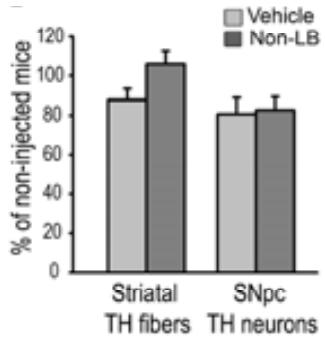


Figure 27. Quantifications of TH-positive striatal terminals by optical densitometry (left) and the number of SNpc TH-positive neurons measured by stereology (right) in mice inoculated with either "non-LB" fractions (F6) containing non-aggregated (soluble or finely granular) α-synuclein, derived from the same PD patients from which LB fractions were extracted, or the corresponding buffer (vehicle), at 4 months post-inoculations. Histogram represent average ± S.E.M. Non-injected mice, N=4-6; vehicle-injected mice, N = 5-6; Non-LB-injected mice, N = 10. No statistically significant differences were found between non-injected, vehicle-injected and animals injected with "non-LB"fractions.

At 4 months, LB-injected animals exhibited astrogliosis in the SNpc, suggestive of ongoing, progressive neurodegeneration (Fig. 28).

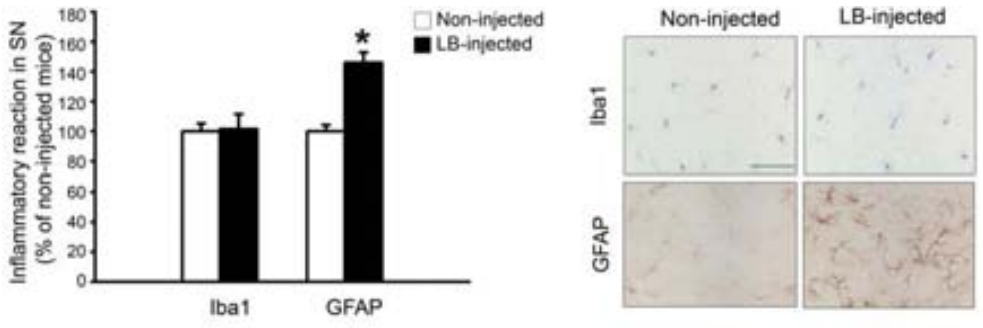


Figure 28. Inflammatory reaction in the substantia nigra (SN) of LB-injected mice. Quantifications (left) and representative photomicrographs (right) of Iba1-positive microglial cells (by stereology) and GFAP-positive astrocytic reaction (by regional densitometry) in the SN of LB-injected mice at 4 months post-inoculations. Histograms represent average ± S.E.M. Non-injected mice, N=8; LB-injected mice, N=9. *$p<0.05$ compared with non-injected animals.

No gross motor or behavioral abnormalities were observed in LB-injected mice up to 17 months. However, at 4 months postinoculation, LB-injected animals exhibited

impaired motor ability when challenged with the pole test, which measures motor coordination and balance (Fig. 29)

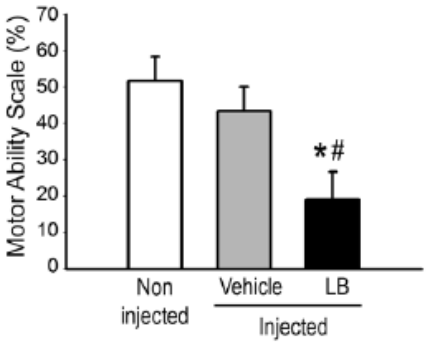


Figure 29. Motor performance of LB-injected mice in the pole test at 4 months post-inoculation. Mice were subjected to the test on three consecutive trials. The average of those three trials was calculated and considered for statistical analyses. *p<0.05 compared with non-injected mice; #p<0-05 compared with vehicle-injected mice. Non-injected mice, N=5; vehicle-injected mice, N=7; LB-injected mice, N=7.

Our results indicate that nigral LB extracts derived from PD patients are able to initiate a slowly progressive nigrostriatal neurodegenerative process affecting initially and more extensively dopaminergic axon terminals in the striatum rather than SNpc cell bodies, which is consistent with the pattern of nigrostriatal degeneration occurring in PD patients[15, 21]

1.3. LB-Induced Pathogenic Effects in Mice are Associated with α-Synuclein Pathology.

We next assessed whether LB-induced neurodegeneration was associated with α-synuclein pathology in injected animals. Using an antibody that recognizes human but not murine α-synuclein, we found that exogenously inoculated LB-linked α-synuclein was internalized by host cells, including TH-positive SNpc neurons, by 24h post-injection, which is consistent with previous studies in which synthetic recombinant α-synuclein was shown to be quickly taken up by cultured neurons via endocytosis (Fig. 30)[235, 238-240]. At this early time point, exogenous human α-synuclein in LB-injected mice was detected as a punctuate, inclusion-like immunolabeling within the cytoplasm and processes of host cells.

No exogenous human α-synuclein immunosignal was detected in LB-inoculated animals at any of the later time-points, from 4 weeks to 17 months postinjection.

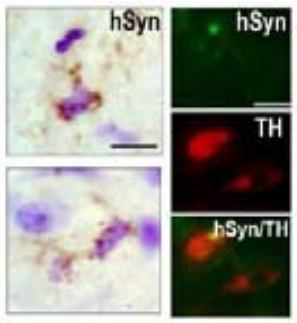


Figure 30. Immunohistochemistry of human-synuclein (hSyn, in brown; thionin staining in purple) and double immunofluorescence for hSyn (green) and TH (red) in the SNpc of LB-injected mice at 24h post-injection.

In contrast, using an antibody that recognizes both human and murine α-synuclein, LB-injected mice exhibited diffuse cytoplasmic accumulations of α-synuclein within SNpc neurons, including TH-positive cells, at 4 months post-injection (Fig. 31). In the absence of any human-specific α-synuclein immunohistochemical signal at this time-point, the observed cytoplasmic accumulation of α-synuclein in LB-injected animals at 4 months can be exclusively attributed to endogenous murine α-synuclein.

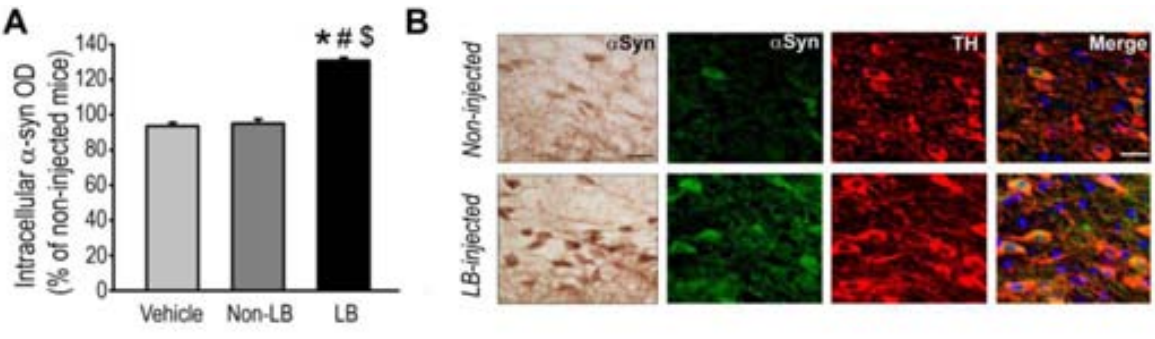


Figure 31. (A) Quantification of intracellular levels of endogenous murine α-synuclein within SNpc neurons by optical densitometry in mice injected with either vehicle, non-LB or LB fractions at 4 months after inoculations, relative to non-injected animals. An average of ~600 neurons per group was analyzed. *$p<0.05$ compared with non-injected mice; #$p<0.05$ compared with vehicle injected mice; \$$p<0.05$ compared with non-LB injected mice. (B) Photomicrographs correspond to representative immunostaining images of endogenous α-synuclein (brown or green) in dopaminergic TH-positive (red) SNpc neurons by confocal microscopy.

Accumulated α-synuclein in LB-inoculated mice became resistant to proteinase K (PK) digestion, indicating that endogenous murine α-synuclein had adopted a pathological insoluble/aggregated β-sheet conformation by 4 months after LB inoculation (Fig. 32).

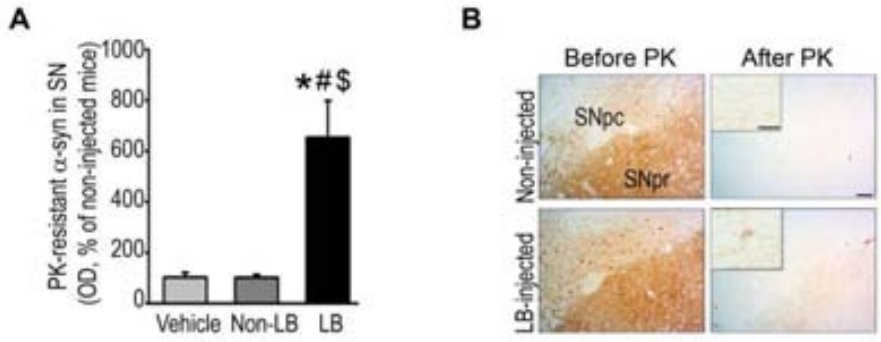


Figure 32. Quantification of regional levels of endogenous murine α-synuclein by optical densitometry in the SN of mice injected with either vehicle, non-LB or LB fractions at 4 months after inoculations, relative to non-injected animals, after proteinase K (PK) digestion. Non-injected mice, N=9; vehicle-injected mice, N=8; Non-LB-injected mice, N=9; LB-injected mice, N=5. *p<0.05 compared with non-injected mice; #p<0.05 compared with vehicle-injected mice; $p<0.05 compared with non-LB injected mice. Photomicrographs correspond to representative immunostaining of endogenous α-synuclein in the SN before and after PK treatment (SNpc, substantia nigra pars compacta; SNpr, substantia nigra pars reticulate).

The pathological nature of accumulated endogenous α-synuclein in LB-injected mice was further corroborated using and antibody recognizing pSyn (phosphorylated at serine 129), which is found abundantly in human LB aggregates and is widely used as a marker for pathological α-synuclein[39], although it can also be found at lower levels in nonpathological tissue[114]. At 4 months postinjection, mice inoculated with LB exhibited numerous pSyn-positive neurons in the SNpc, including TH-positive cells (Fig. 33). In affected neurons, pSyn immunolabeling mostly appeared as weakly diffuse within the neuronal cytoplasm, although in a few instances it adopted a more punctuate, inclusion-like conformation. No pSyn-positive cells were detected at later time-points (i.e. 17months after LB inoculation), once SNpc dopaminergic cell death was full established. In addition, no pathological alterations of murine α-synuclein (i.e. cytoplasmic accumulation, PK resistance or phosphorylation) were found in the SNpc of mice injected with vehicle or non-LB fractions.

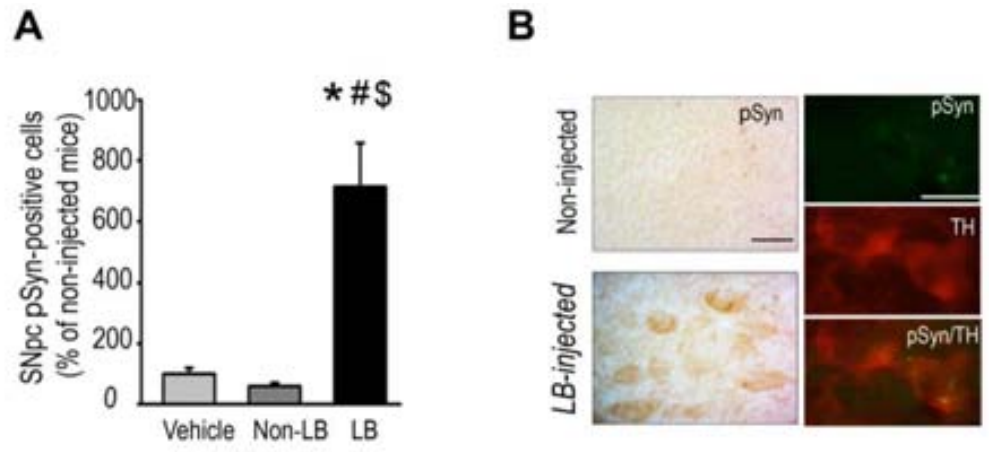


Figure 33. (A) Number of phosphorylated α-synuclein (pSyn)-immunopositive cells in the SNpc of mice injected with either vehicle, non-LB or LB fractions at 4 months after inoculations, relative to non-injected animals. Non-injected mice, N=10; vehicle-injected mice, N=10; Non-LB-injected mice, N=9; LB-injected mice, N=5. *p<0.05 compared with non-LB-injected mice. (B) Photomicrographs correspond to representative immunostaining of pSyn (brown) in the SNpc of non-injected and LB-injected mice 4 months after inoculation. In LB-injected mice, some pSyn immunofluorescence signal (green) was detected within dopaminergic TH-positive neurons (red). Histogram represents average ± S.E.M. Scale bar = 30μm.

As previous studies suggested that α-synuclein pathology spreads into other brain regions, we studied the presence of pathological phosphorylated form of α-synuclein in other brain areas. Remarkably, by 4 months post-inoculation, LB-injected mice also displayed increased levels of pSyn in distant brain regions such as striatum and neocortical areas (i.e. cingulate, motor and somatosensory cortices) (Fig. 34). These increases were revealed by regional densitometry of pSyn immunolabeling and corresponded mostly to presynaptic terminals innervating the affected regions, rather than cell bodies intrinsic of these regions.

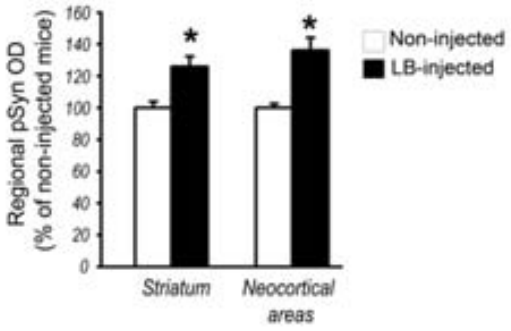


Figure 34. Quantification of regional level of pSyn by optical densitometry in the striatum and neocortical areas (i.e. cingulate, motor and somatosensory cortices) of LB-injected mice relative to non-injected animals at 4 months after LB inoculations. Non-injected mice, N=12; LB-injected-mice, N=8, *p<0.05 compared with non-injected mice. Histogram represents average ± S.E.M.

1.4. LB-Induced Pathogenic Effects in Mice are Dependent on Both Exogenous α-Synuclein Present in LB and Host (Endogenous) Expression of α-Synuclein

Because LB extracts contain many components other than α-synuclein[257], we next determined whether the pathogenic effects induced by these extracts were mediated by α-synuclein. Supporting the later, the presence of human α-synuclein within LB extracts appeared as a requirement to induce the pathological conversion of murine α-synuclein, as no pSyn-positive cells were observed in mice inoculated with LB fractions that had been depleted of α-synuclein by sequential immunoprecipitation prior to injection (Fig. 35)

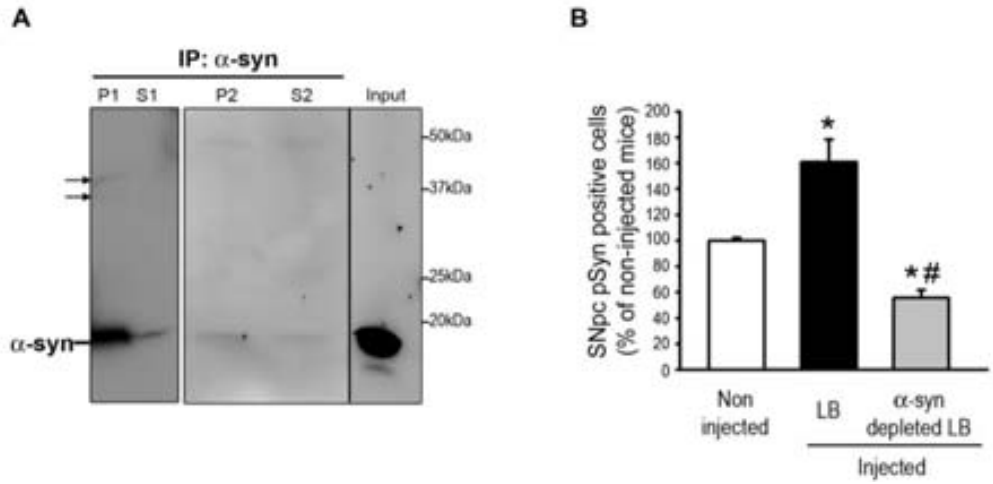


Figure 35. (A) Immunoblots for α-synuclein in α-synuclein-immunodepleted LB fractions detected by sequential immunoprecipitation. P1 and P2 correspond to eluted pellet fractions after the first and second depletion steps. S1and S2 correspond to immunodepleted supernatant fractions after the first and second depletion steps. Inputs: intact LB extract. Arrows indicate oligomeric α-synuclein species. (B) Number of phosphorylated (pSyn)-immunopositive cells in the SNpc of mice injected with either LB fractions or α-synuclein-immunodepleted LB fractions, relative to non-injected animals. N = 4 mice per group. * $p < 0.05$ compared with non-injected mice; # $p<0.05$ compared with LB-injected mice. Histogram represents average ± S.E.M.

In addition, host expression of soluble α-synuclein was a prerequisite for the pathogenic effects of LB fractions, as inoculation of these fractions into α-synuclein-deficient mice did not produce any α-synuclein pathology or evidence of nigrostriatal lesion in these animals (Fig. 36)

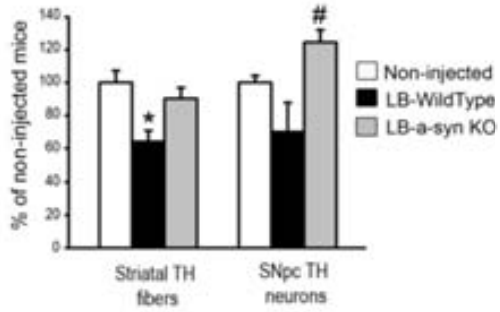


Figure 36. Quantification of striatal TH terminals by optical densitometry, and number of SNpc TH-positive neurons by stereology in LB-injected wild-type or α-synuclein deficient (KO) mice relative to non-injected wild-type animals. N = 3-4 for α-synuclein-deficient mice and 8-10 for wild-type mice. *p<0.05 compared with non-injected wild-type mice; #p<0.05 compared with LB-injected wild-type mice. Histogram represents average ± S.E.M.

Overall, our results indicate that LB-linked α-synuclein derived from PD patients is able to trigger the pathological conversion of endogenous murine α-synuclein, which correlates with PD-like, α-synuclein-dependent nigrostriatal degeneration.

1.5. Intracerebral Inoculation of Human LB Extracts Induce Nigro-Striatal Dopaminergic Neurodegeneration and α-Synuclein Pathology in Monkeys

To assess whether the pathogenic effects of PD-derived LB extracts observed in mice could also be exerted in an experimental setting more relevant to humans, the same LB fractions used in the rodent experiments described above were inoculated in a total of four rhesus monkeys (*Macaca fascicularis)*. Two monkeys received LB inoculations into the striatum, a region interconnected with multiple CNS nuclei including the SNpc, whereas two additional animals were inoculated directly into the SNpc (Fig. 37). The total injected volume was 100 μl for the striatum (derived between two different sites) or 10 μl for the SNpc. To help determine the potential contribution of the dopaminergic nigrostriatal projection in the eventual spread of LB-induced α-synuclein pathology, one monkey of each group was treated with the neurotoxin MPTP to produce a severe lesion of this pathway prior to LB inoculations. Brains from four additional untreated control animals

and three additional MPTP-treated monkeys (not receiving LB inoculations) were used as control when appropriate.

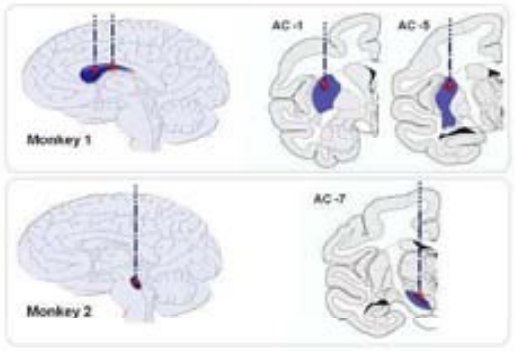


Figure 37. Schematic diagram indicating the site of LB inoculations in monkey

LB-injected monkeys were examined at different times post-injection by PET with the radioligand ^{11}C(+)-DTBZ, a marker of striatal vesicular monoamine transporter density that reflects nigrostriatal dopaminergic innervations. In particular, PET scan was performed 3, 9 and 12 months after LB inoculation. A basal PET scan was performed before LB inoculation (Fig. 38).

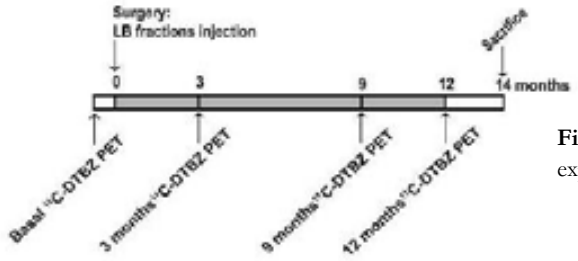


Figure 38. Schematic diagram indicating the overall experimental design in monkeys.

Using this method, the monkey receiving striatal LB injections exhibited a ~34% reduction in striatal dopaminergic innervations starting at 9 months after LB injection that remained stable up to 12 months (Fig. 39).

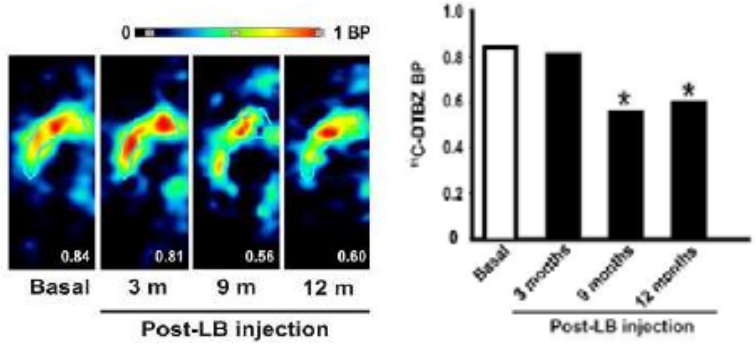


Figure 39. Representative images (coronal planes) and quantification of PET scans performed in LB-injected monkey 1 using ^{11}C-(+) DTBZ. *p<0.05 compared with basal levels.

This was confirmed by neuropathological examination, performed 14 months post-injection, by quantitative analysis of striatal TH immunoreactivity (Fig. 40). A similar degree of striatal dopaminergic terminal loss was also observed in the monkey receiving LB inoculations directly into the SNpc. No behavioral changes were detected in any of these animals over the 14-months post-injection period, which is consistent with a degree of nigrostriatal lesion below the threshold for appearance of parkinsonian signs[267]. Concomitant to striatal dopaminergic terminal loss, monkeys injected in either the striatum or SNpc exhibited dopaminergic cell loss in the SNpc (~40% and ~15%, respectively), as assessed by stereological counting of SNpc TH-positive neurons. The relatively sparse loss of nigral dopaminergic neuron cell bodies in SNpc-injected monkeys compared to their striatally injected counterparts suggests that the LB-induced neurodegenerative process may be progressing more slowly in these animals.

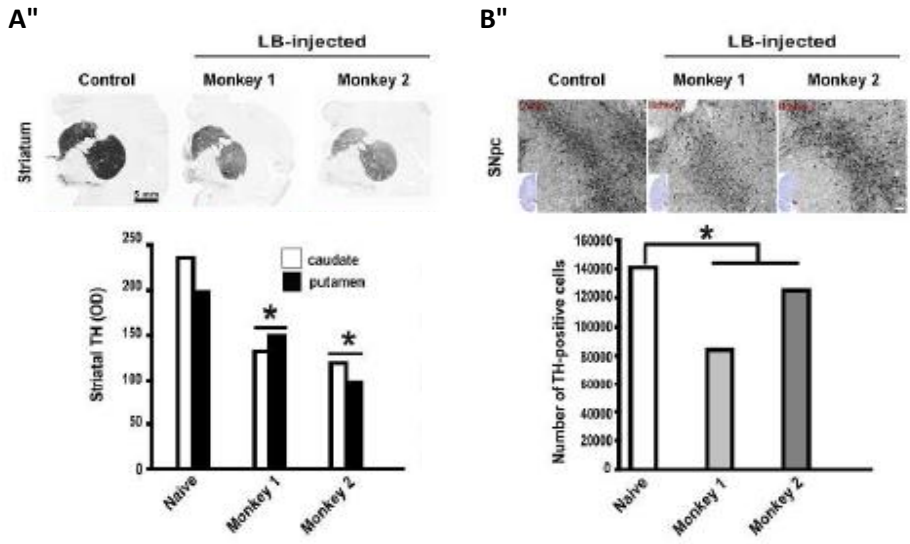


Figure 40. Illustrative images (top) and quantifications (bottom) of TH-positive striatal terminals by optical densitometry (A) or of TH-positive SNpc neurons (B) by stereology, in non-injected and LB-injected monkeys. *p<0.05 compared with non-injected animals. Scale bars: 5mm (Striatum) and 250 μm (SNpc).

LB-injected monkeys were subsequently analyzed for potential α-synuclein pathology using and antibody (Syn211) that recognizes amino acid residues 121-125 in both soluble and pathological (LB-linked) α-synuclein[274]. In the monkey receiving striatal injections of LB, α-synuclein immunohistochemical signal measured by regional

densitometry was increased in several brain regions along the rostrocaudal axis that project to, or receive input from, the inoculation site, and which show α-synuclein accumulation in PD[211], including the putamen, SNpc, globus pallidus, precentral gyrus, superior frontal gyrus and entorhinal area in the temporal cortex (Fig. 41 A-B). In these regions, increased α-synuclein immunolabeling mostly corresponded to presynaptic terminals, although some

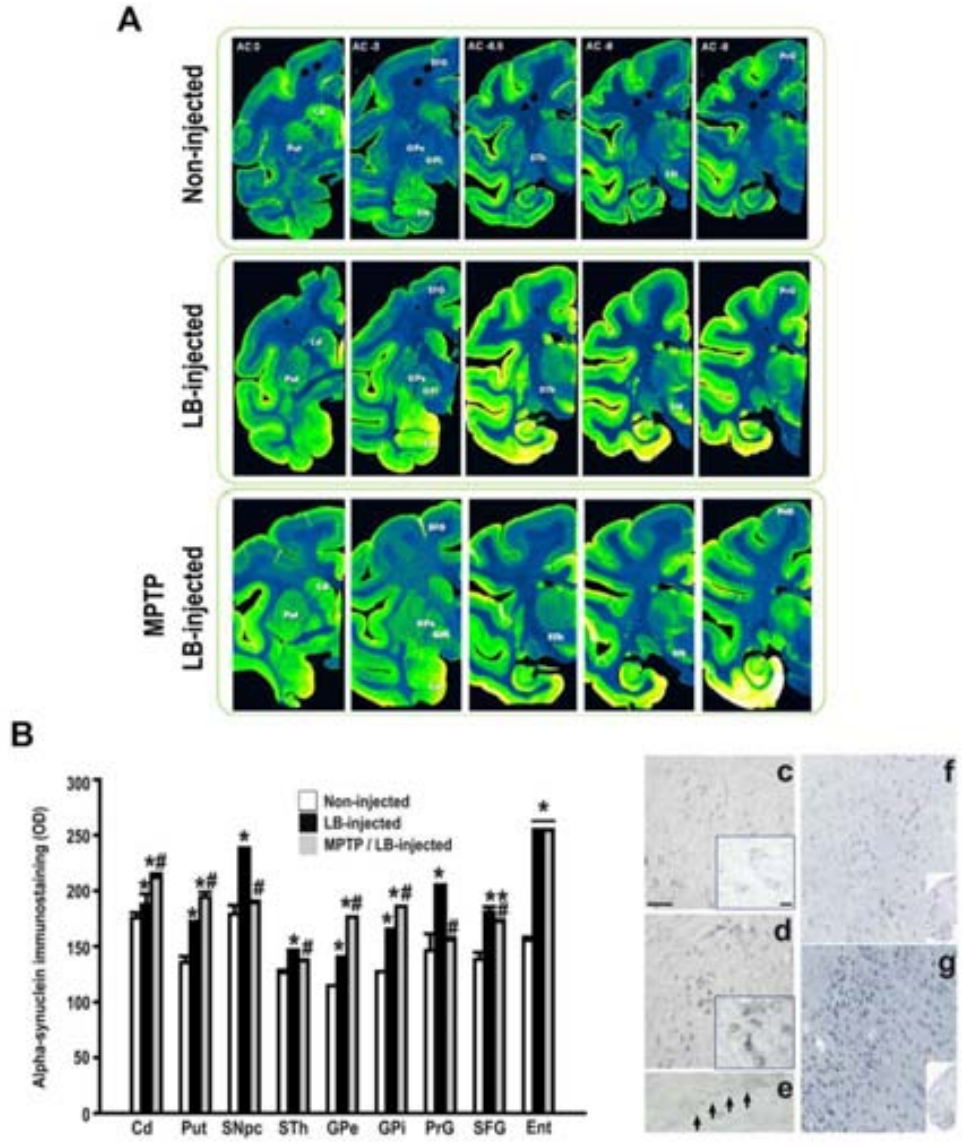


Figure 41. (A) Representative coronal brain sections of endogenous α-synuclein immunostaining (green) in non-injected and striatal LB-injected monkeys, with or without MPTP treatment. (B) Quantification of regional levels of endogenous α-synuclein by optical densitometry in the caudate (Cd), putamen (Put), SNpc, subthalamic nucleus (STh), external Globus pallidus (GPe), internal globus pallidus (GPi), precentral gyrus (PrG), superior frontal gyrus (SFG) and entorhinal cortex (Ent) in non-injected and striatal LB-injected monkeys, with or without MPTP treatment. Histograms represent average ± S.E.M. of the α-synuclein level per region. *p<0.05 compared with non-injected monkeys, # p<0.05 compared with striatal LB-injected monkey without MPTP. (C-G) Illustrative photomicrographs of endogenous α-synuclein (c-e) and pSyn (f-g) immunostaining (blue) in the SNpc (c-e) and Ent (f-g) of non-injected (c,f) and striatal LB-injected (d,e,g) monkeys. Arrows in (e) indicate an α-synuclein-positive neurite. Scale bars: 80μm (c) and 20 μm (c, inset).

of these regions, such as the SNpc and entorhinal cortex, also exhibited enlarged α-synuclein-positive neurites and intracytoplasmic diffuse accumulations of α-synuclein that were associated with concomitant increases in phosphorylated α-synuclein species, without the formation of definite LB-like inclusions (Fig. 41C-G). Supporting the retrograde transmission of the α-synuclein pathology from the LB-injected striatum to the SNpc, striatal LB inoculation in the MPTP-treated monkey did not lead to any nigral α-synuclein pathology, resulting instead in further increases in α-synuclein within the injection site (striatum) and its efferently connected areas, such as the globus pallidus (Fig. 41 A-B).

The increase of α-synuclein in these areas in the MPTP-treated monkey after LB inoculation were not due to MPTP-inoculation, as no increments of α-synuclein in these areas were observed in MPTP-treated monkey without LB injection (Fig. 42). These results

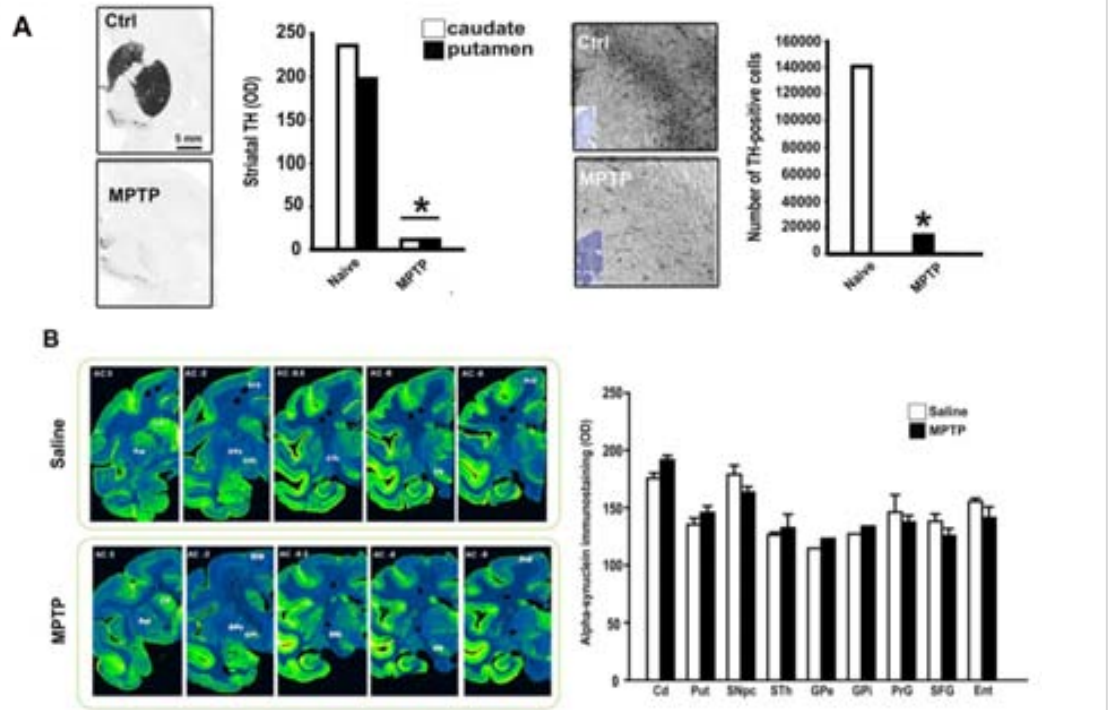


Figure 42. (A) Illustrative images (top panels) and quantifications (bottom panels) of TH-positive striatal terminals by optical densitometry (A) and SNpc TH-positive neurons measured by stereology (B) in MPTP-treated monkeys. (B,Left) Representative coronal brain sections of endogenous α-synuclein immunostaining (green) in saline- and MPTP-treated monkey in absence of LB inoculations at 12 months post-intoxication. (B,Right) Quantification of regional levels of endogenous α-synuclein by optical densitometry (OD) in caudate (Cd), putamen (Put), SNpc, subthalamic nucleus (STh), external globus pallidus (GPe), internal globus pallidus (GPi), precentral gyrus (PrG), superior frontal gyrus (SFG) and entorhinal cortex (Ent) in saline- and MPTP-treated monkey. *$p<0.05$ compared with saline-treated animals. Histograms represent average ± S.E.M. of levels of α-synuclein in each anatomical region

indicated that LB-induced α-synuclein pathology can extend to brain regions distant to the injection site.

In contrast to the widespread increases in α-synuclein signal observed in striatally-injected monkey, inoculation of LB fractions into the primate SNpc mostly resulted in local intracellular diffuse accumulations of α-synuclein within the SNpc, with the spread of α-synuclein pathology mostly limited to the entorhinal area of the temporal cortex (Fig. 43). The more conspicuous spreading effect observed in striatally-injected monkeys, compared to their SNpc-injected counterparts, may correspond to the higher anatomical connectivity of the striatum and/or to a slower progression of the neurodegenerative process in SNpc-injected animals.

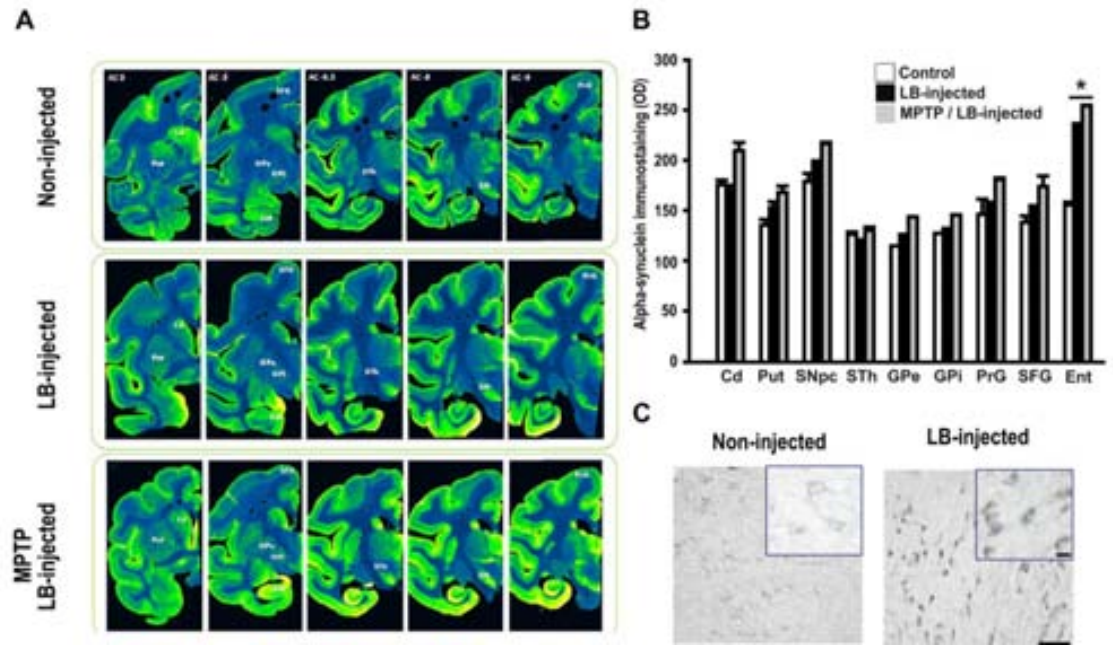


Figure 43. (A) Representative coronal brain section of endogenous α-synuclein immunostaining (green) in non-injected and nigral LB-injected monkeys, with or without MPTP treatment. (B) Quantification of regional levels of endogenous α-synuclein by optical densitometry in the caudate (Cd), putamen (Put), SNpc, subthalamic nucleus (STh), external globus pallidus (GPe), internal globus pallidus (GPi), precentral gyrus (PrG), superior frontal gyrus (SFG) and entorhinal cortex (Ent) in non-injected and nigral LB-injected monkeys, with or without MPTP treatment. Histograms represent average ± S.E.M. of the α-synuclein level per region. *$p<0.05$ compared with non-injected monkeys. (C) Illustrative photomicrographs of endogenous α-synuclein immunostaining (blue) in the SNpc of non-injected (top) and nigral LB-injected (bottom) monkeys. Scale bars: 80μm (C) and 20μm (C, inset).

Although more extensive studies will be needed using a larger number of animals, our results in monkeys represent a proof-of-concept of the data generated in mice by showing that LB extracts derived from PD patients are also able to induce pathogenic effects in nonhuman primates, including nigrostriatal dopaminergic neurodegeneration and spreading of α-synuclein pathology to distant brain regions, especially when inoculated into highly interconnected areas such as the striatum.

2. SELECTIVE SILENCING OF α-SYNUCLEIN IN SNpc DOPAMINERGIC NEURONS IN VIVO BY THE INTRANASAL DELIVERY OF TARGETED SMALL INTERFERING RNA

2.1 Screening of Various siRNAs Directed to Downregulate Endogenous or Overexpressed α-Synuclein mRNA Levels in M17 Cells

First, we assessed the ability of three different siRNA sequences directed against α-synuclein (SNCA499-siRNA, MAYO2-siRNA, SNCA2-siRNA) to downregulate endogenous α-synuclein in M17 human neuroblastoma cells by quantitative real-time PCR (RT-qPCR). Cells were transfected with either (i) lipofectamine alone as a control, (ii) lipofectamine + non-sense siRNA (NS-siRNA), and (iii) lipofectamine + α-synuclein directed siRNAs: SNCA499-siRNA; MAYO2-siRNA and SNCA2-siRNA. The analysis of α-synuclein expression by RT-qPCR revealed that all the siRNA sequences suppressed α-synuclein mRNA expression in M17 neuroblastoma cells, 24h after transfection (Fig. 44). In particular, SNCA499-siRNA induced a 78% downregulation, SNCA2-siRNA 76% and MAYO2-siRNA 86%. In contrast, NS-siRNA did not affect the expression of α-synuclein expression. Similarly, lipofectamine treatment alone did not induce changes in α-synuclein expression

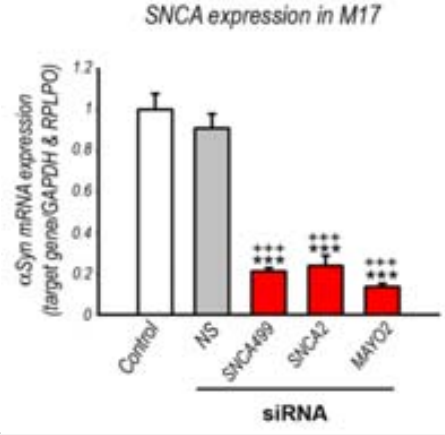


Figure 44. Quantification of α-synuclein mRNA expression by RT-qPCR in M17 cells 24h after transfection with various siRNA directed against α-synuclein: SNCA499-siRNA, SNCA2-siRNA and MAYO2-siRNA. Controls cells were transfected with lipofectameine alone (control) or with NS-siRNA. Control cells, N=39; NS-siRNA, N=34; 499-siRNA, N=13; MAYO2-siRNA, N=10; SNCA2-siRNA, N=11. ***p<0.001 compared with control cells; +++ p<0.001 compared with NS-siRNA. Histogram represents average ± S.E.M.

Because we wanted to downregulate α-synuclein expression after intranasal administration without decreasing β- and γ-synuclein expression, the specificity of our three siRNAs candidates was studied in vitro by RT-qPCR (Fig. 45). The results obtained revealed that MAYO2-siRNA significantly suppressed γ-synuclein expression (58% downregulation). Although no statistically different, MAYO2-siRNA also suppressed the expression of β-synuclein (67% downregulation), 24h post-transfection. Transfection with SNCA499-siRNA and SNCA2-siRNA did not downregulated the expression of either β- or γ-synuclein Indeed, SNCA499-siRNA increased the expression of β-synuclein, probably as a compensatory effect.

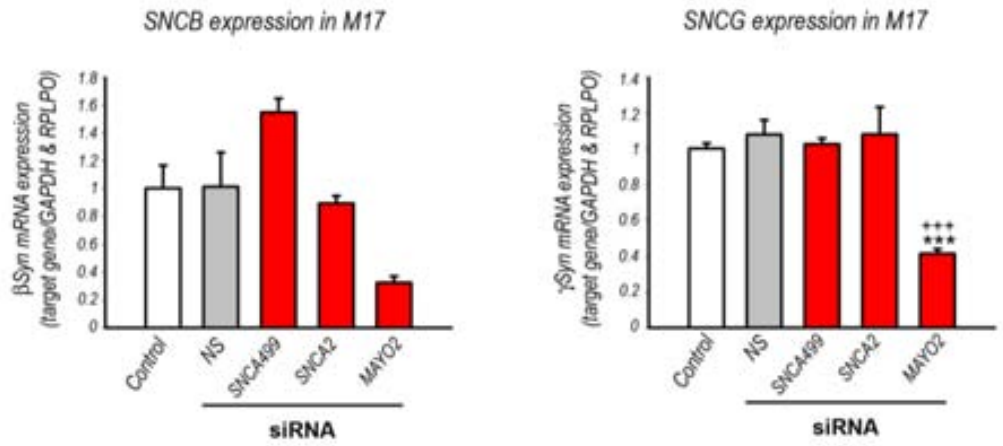


Figure 45. Quantification of β- (left) and γ-synuclein (right) mRNA expression in M17 cells by RT-qPCR, 24h after the transfection with: (i) lipofectamine alone (control), (ii) siRNA-NS, (iii) α-synuclein directed siRNA: SNCA499; SNCA2 and MAYO2. Control cells, N=14-28; NS-siRNA transfected cells, N=16-25; SNCA499-siRNA transfected cells N=4-6; MAYO2-siRNA transfected cells, N=5-11; SNCA2-siRNA transfected cells, N=5-11. ***$p<0.001$ compared with control cells; +++$p<0.001$ compared with NS-siRNA. Histogram represents average ± S.E.M.

Since PD is associated with increased levels of α-synuclein, we wanted to validate that our siRNA sequences would be able to downregulate increased α-synuclein expression into basal levels in a model with increased levels of α-synuclein, which would be closer to a pathological PD situation. For these reason, we studied the effect of the different siRNAs in the neuroblastoma M17 overexpressing wild-type α-synuclein (M17 α-Syn). M17 α-Syn

cells highly overexpress α-synuclein compared to wild-type M17 at both mRNA and protein levels (Fig. 46).

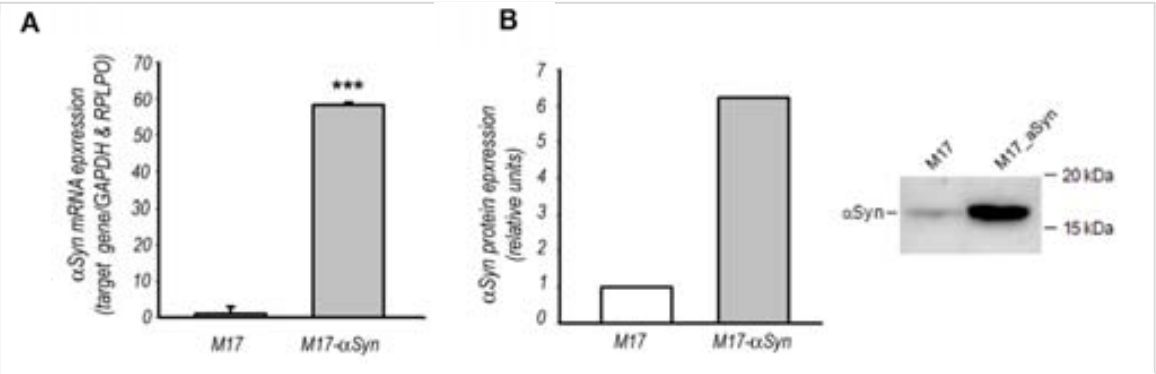


Figure 46. (A) α-Synuclein mRNA expression of M17 overexpressing α-synuclein(M17-α-syn) compare to wild-type M17 (M17) assessed by RT-qPCR; M17 cells, N=5 ; M17-α-syn cells, N=4; ***p<0.001 compared with M17 cells; (B) Quantification (left) and immunoblot (right) of α-synuclein protein levels expression in wild-type M17 cells and M17-α-syn. Histogram represents average ± S.E.M.

RT-qPCR analysis revealed that all the siRNA sequences were able to downregulate the increased expression of α-synuclein in M17 α-Syn cells, 24h after transfection: SNCA499-siRNA induced a 74% of downregulation, MAYO2-siRNA 74% and SNCA2-siRNA 69% (Fig. 47). As in wild-type cells, MAYO2-siRNA decreased the expression of β- and γ-synuclein in M17 α-Syn cells. Although no statistically significant, SNCA2-siRNA also decreased the expression of γ-synuclein (25% downregulation). In contrast, SNCA499-

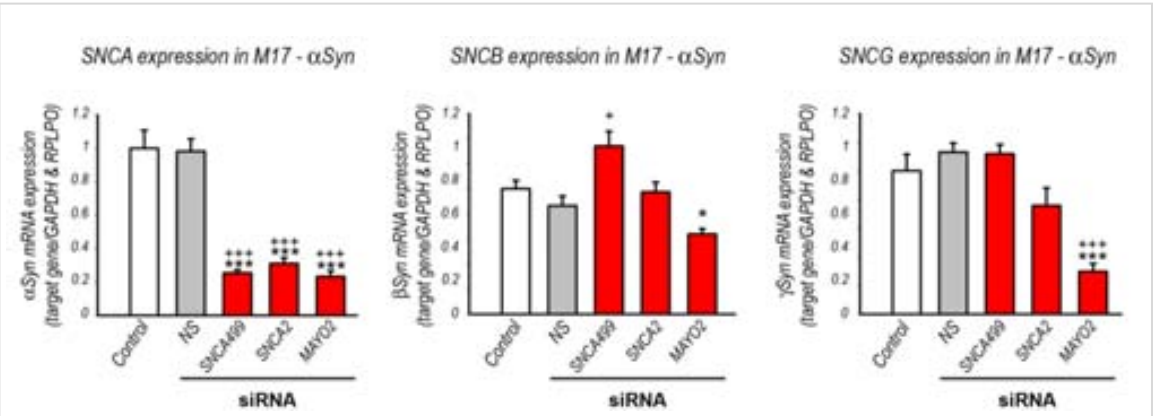


Figure 47. Quantification of α- (left), β- (middle) and γ-synuclein (right) mRNA expression by RT-qPCR in M17 cell line overexpressing α-synuclein (M17 α-Syn), 24h after the transfection with: (i) lipofectamine alone (control), (ii) NS-siRNA, (II) α-synuclein directed siRNA: SNCA499; SNCA2 and MAYO2. Control, N=19-34; NS-siRNA transfected cells, N=13-24; SNCA499-siRNA, N=6-19; SNCA2-siRNA, N=5-12; MAYO2-siRNA, N=6-8. ***p<0.001 compared with control; +++p<0.001 compared with NS-siRNA. Histogram represents average ± S.E.M.

siRNA did not decrease the expression of either β- or γ-synuclein. According to these results, SNCA499-siRNA was the only sequence able to specifically downregulate α-synuclein expression without decreasing β- and γ-synuclein expression. Therefore, SNCA499-siRNA was selected for further analyses in vivo.

2.2 Selective α-Synuclein Silencing by Local Infusion of SNCA499-siRNA in Mice

The candidate SNCA499-siRNA was administrated locally in SN/VTA to confirm that it could downregulate the expression of basal α-synuclein levels in vivo. Adult mice received ipsilaterally: (i) vehicle, (ii) NS-siRNA or (iii) SNCA499-siRNA at 10 or 20 μg/μl for 2 consecutive days into right SNc/VTA and were euthanized 24h after last administration (Fig. 48).

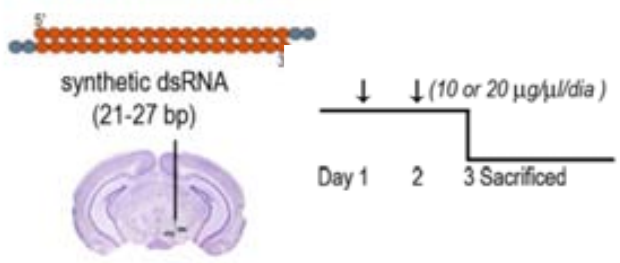


Figure 48. Schematic representation of siRNA local administration in the mice substantia nigra. Adult mice (C57BL/6NCrl) received ipsilaterally: i)vehicle, ii) NS-siRNA or iii) SNCA499-siRNA at 10 or 20 μg/μl for 2 days into right SNc/VTA and were euthanized 24h after last administration.

Local infusion of SNCA499-siRNA was able to downregulated α-synuclein mRNA expression(~25-30%), as assessed by in situ hybridization (Fig. 49). No significant differences were obtained between both doses of siRNA. In contrast, no α-synuclein suppression was obtained after the administration of either vehicle or NS-siRNA (Fig. 49).

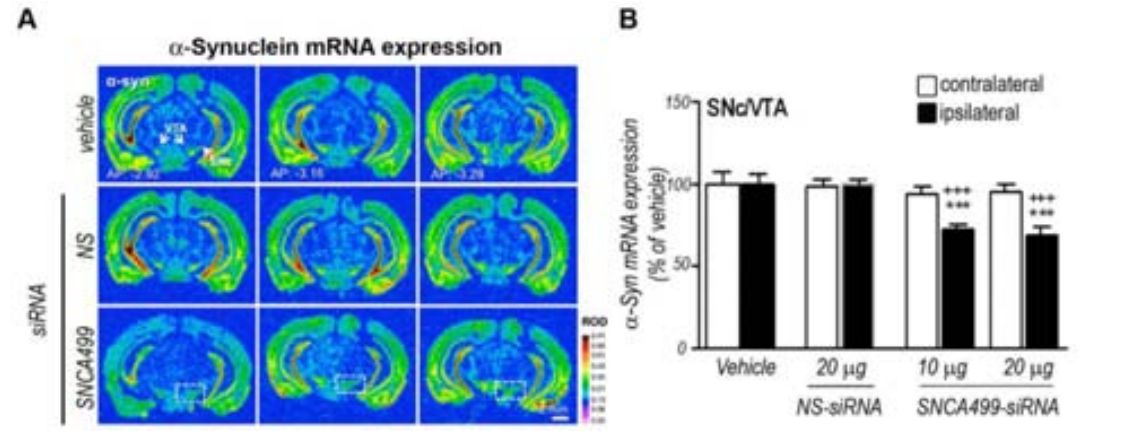


Figure 49. (A) Representative coronal brain sections showing the reduction of α-synuclein expression in SNc/VTA at different anteroposterior coordinates (AP -2.92 to -3.98 mm, taken from bregma) after local infusion of the SNCA499-siRNA in the SN assessed by in situ hybridization. The boxes indicate areas of SNc/VTA shown the decreased α-synuclein mRNA levels. Scale bar: 2mm. (B) Densitometric quantification of α-synuclein mRNA levels in SNc/VTA of mice (n=4 mice/group). ***p<0.001 versus ipsilateral SNc/VTA of vehicle and NS-siRNA-treated mice; +++p<0.001 versus contralateral SNc/VTA of SNCA499-siRNA-treated mice. Histograms represent average ± S.E.M. Results provided by Analia Bortolozzi (IDIBAPS – CSIC)

In addition, the local infusion of 10 μg or 20 μg of SNCA499-siRNA into SN/VTA did not induce changes in β- or γ-synuclein expression (Fig. 50). These results confirmed that SNCA499-siRNA suppressed specifically the expression of α-Synuclein expression in mice when injected directly into the SN.

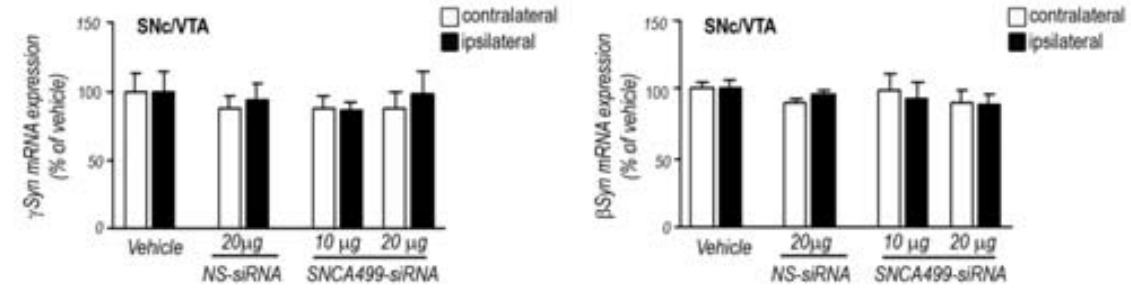


Figure 50. Densitometric quantification of γ- (left) and β-synuclein (right) mRNA levels in the SNc/VTA of mice (n=4 mice/group) after local infusion of SNCA499-siRNA in the SN. No group differences were detected for γ- and β-synuclein mRNA expression in the SNc/VTA (n=4 mice/group). Histograms represent average mean ± S.E.M. Results provided by Analia Bortolozzi (IDIBAPS – CSIC)

2.3 Indatraline Targeting Validation in Rat Ventral Midbrain Primary Culture

Since we wanted to downregulate α-synuclein expression specifically in affected PD brain regions and not in the whole brain, siRNA sequences were conjugated to the cell-specific ligand indatraline, which has a high affinity for DA, NE and 5-HT transporters (DAT, NET and SERT), in order to promote their selective delivery into PD vulnerable neuronal populations, including SNpc, LC and DR. This selective delivery was tested in vitro using rat postnatally-derived ventral midbrain primary culture. This primary culture consists in a monolayer of glial cells above which ventral midbrain neurons are grown. Although several neuronal populations are found in this primary culture, the protocol used allowed us to obtain 20-70% of dopaminergic neurons. The primary culture was incubated with 100nM of siRNA conjugated with both indatraline and the fluorescent dye Cy3 (IND-siRNA-Cy3). Control cultures were treated with siRNA-Cy3 without the Indatraline target. Three hours after incubation, cultures were examined using an inverted fluorescent microscope (Fig. 51). Cultures treated with IND-siRNA-Cy3 exhibited Cy3-positive neuronal cells, suggesting that IND-siRNA-Cy3 was taken up by these neurons. In contrast, glial cells were not positive for Cy3. In addition, siRNA-Cy3 (without targeting) was not internalized by neurons, as no Cy3-positive neurons were detected after siRNA-

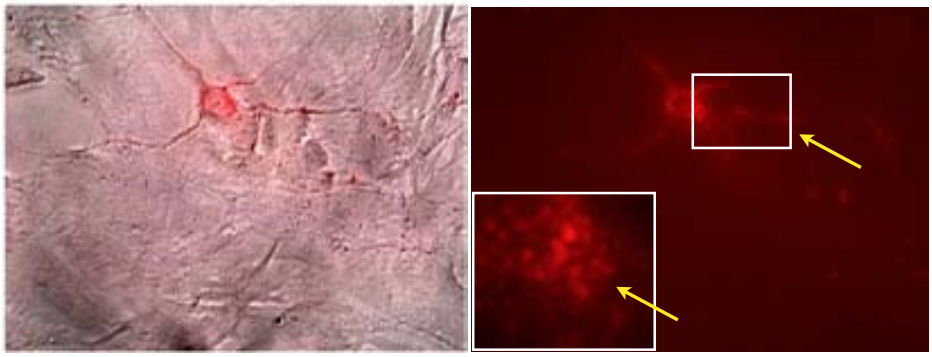


Figure 51. Rat postnatally-derived ventral midbrain primary culture was incubated with siRNA conjugated to both indatraline and Cy3 (IND-siRNA-Cy3). The images illustrated the uptake of the IND-siRNA-Cy3 (red) in neurons 3 hours after incubation

Cy3 treatment. These results in vitro demonstrated that the ligand indatraline promotes the specific siRNA penetrance in neurons but not in glial cells.

2.4 Intranasal Administration of IND-499-siRNA Suppresses α-Synuclein Expression Selectively in SN Dopaminergic Neurons

Once demonstrated that the SNCA499-siRNA sequence downregulated α-synuclein expression both in vitro and in vivo and that Indatraline promoted the selective penetrance of the siRNA into neurons, IND-SNCA499-siRNA was intranasally administrated in mice. A 5µl drop of PBS or conjugated siRNA (IND-SNCA499-siRNA) was applied alternatively to each nostril once daily. A total of 10 µl containing 30 µg (2.1 nmol/day) of conjugated siRNA was delivered for 4 consecutive days and mice were euthanized at 1, 3 or 7 days after last administration (Fig. 52).

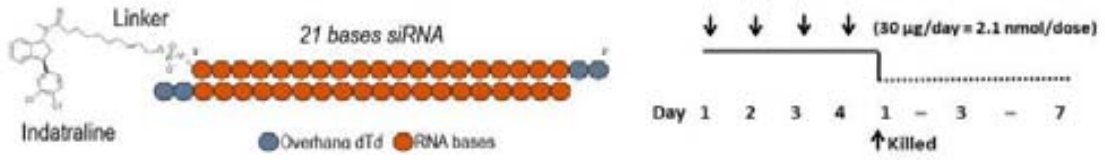


Figure 52. (Left) Schematic representation of the siRNA linked to indatraline administrated intranasally. (Right) Schematic representation of the intranasal administration treatment. Adult mice (C57BL/6NCrl) received i.n: i) vehicle, ii)IND-NS-siRNA or iii) IND-SNCA499-siRNA at 30 µg/day for 4 days and were killed a 1, 3 or 7 days after last administration.

The downregulation of α-synuclein expression within SN after intranasal administration was confirmed at mRNA level by *in situ* hybridization (Fig. 53). This technique revealed that IND-SNCA499-siRNA administrated intranasally induced the suppression of α-synuclein mRNA expression (~20%) within the SN 1 day after last administration. After this time point, α-synuclein mRNA expression levels were

subsequently recovered to basal levels. Intranasal administration of IND-NS-siRNA or PBS did not induce changes in the mRNA expression of α-synuclein (Fig. 53)

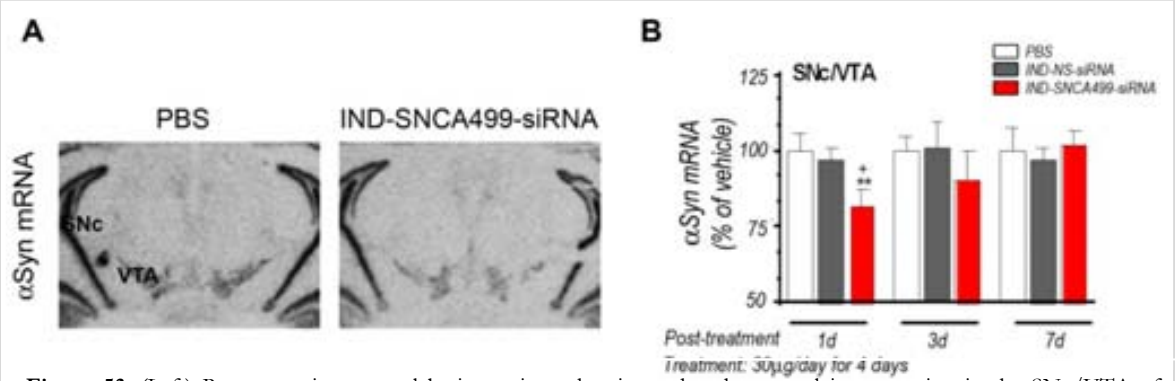


Figure 53. (Left) Representative coronal brain sections showing reduced α-synuclein expression in the SNc/VTA of mice treated with intranasal administration of PBS or IND-SNCA499-siRNA (four doses) assessed by in situ hybridization. Scale bar: 500μm. (Right) Bar graphs showing densitometric quantification of α-synuclein mRNA levels in SNc/VTA of mice treated with either (i) PBS, (ii) IND-NS-siRNA and (iii) IND-SNCA499-siRNA (n=4-5 mice/group). *p<0.01 versus PBS-treated mice, +p<0.05 versus IND-NS-siRNA-treated mice. Histograms represent average mean ± S.E.M. Results provided by Analia Bortolozzi (IDIBAPS – CSIC).

Consistent with the mRNA results, siRNA administrated intranasally was also able to downregulate α-synuclein protein expression within the SN, as assessed by immunohistochemistry. Twelve sections representative of all the SN for each animal were stained with an antibody against α-synuclein (Table 6, page 82) and the number of α-synuclein-positive cells within SN and the intracellular α-synuclein levels (by densitometry) were analyzed at 1, 3 and 7 days after last siRNA administration. One day post-administration, animals treated with siRNA exhibited a significant decrease in the number of SNpc α-synuclein positive cells (22% decrease) (Fig. 54A-B). Three days post-administration, animals still exhibited a reduction in the number of α-synuclein-positive cells, although it was no longer statistically significant. Similarly to the mRNA results, the number of α-synuclein positive cells was recovered to basal levels 7 days after last administration. Mice treated with IND-SNCA499-siRNA also exhibited a decrease in the intracellular α-synuclein levels within SN neurons at day 3 after last intranasal

administration (23% of decrease) (Fig. 54C). Seven days after last administration, intracellular α-synuclein OD was recovered to basal levels.

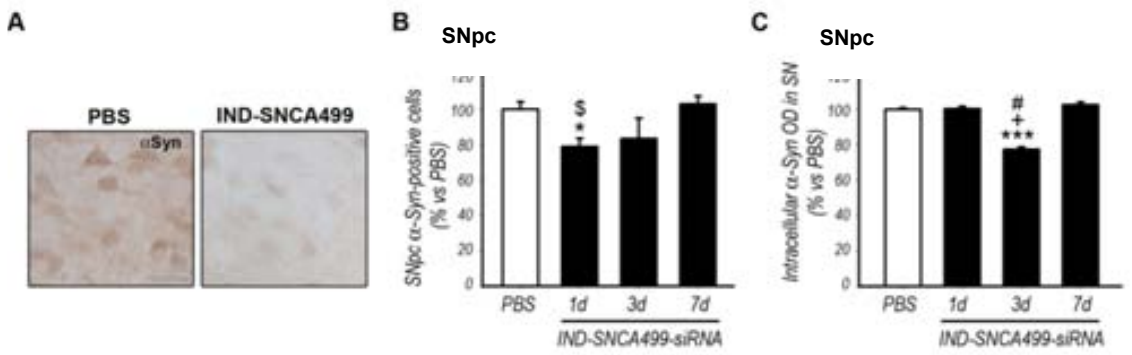


Figure 54. (A) Representative immunostaining images of α-synuclein (brown) positive cells in the SNpc of PBS and IND-499-siRNA mice, 1 day after last siRNA-administration. Scale bar = 30μm. (B) Bar graphs showing the number of α-synuclein immunopositive cells in the SNpc of mice injected with either PBS or IND-499-siRNA 1 day, 3 days or 7 days after last administration. PBS, N=15; IND-SNCA499-siRNA 1day, N=5; IND-SNCA499-siRNA 3 days, N=3; IND-SNCA499-siRNA 7days, N=5. *p<0.05 compared with PBS; $ p<0.05 compared with IND-SNCA499-siRNA 7 d. (C) Quantification of intracellular levels of α-synuclein within SNpc neurons by optical densitometry in mice administrated with either PBS or IND-SNCA499-siRNA 1 day, 3 days or 7 days after last administration. An average of ~1200 neurons per group was analyzed. ***p<0.01 compared with PBS; # p<0.001 compared with IND-499-siRNA 3days; +p<0.001 compared with IND-SNCA499-siRNA 7days. Histograms represent average ± S.E.M.

The downregulation of α-synuclein protein levels after intranasal IND-SNCA499-siRNA was confirmed by Western Blot (Fig. 55). In particular, IND-SNCA499-siRNA produced a significant decrease in α-synuclein protein levels within SN at day 1 after last administration (29% decrease), and α-synuclein levels were recovered 3 days after last administration (Fig. 55). In contrast, IND-NS-siRNA or PBS did not reduce the expression of α-synuclein at any time (Fig. 55).

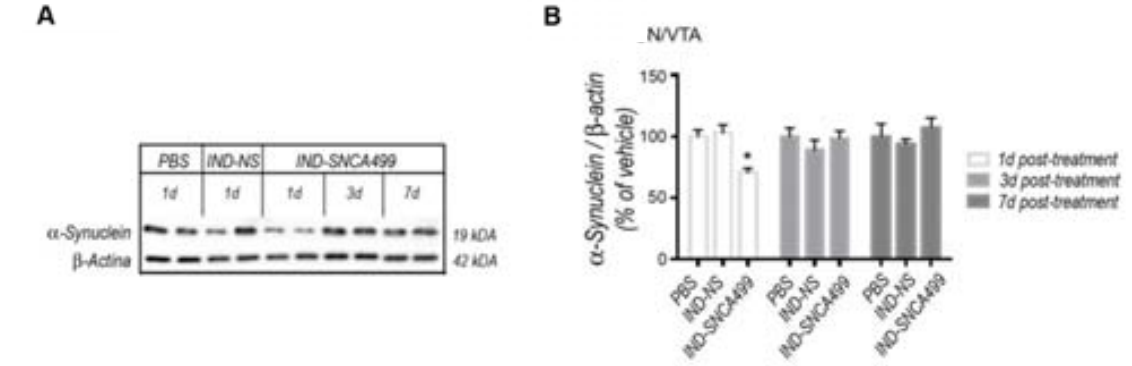


Figure 55. (Left) Representative immunoblot images of α-synuclein and β-actin protein levels in the SN of mice treated with intranasal administration of: (i) PBS, (ii) IND-NS-siRNA and (iii) IND-SNCA499-siRNA mice, 1day (d), 3d and 7d after last administration. (Right) Densitometric quantification of the immunoblot. N = 6-8 in each group. *p<0.05 compared with PBS;.Histograms represent average ± S.E.M. Results provided by Raquel Revilla (nLife

2.5. Intranasal Administration of IND-SNCA499-siRNA Did Not Induce Nigrostriatal Dopaminergic Degeneration in Mice

Because other studies have shown that knockdown of α-synuclein basal levels might be associated with dopaminergic cell loss[284-286], we assessed whether downregulation of α-synuclein by intranasal administration of IND-SNCA499-siRNA might affect the integrity of the dopaminergic nigrostriatal pathway. No loss of striatal TH-positive fibers was observed after the administration of intranasal siRNA administration up to 7 days, as assessed by immunohistochemistry (Fig. 56A). Consistent with the striatal results, no loss of cell bodies in the SNpc was observed up 7 days post-administration (Fig. 56B). These results indicate that downregulation of α-synuclein levels by IND-SNCA499-siRNA intranasal administration does not affect the integrity of the nigrostriatal system in wildtype mice.

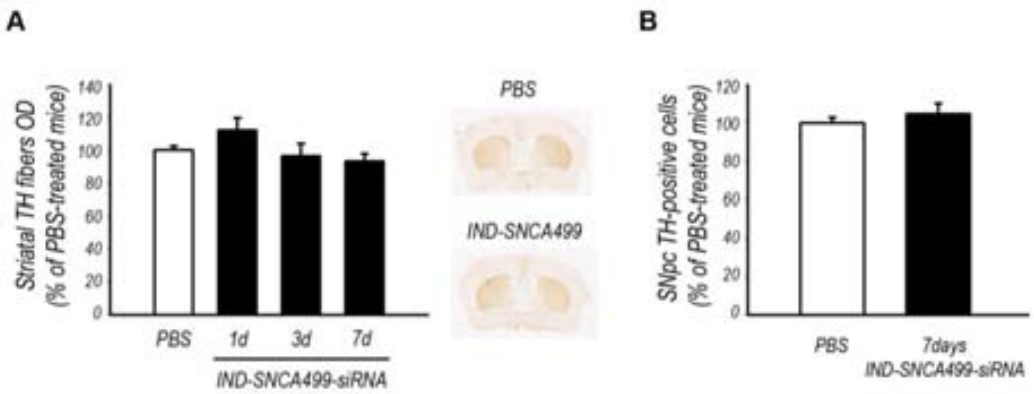


Figure 56. Integrity of the dopaminergic nigrostriatal system after IND-SNCA499-siRNA intranasal administration. (A-B) Quantification of TH-positive striatal terminals by optical densitometry (A, left) and number of TH-positive SNpc neurons by stereology (B) in PBS and IND-SNCA499-siRNA treated mice mice, relative to PBS-treated mice. No group differences were detected between groups. N=5 mice/group. Histograms represent average mean ± S.E.M. (A, right) Representative photomicrographs of TH-positive striatal terminals in PBS and IND-SNCA499-siRNA-treated mice at different 7days after administration.

Overall, our results set the stage for the use of the intranasal IND-SNCA499-siRNA delivery as a potential therapeutic strategy in *in vivo* models of PD associated with increased levels of α-synuclein, such as MPTP or α-synuclein overexpressing models (i.e. transgenic and/or AAV-models).

DISCUSSION

1- Lewy body extracts from Parkinson's disease brains trigger α–synuclein pathology and neurodegeneration in mice and monkeys.

Our study unravels a pathogenic effect of disease-associated, LB-linked human α-synuclein derived from sporadic PD patients in both rodents and non-human primates. In particular, we show here that LB-derived α-synuclein can trigger the pathological conversion of endogenous α-synuclein – including phosphorylation, proteinase K resistance and diffuse cytosolic accumulation. LB-induced α-synuclein pathology is dependent on both the exogenous α-synuclein contained in LB and on host (endogenous) α-synuclein expression, and is associated with progressive PD-like neurodegeneration. In addition, the LB-induced α-synuclein pathology can spread to brain regions distant to the injection site, especially in striatally-inoculated animals.

These results confirm that the intracerebral injection of LB-linked human α-synuclein causes the pathogenic effect seen with recombinant synthetic α-synuclein fibrils as previously reported[247, 248]. However, in contrast to those previous studies, the formation of definite LB-type inclusions/aggregates was not observed in LB-injected animals, but rather what was seen was diffuse cytosolic and presynaptic accumulations of pathological (i.e. phosphorylated or aggregated/PK-resistant) α-synuclein. This could potentially be attributed to the much lower amount of LB-derived fibrillar α-synuclein injected into our animals compared with the amount of recombinant synthetic α-synuclein used in previous studies[247, 248]. Despite the much lower levels of pathological α-synuclein used in this study, the extent and temporal dynamics of α-synuclein pathology and neurodegeneration observed in LB-injected animals was remarkably similar to that obtained previously with recombinant synthetic α-synuclein fibrils[247, 248], thus suggesting that PD-derived

pathological α-synuclein may in fact be more pathogenic than recombinant synthetic α-synuclein fibrils as it may harbor certain "pathologic" physico-chemical features not fully mimicked by synthetic α-synuclein. Supporting this concept, brain lysates from symptomatic mice containing small amounts of aggregated α-synuclein propagated the pathology with the same potency as high doses of synthetic α-synuclein fibrils, suggesting that pathological α-synuclein generated *in vivo* has enhanced templating effiency[245]. Alternatively, additional components present in LB other than α-synuclein might contribute to the pathogenic effects of LB-inoculations.

Some contradictory results regarding α-synuclein-induced neurodegeneration were forthcoming from this study. Luk KC et al.,[247] first demonstrated that striatal injections of synthetic α-synuclein fibrils induced cell loss in the SN. In contrast, the injection of α-synuclein fibrils directly into the SN did not induce nigrostriatal degeneration[248]. Masuda et al justified these contradictory results on the basis of differences in the injection site. However, our results demonstrate that both striatal and nigral injections of LB-linked human α-synuclein induced nigrostriatal degeneration in mice and monkeys. It is worth noting, nevertheless, that the striatal injection of LB into monkeys induced a more important SN cell loss compared with the nigral LB injection, supporting the concept that differences in the injection site might have an effect upon induction of the neurodegenerative process. Another possibility could be related with the amount of LB injection, as monkeys injected in SN received 10 μl of LB-fraction, while monkeys injected in striatum receive a ten-fold higher dose of LB-fraction (i.e., 100 μl).

The time-course and pattern of LB-induced neurodegeneration induced by either PD-derived material or recombinant α-synuclein fibrils are compatible with pathological α-synuclein triggering a slowly progressive neurodegenerative process instead of acute

neuronal loss, probably by disrupting neuronal function. Suggestive of the latter, we found here that, whether injected into the striatum or the SNpc, LB-induced neurodegeneration was detected earlier and more extensively at the level of striatal dopaminergic axon terminals compared with SNpc cell bodies. This is consistent with the pattern of nigrostriatal degeneration that is believed to take place in PD patients as evidenced by human postmortem studies, functional neuroimaging, genetic causes of the disease, and neurotoxin animal models[15, 21]. In agreement with this, the LB-induced accumulation of pathological α-synuclein in different regions of the brain in mice and monkeys was mostly observed in presynaptic terminals, which might be potentially linked to synaptic pathology. Supporting the latter, it has been reported that the majority of α-synuclein aggregates in the cortex of patients with DLB are located in the presynaptic terminals, which correlates with severe synaptic dysfunction (i.e. loss of dendritic spines in the postsynaptic area) and cognitive impairment in these patients[280]. Along this line, the widespread accumulation of pathological α-synuclein in different brain regions of PD patients is not necessarily associated with cell loss[43], but may instead cause impaired neuronal function[287]. These observations support the concept that novel therapeutic strategies for PD should be aimed preferentially at the maintenance of synaptic structure and function rather than at the mere preservation of neuronal cell bodies.

Since this project commenced, two different groups have published the results of studies in which samples derived from patients with α-synucleinopathies were injected into mice. Firstly, Masuda et al.,[248] injected samples obtained from DLB patients into wild-type mice, while more recently the group of Nobel laureate Professor S. Prusiner injected samples from multiple system atrophy (MSA) patients into α-synuclein transgenic mice expressing human α-synuclein containing the familial PD-associated A53T mutation[288]. PD, DLB and MSA are all neurodegenerative diseases characterized by intracellular protein

deposits containing aggregated α-synuclein – the so-called "synucleinopathies". The injection of DLB and MSA homogenates triggered α-synuclein pathology in mice. While the DLB homogenate did not induce a glial response or neuronal loss, mice injected with MSA exhibited prominent astrocytic and microglial activation and developed progressive signs of neurologic disease as early as ~100 days post-inoculation. These contradictory results concerning human α-synuclein-induced neurodegeneration might be explained by differences in: (i) mouse strain (wild-type vs. transgenic), (ii) injection site (SN vs. parietal lobe) and (iii) sample sonication (non-sonicated vs. sonicated). Bearing in mind the results of the present study, a further possibility is that different α-synuclein strains might exist in each disease, thus explaining the differences observed after the injection of each synucleinopathic sample. In addition, the existence of various α-synuclein strains may explain differences between PD patients. Supporting this concept, distinct α-synuclein strains generated through repetitively seeded fibrillization *in vitro* (i.e. strain A and B) exhibited different seeding properties both *in vitro* (i.e. primary hippocampal neurons from mouse embryos overexpressing human mutant P301S tau) and *in vivo* (i.e. mice overexpressing human P301S mutant tau). In particular, strain B displayed an enhanced capacity for promoting tau aggregation compared to strain A. On the other hand, strain A induced a higher α-synuclein pathology (i.e. α-synuclein aggregation) compared to strain B both *in vitro* and *in vivo*. Moreover, strain A was more toxic than strain B to primary hippocampal neurons as evidenced by significantly increased lactate dehydrogenase (LDH) release and reduced metabolic activity. Importantly, two conformational variants of pathological α-synuclein have been described in PDD brains[289]. Taken together, these studies indicate that human disease-associated α-synuclein is pathogenic when injected intracerebrally into mice.

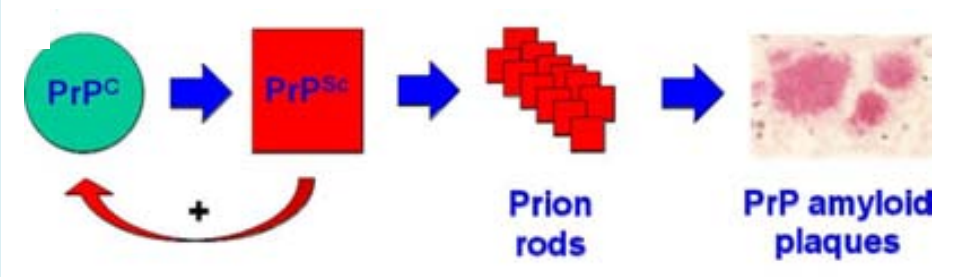
Interestingly, intracerebrally injectied non-LB fraction, containing soluble or finely granular α-synuclein but lacking LB-linked α-synuclein aggregates, obtained from the same PD patients did not induce nigrostriatal degeneration or α-synuclein pathology. This is in agreement with previous work[248] in which the intracerebral injection of soluble α-synuclein into the SN did not induced α-synuclein pathology, in contrast to findings obtained with synthetic recombinant fibrils. These results suggest that the structural conformation of α-synuclein is critical for its pathogenic effect, and support the idea that oligomeric or aggregated α-synuclein species rather than soluble α-synuclein are the toxic forms of this protein.

Although various studies have been published demonstrating the pathogenic role of α-synuclein in mice, we show here for first time that human pathological α-synuclein could also trigger a pathogenic process in non-human primates, including nigrostriatal dopaminergic neurodegeneration and spread of the α-synuclein pathology to distant brain regions. Thus, an important step forward has been taken in our understanding of the pathological role of α-synuclein in PD. However, it is important to note that the results in monkeys only represent a proof-of-concept of the data generated in mice, as a limited number of monkeys were used and therefore, extensive studies are needed using a larger number of animals. Our group, in collaboration with the group of Erwan Bezard in Bordeaux, have started a new project funded by the Michael J. Fox Foundation in which a total of 50 monkeys are being used. In particular, monkeys receive injections of LB into the: (i) striatum, (ii) cortex, in order to study if the spread of α-synuclein pathology is specific to the nigrostriatal track, and (iii) duodenum ventral wall, to study the effects of local LB-injection in the PNS and the possible progression of the LB-induced α-synuclein pathology from the PNS into the CNS. After injection, the behavior of the animals is

followed up every three months, with monkeys sacrificed at different time-points, from 6 to 24 months.

Based on the results presented here and in previous studies of a similar nature, several groups have proposed that α-synuclein might be a prion and that PD is a prion-like disease (Box 12). This prion-like hypothesis implies that native α-synuclein, under certain unknown conditions, undergoes a conformational change that promotes misfolding of the wild-type protein in a chain reaction, thus leading to the formation of toxic oligomers that polymerize to form amyloid plaques, eventually causing neuronal dysfunction and neurodegeneration. Furthermore, aggregated α-synuclein from affected neurons could be transferred to unaffected neurons, thus contributing to the spread of the neurodegenerative process, and ultimately leading to the clinical features of PD. Supporting this hypothesis, α-synuclein, as for the PrP^{C} prion protein, assumes an alpha-helical configuration when bound to membranes; this causes it to misfold and form beta-rich sheets, resulting in toxic

Box 12. Prion Disorders



Prions are infectious protein particles that lack nucleic acid and are comprised solely of aberrantly folded proteins. They cause disease by misfolding into a β-sheet-rich conformation with the formation of toxic oligomers/filaments and amyloid agrgregates. The misfolded protein acts as a template to promote conformation change in the wild-type protein, causing a chain reaction that ultimately leads to neurodegeneration. Further, the abnormal protein can be transmitted from affected to healthy unaffected nerve cells, in which the sequence is repeated, thereby extending the neurodegenerarive process. In mammals, the most well-studied prion protein is PrP^{Sc}. It is formed from the precursor protein PrP^{C} by a poorly understood process in which the α-helix-rich native protein is refolded into the PrP^{SC} protein with a high β-sheet conformation. The accumulation of PrP^{SC} triggers the additional misfolding of host PrP^{C} (a prion conformer reaction), which join to form toxic oligomers/filaments, polymerize to form amyloid plaques, and ultimately lead to neurodegeneration with neurological dysfunction. Figure from Olanow W. and Prusiner SB. (2009) *PNAS*

oligomers and aggregates that are associated with neurodegeneration. These aggregates polymerize to form amyloid plaques (i.e. LB) and appear to spread from affected to unaffected neurons. While, there are several similarities between prion disease and PD (Table 7), several important questions however remain to be solved. For instance: (i) it is currently unknown whether the pathological conversion of endogenous α-synuclein triggered by PD-derived material or recombinant α-synuclein fibrils actually occurs directly through a seeding process or indirectly as a general response to cellular stress[290]; (ii) the association between pathological α-synuclein accumulation and neuron cell death remains so far correlative, with further mechanistic studies needed to demonstrate a potential cause-effect relationship; (iii) besides any potential pathogenic effect of intraneuronal α-synuclein, extracellular pathological α-synuclein (or other LB components) may be able to activate a deleterious microglial response that could contribute to overall cell death and extension of the PD pathological process[291]. In addition, in contrast to prion diseases, there is no evidence to support the idea that PD can spread from one person to another. A retrospective, postmortem study of recipients of cadaver-derived human growth hormone (hGH) found no reported incidence of PD, although the donors of pituitary glands used for hGH preparation probably included people with PD, and immunohistochemical examination revealed frequent accumulation of pathological α-synuclein in the postmortem pituitary glands of people with PD[292]. In contrast, hGH administration has resulted in worldwide outbreaks of iatrogenic Creutzfeldt-Jakob disease (CJD)[293] caused, presumably, by PrP^{SC} contamination of the hGH preparation, despite the extremely low rate of CJD incidence in the population.

Overall, the results presented here indicate that the insoluble α-synuclein forms contained in PD-derived LB are pathogenic. In this context, LB formation in PD may represent a protective cellular mechanism to trap pathological α-synuclein species, thereby

Both prion disease and PD are age-dependent neurodegenerative disorders.
Both prion disease and PD illustrate selective vulnerability affecting specific subgroups of neurons
Both disease are 90% sporadic and 10% familial aprox.
Point mutations in the α-synuclein and PrP genes causes inherited forms of PD and prion diseases.
Familial prion diseases can be caused by expanded octorepeat inserts in the PrP genes, and familial PD can be caused by duplication and triplication of the wild-type α-synuclein gene.
Protein deposits are characterisic features of both disorders.
Proteins in both diseases undergo an α→β conformation transition (α-synuclein in PD and PrP in prion diseases)
Proteins in the disease state acquire a high β-sheet conformation and readily polymerize into fibrils, Lewy bodies in PD, and amyloid plaques in prion diseases.
Misfolded proteins can be taken up by neurons and transmitted to unaffected neurons.
Misfoled proteins are thought to migrate and spread across the neuraxis in both diseases: PrPsc prion migrate along peripherial nerves and up the spinal cord, whereas it is postulated that α-synuclein migrates from the gut and olfactory regions to affect specific regions of the CNS.

Table 7. Similarities between prion diseases and Parkinson's disease. Adapted from Olanow W. and Brundin P. (2013) *Mov. Disorders.*.

reducing the accessible toxic surface of these species and potentially removing them from more deleterious locations such as presynaptic terminals[280]. However, such pathogenic α-synuclein species could be eventually released or secreted from LB-containing neurons or extracellular LB material, which are observed in abundance in PD patients, and trigger a pathogenic process in unaffected neighboring cells, either directly or indirectly through activation of a deleterious microglial response, thereby contributing to the overall progression of the disease. The release of LB-derived α-synuclein pathogenic species into the cerebrospinal fluid and blood, as has been shown in PD patients[294 295], may represent additional routes for the potential spread of the α-synuclein pathology within affected individuals. These results may have important implications for the development of disease-modifying therapies for PD aimed at targeting expression levels, pathological conversion and/or cell-to-cell transmission of α-synuclein species. Such approaches could include immunization therapy (i.e. active and passive immunization). Supporting this idea, it has

been demonstrated that passive immunization with α-synuclein antibody promotes the clearance of extracellular α-synuclein via microglial cells, thus preventing the cell-to-cell transmission of aggregates in α-synuclein transgenic mice, resulting in reduced neurodegeneration and behavioral deficits associated with α-synuclein overexpression[282]. Furthermore, active immunization with full-length recombinant human α-synuclein has also been demonstrated to ameliorate the α-synuclein pathology (i.e. decreased accumulation of α-synuclein and reduced neurodegeneartion) in transgenic mice expressing human α-synuclein under the control of the platelet-derived growth factor-β (PDGFβ) gene promoter[296]. However, active immunization with full-length molecules could cause α-synuclein-specific T cell and autoimmune responses. More recently, active immunization with short peptides-AFFITOPEs® (i.e. short peptides that are too short to induce a T cell response) resulted in decreased accumulation of α-synuclein oligomers in axons and synapses, reduced degeneration of TH fibers in the striatum and improvements in motor and memory deficits in two different transgenic mouse models of synucleinopathies (i.e. PDGFβ- and mThy1-α-synuclein transgenic mice), by promoting microglial α-synuclein clearance and the production of anti-inflammatory cytokines[297]. In fact, AFFITOP® vaccine candidate P01 directed aginst α-synuclein is being currently assessed in a monocentric phase I study in early PD patients (https://www.michaeljfox.org/foundation/grant-detail.php?grant_id=896).

In addition to immunization therapy, agents that facilitate the clearance of misfolded α-synuclein could serve as potential therapies for PD. Supporting this idea, overexpression of the enhancer of lysosomal biogenesis, called transcriptional factor EB (TFEB), or its activation by trehalose, attenuated MPP+-induced cell death in an M17 cell line by enhancing the number of lysosomes[196]. Moreover, it has been reported that

rapamycin, which induces autophagy, also attenuated cell death *in vitro* (i.e. in M17 cells and primary mesencephalic dopaminergic neurons) and *in vivo* (i.e. wild-type mice) induced by MPP+ and MPTP, respectively[196, 201, 298].

Finally, a third approach to inhibit or delay the progression of the disease could be the use of siRNAs or oligonucleotides that restore the host α-synuclein expression to basal levels. To validate this approach, our group, in collaboration with the laboratory of Dr. Analia Bortolozzi (IDIBAPS, Barcelona, Spain) and nLife therapeutics (La Coruña, Spain) are studying the effectiveness of siRNAs directed against α-synuclein administrated intranasally and chemically modified to selectively penetrate in affected regions of the PD brain as a potential therapy for this disease.

2- Selective silencing of α-synuclein in SNpc dopaminergic neurons in vivo by the intranasal delivery of targeted small interfering RNA.

Our results demonstrate that the SNCA499-siRNA sequence conjugated to the cell-specific target indatraline downregulate α-synuclein expression in the SN in a time-dependent manner after intranasal administration in wild-type mice.

SNCA499-siRNA was selected as the best candidate among three differences sequences directed against α-synuclein (SNCA499, SNCA2 and MAYO2) after an *in vitro* screening process using wild-type or α-synuclein-overexpressing M17 cells. Although the three α-synuclein-directed sequences were able to induce an important downregulation of α-synuclein expression in both wild-type and α-synuclein-overexpressing M17 cells, only SNCA499-siRNA specifically downregulated α-synuclein expression without decreasing the expression of β- or γ-synuclein. By local infusion directly into the SN of wild-type mice, we validated the fact that SNCA499-siRNA was also able to downregulate the

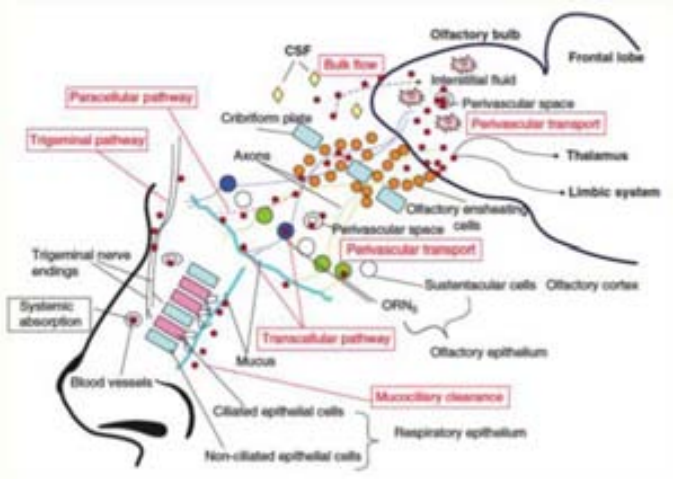
expression of α-synuclein without affecting β- or γ-synuclein expression *in vivo*. The specificity of this siRNA might have important therapeutic implications as it may avoid potential side effects induced by the downregulation of either β- or γ-synuclein.

Indatraline, a non-selective monoamine transporter inhibitor with high affinity for DAT, SERT and NET, was selected as a cell-specific target to promote specific delivery of the siRNA into affected PD brain regions such as the SN (which contains DA neurons), LC (i.e. NE neurons) and DR (i.e. 5-HT neurons). The indatraline target was validated both *in vitro* and *in vivo*. Using rat ventral midbrain primary cultures, which are enriched in dopaminergic neurons, we observed that siRNA conjugated with indatraline was mostly uptaken by neurons rather than glial cells. This is consistent with the fact that glial cells do not express (or at least not in detectable amounts) DAT[299-301] or NET[302], and only express low levels of SERT[301, 303-307] compared to neurons. In contrast, siRNA without indatraline was taken up far less by neurons compared with the conjugated siRNA.

Once the sequence and the target had been validated, the candidate IND-SNCA499-siRNA was administrated intranasally into wild-type mice (Box 13). Intranasal administration of IND-SNCA499-siRNA during four consecutive days specifically downregulated α-synuclein expression within the SN at both the protein and mRNA levels, without affecting the expression of γ-synuclein. In addition, the IND-SNCA499-siRNA-induced α-synuclein downregulation was not associated with neuronal loss within the nigrostriatal system. These results further demonstrate that indatraline promotes a selective targeting to affected PD areas. Taken together, these results validate the feasibility and safety of IND-SNCA499-siRNA administrated intranasally, and set the stage for testing these molecules as potential disease-modifying agents in animal models of PD accompanied by pathogenic increases in α-synuclein.

Three other groups have published studies related to *in vivo* α-synuclein knockdown using siRNA technology. Two of them used viral-vector technology[284-286, 308] to downregulate α-synuclein expression. The group of M. C. Bohn coinjected human *SNCA* (hSNCA) AAV2/8 (which induced forelimb motor deficit and loss of TH neurons in the SN at one month) and a short hairpin (sh) RNA targeting hSNCA into the rat SN. Although AAV-shRNA-SNCA silenced hSNCA and protected against the forelimb deficit, this approach also led to DA neuron loss[284]. To reduce this toxicity, they embedded the silent sequences in a mir30 transcript[285]. Although the mir30-embedded hSNCA was less toxic, they still reported several negative effects including the incomplete protection of TH neurons in the SN, an initial toxic effect on TH fibers in the striatum, and the presence of inflammation in the SN, as well as reduced total TH expression in the SN and reduced total ser40 phosphorylated TH in the striatum. Thus, they concluded that hSNCA-specific

Box 13: Intranasal administration



Intranasal administration is a practical, safe, convenient and non-invasive drug delivery technique with several advantages such as: circumvention of the BBB, and avoidance of hepatic first-pass metabolism. While the precise mechanisms underlying intranasal drug delivery to the CNS are not entirely understood, an accumulating body of evidence demonstrates that it could involve several pathways:

- Olfactory nerve pathway. The different modes of drug transport across the nasal olfactory epithelium are: transcellular passive diffusion, paracellular passive diffusion, carrier-mediated transport, transcytosis and efflux transport.
- Trigeminal nerve pathway, which innervates the respiratory and olfactory epithelium of nasal passages and enters the CNS via the pons.
- Pathways involving cerebrospinal fluid and nasal lymphatics:

mir30-SNCA did not hold potential for development as a clinical therapy. Gorbatyu et al. reported that the injected α-synuclein siRNAs embedded as short-hairpin RNAs (shRNAs) in AAV induced nigrostriatal degeneration as well as behavioral effects as soon as four weeks after inoculation[286]. In contrast, Lewis et al.,[309] downregulated α-synuclein expression in mice by using naked siRNA without viral vector technology. They infused siRNA into the hippocampus for 15 days using an osmotic pump, and found that α-synuclein was reduced for at least one week following completion of the infusion and recovered to normal levels by week 3, without obvious toxicity in the treated area.

Differences in neuronal loss induced by α-synuclein knockdown might be explained by differences in the brain area where α-synuclein expression is downregulated. It has been suggested that the SN is preferentially susceptible to reduced α-synuclein[286]. However, we have demonstrated here that downregulation of α-synuclein expression in the SN by intranasal administration of IND-SNCA499-siRNA did not induce neuronal loss. Indeed, all the studies that reported neurodegeneration have, as a common thread, the use of viral vectors to downregulate the α-synuclein expression. This suggests that the use of viral vectors is a possible cause of the neurodegeneration process induced by α-synuclein downregulation. The effect could be because the virus induced a chronic reduction of α-synuclein expression, in contrast to the time-limited reduction obtained by osmotic pumps and intranasal administration, in which α-synuclein expression levels had recovered within 3 weeks or 7 days respectively. Another possible explanation for the differences in neurodegeneration concerns the percentage of α-synuclein downregulation achieved. Following IND-SNCA499-siRNA intranasal administration performed here, 22-29% and 25% downregulation of α-synuclein expression were found at the protein and mRNA levels, respectively. Gorbatyuk et al., reported 68-86% of α-synuclein downregulation at

the protein levels and a 90-95% downregulation at the mRNA levels. These results suggest that there might exist a therapeutic window of α-synuclein downregulation, and that a moderate α-synuclein downregulation could be beneficial, whereas a strong α-synuclein downregulation is toxic.

Compared with the previous works, one of the weaknesses of IND-SNCA499-siRNA intranasal administration is the quick recovery of α-synuclein expression to basal levels. In fact, seven days after the last administration, α-synuclein levels, at both the protein and mRNA levels, had fully recovered. These results suggest that IND-SNCA499-siRNA would need to be administrated chronically; but it should be kept in mind that frequent use of intranasal mode of administration can cause degradation or irritation to the nasal mucosa[310-312]

In summary, given the fact that PD patients have increased levels of α-synuclein expression, we hypothesized that knocking down upregulated α-synuclein back to basal levels by intranasal administration of IND-SNCA499-siRNA might provide therapeutic benefit. However, the relatively modest 25-30% reduction in α-synuclein expression obtained here by IND-SNCA499-siRNA may prove insufficient to attain a therapeutic effect. While further studies may be needed to try to enhance IND-SNCA499-siRNA effect (e.g. by increasing the dose), it is worth noting that several studies estimate that PD patients exhibit a 25-50% increases of α-synuclein protein levels at early stages of this disease[313, 314]. Therefore, the 25-30% α-synuclein downregulation that we achieve after intranasal administration of IND-SNCA499-siRNA might be sufficient to virtually restore α-synuclein expression back to basal levels in PD patients, with the additional advantage of not causing any detrimental effect to dopamine neurons.

In any event, the potential beneficial effect of IND-SNCA499-siRNA will be tested in future studies using experimental PD models coursing with increased α-synuclein levels (such as MPTP or α-synuclein transgenic mice and rats). If successful, we expect that this strategy might be translated into a disease-modifying therapy for PD.

CONCLUSIONS

1. Lewy body extracts from Parkinson's disease brains trigger α–synuclein pathology and neurodegeneration in mice and monkeys.

1a. Nigral LB extracts from postmortem PD brains are pathogenic when injected intracerebrally into rodents and non-human primates.

1b. Intracerebral LB inoculations trigger the pathological conversion (i.e. phosphorylation, proteinase K resistance and diffuse cytosolic accumulation) of endogenous α-synuclein in host mice and monkeys, which is associated with progressive PD-like nigrostriatal degeneration starting at the striatal terminals.

1c. Intracerebral injection of LB does not induce the formation of definite, LB-like α-synuclein inclusions but rather diffuse α-synuclein accumulations, both in cell bodies and pre-synaptic terminals.

1d. PD-like pathogenic effects induced by LB inoculations are dependent on both exogenous α-synuclein present in LB and on host (endogenous) α-synuclein expression.

1e. LB-induced α-synuclein pathology can spread to brain regions distant to the injection site both anterogradely and retrogradely, especially in striatally-inoculated animals.

2. Selective silencing of α-synuclein in SNpc dopaminergic neurons in vivo by intranasal delivery of targeted small interfering RNA.

2a. All the siRNA candidates directed against α-synuclein (SNCA499-siRNA, SNCA2-siRNA, Mayo2-siRNA) were able to downregulate the expression of α-synuclein in both wild-type and α-synuclein-overexpressing M17 cells. However, only the SNCA499-siRNA

candidate was able to specifically downregulate α-synuclein expression without decreasing β- or γ-synuclein expression.

2b. SNCA499-siRNA was able to downregulate the mRNA expression of α-synuclein in mice after local infusion in the SN, without affecting β- or γ-synuclein expression.

2c. Conjugation of siRNA with the cell-specific ligand indatraline (IND) promoted its selective penetrance into SNpc dopaminergic neurons.

2d. The intranasal administration of IND-SNCA499-siRNA produced a transient, time-dependent downregulation of endogenous α-synuclein in SNpc at both protein and mRNA levels.

2e. Downregulation of endogenous α-synuclein levels in the SNpc by intranasal administration of IND-SNCA499-siRNA is not associated with nigrostriatal degeneration.

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ANNEX

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Neurobiology of Disease

Pathogenic Lysosomal Depletion in Parkinson's Disease

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Mounting evidence suggests a role for autophagy dysregulation in Parkinson's disease (PD). The bulk degradation of cytoplasmic proteins (including α-synuclein) and organelles (such as mitochondria) is mediated by macroautophagy, which involves the sequestration of cytosolic components into autophagosomes (AP) and its delivery to lysosomes. Accumulation of AP occurs in postmortem brain samples from PD patients, which has been widely attributed to an induction of autophagy. However, the cause and pathogenic significance of these changes remain unknown. Here we found in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of PD that AP accumulation and dopaminergic cell death are preceded by a marked decrease in the amount of lysosomes within dopaminergic neurons. Lysosomal depletion was secondary to the abnormal permeabilization of lysosomal membranes induced by increased mitochondrial-derived reactive oxygen species. Lysosomal permeabilization resulted in a defective clearance and subsequent accumulation of undegraded AP and contributed directly to neurodegeneration by the ectopic release of lysosomal proteases into the cytosol. Lysosomal breakdown and AP accumulation also occurred in PD brain samples, where Lewy bodies were strongly immunoreactive for AP markers. Induction of lysosomal biogenesis by genetic or pharmacological activation of lysosomal transcription factor EB restored lysosomal levels, increased AP clearance and attenuated 1-methyl-4-phenylpyridinium-induced cell death. Similarly, the autophagy-enhancer compound rapamycin attenuated PD-related dopaminergic neurodegeneration, both *in vitro* and *in vivo*, by restoring lysosomal levels. Our results indicate that AP accumulation in PD results from defective lysosomal-mediated AP clearance secondary to lysosomal depletion. Restoration of lysosomal levels and function may thus represent a novel neuroprotective strategy in PD.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder of unknown origin mainly characterized by the loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) and the presence, in the affected brain regions, of ubiquitinated intraneuronal proteinacious cytoplasmic inclusions called Lewy bodies (Dauer and Przedborski, 2003). The presence of Lewy bodies in PD suggests that defective protein handling may contribute to the pathogenesis of the disease. Proteasomal and autophagic proteolysis are the two major pathways for degradation of cellular constituents in eukaryotic cells. While initial reports focused on the proteasomal system, mounting evidence indicates that alterations in autophagic pathways may be preferentially involved in neurodegenerative diseases, including PD (Levine and Kroemer, 2008). Autophagy refers to the global process by which intracellular components are degraded by lysosomes, i.e., cytoplasmic membrane-enclosed organelles that contain a wide variety of hydrolytic enzymes and control the intracellular turnover of macromolecules (Luzio et al., 2007). While chaperone-mediated autophagy (CMA) involves the selective targeting of particular proteins directly to lysosomes, the bulk degradation of cytosolic proteins and organelles is largely mediated by macroautophagy. Macroautophagy involves the formation of double-membrane-bounded structures known as autophagosomes (AP) that fuse with lysosomes to form single-membrane-bound autophagolysosomes, which contents is then degraded by acidic lysosomal hydrolases.

While alterations in CMA have been linked to PD because of the ability of mutant and posttranslationally modified α-synuclein to block CMA *in vitro* (Cuervo et al., 2004; Martinez-Vicente et al., 2008), macroautophagy dysregulation is increasingly recognized as a potential pathogenic factor in neurodegeneration. For instance, constitutive macroautophagy is essential for neuronal survival, as its genetic inactivation selectively in neurons leads to the formation of ubiquitinated intracellular inclusions and neuron cell loss in mutant mice (Hara et al., 2006; Komatsu et al., 2006). Relevant to PD, macroautophagy is the primary mechanism by which long-lived proteins, such α-synuclein, are degraded and is the only mechanism by which entire organelles, such as mitochondria, are recycled (C. T. Chu et al., 2007; Mizushima, 2007; Vogiatzi et al., 2008). Both mitochondrial dysfunction and α-synuclein accumulations play major pathogenic roles in PD (Dauer and Przedborski, 2003;

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Optic atrophy 1 mediates mitochondria remodeling and dopaminergic neurodegeneration linked to complex I deficiency

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Mitochondrial complex I dysfunction has long been associated with Parkinson's disease (PD). Recent evidence suggests that mitochondrial involvement in PD may extend beyond a sole respiratory deficit and also include perturbations in mitochondrial fusion/fission or ultrastructure. Whether and how alterations in mitochondrial dynamics may relate to the known complex I defects in PD is unclear. Optic atrophy 1 (OPA1), a dynamin-related GTPase of the inner mitochondrial membrane, participates in mitochondrial fusion and apoptotic mitochondrial cristae remodeling. Here we show that complex I inhibition by parkinsonian neurotoxins leads to an oxidative-dependent disruption of OPA1 oligomeric complexes that normally keep mitochondrial cristae junctions tight. As a consequence, affected mitochondria exhibit major structural abnormalities, including cristae disintegration, loss of matrix density and swelling. These changes are not accompanied by mitochondrial fission but a mobilization of cytochrome *c* from cristae to intermembrane space, thereby lowering the threshold for activation of mitochondria-dependent apoptosis by cell death agonists in compromised neurons. All these pathogenic changes, including mitochondrial structural remodeling and dopaminergic neurodegeneration, are abrogated by OPA1 overexpression, both *in vitro* and *in vivo*. Our results identify OPA1 as molecular link between complex I deficiency and alterations in mitochondrial dynamics machinery and point to OPA1 as a novel therapeutic target for complex I cytopathies, such as PD.
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Reduced activity in mitochondrial complex I (NADH/ubiquinone oxidoreductase) impairs mitochondrial respiration and is associated with a wide spectrum of neurodegenerative diseases, including Parkinson's disease (PD).[1] Reduced complex I activity has been found in autopsy brain tissues and platelets of patients affected with sporadic PD.[1] In addition, complex I inhibitor 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine (MPTP) reproduces some of the clinical and neuropathological hallmarks of PD in monkeys and humans, including degeneration of dopaminergic neurons of the substantia nigra pars compacta (SNpc).[1] In rodents, complex I inhibition with MPTP or rotenone leads to SNpc dopaminergic cell death through the activation of mitochondria-dependent apoptotic pathways.[2–6] Growing evidence indicate, however, that the potential role of mitochondria in PD may extend well beyond a sole deficit in respiration.[7] Genetic- and neurotoxic-based experimental systems related to PD have indeed unraveled perturbations in several aspects of mitochondria dynamics, including alterations in mitochondria fusion/fission balance, abnormalities in mitochondria morphology/structure or defects in mitochondria turnover.[7] It is currently unknown whether and how these novel mechanisms by which mitochondria may also be linked to the pathogenesis of PD are actually related to the previously reported complex I defects occurring in this disease.

Optic atrophy 1 (OPA1) is a dynamin-related GTPase residing in the inner mitochondrial membrane (IMM) that participates in both the regulation of mitochondrial fusion and the pro-apoptotic remodeling of mitochondria.[8,9] OPA1 promotes the IMM fusion whereas other GTPases, such as Mitofusins 1 and 2 (Mfn1, Mfn2), are involved in the fusion of the outer mitochondrial membrane (OMM).[8] In addition, OPA1 has been shown to regulate mitochondria structural alterations occurring during apoptosis independently of its role in mitochondrial fusion.[9] Biochemical analyses identified an

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Keywords: Parkinson's disease; MPTP; rotenone; apoptosis
Abbreviations: AAV, adenoassociated viral; Drp1, dynamin-related protein 1; EDC, ethyl-3-(3-dimethylaminopropyl) carbodiimide; GFP, green fluorescent protein; IMM, inner mitochondrial membrane; IMS, mitochondria intermembrane space; Mfn1/2, Mitofusins 1 and 2; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-pheny-1,2,3,6-tetrahydropyridine; OMM, outer mitochondrial membrane; OPA1, Optic atrophy 1; PARL, presenilin-associated rhomboid-like protease; PD, Parkinson's disease; PINK1, PTEN-induced putative kinase-1; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; TOM, translocase of the mitochondrial outer membrane; UT, untreated
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BASIC RESEARCH PAPER

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BAX channel activity mediates lysosomal disruption linked to Parkinson disease

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Keywords: Parkinson disease, neurodegeneration, BAX channel inhibitor, lysosome, mitochondria, MPTP

Abbreviations: AP, autophagosome; BAX, BCL2-associated X protein; Bci, BAX channel inhibitor; LAMP1, lysosomal-associated membrane protein 1; LC3-II, microtubule-associated protein 1A/1B-light chain 3-II; LMP, lysosomal membrane permeabilization; MOMP, mitochondrial outer membrane permeabilization; MPP^+, 1-methyl-4-phenylpyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson disease; PTPC, permeability transition pore complex; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TOMM20, translocase of outer mitochondrial membrane 20 homolog (yeast)

Lysosomal disruption is increasingly regarded as a major pathogenic event in Parkinson disease (PD). A reduced number of intraneuronal lysosomes, decreased levels of lysosomal-associated proteins and accumulation of undegraded autophagosomes (AP) are observed in PD-derived samples, including fibroblasts, induced pluripotent stem cell-derived dopaminergic neurons, and post-mortem brain tissue. Mechanistic studies in toxic and genetic rodent PD models attribute PD-related lysosomal breakdown to abnormal lysosomal membrane permeabilization (LMP). However, the molecular mechanisms underlying PD-linked LMP and subsequent lysosomal defects remain virtually unknown, thereby precluding their potential therapeutic targeting. Here we show that the pro-apoptotic protein BAX (BCL2-associated X protein), which permeabilizes mitochondrial membranes in PD models and is activated in PD patients, translocates and internalizes into lysosomal membranes early following treatment with the parkinsonian neurotoxin MPTP, both in vitro and in vivo, within a time-frame correlating with LMP, lysosomal disruption, and autophagosome accumulation and preceding mitochondrial permeabilization and dopaminergic neurodegeneration. Supporting a direct permeabilizing effect of BAX on lysosomal membranes, recombinant BAX is able to induce LMP in purified mouse brain lysosomes and the latter can be prevented by pharmacological blockade of BAX channel activity. Furthermore, pharmacological BAX channel inhibition is able to prevent LMP, restore lysosomal levels, reverse AP accumulation, and attenuate mitochondrial permeabilization and overall nigrostriatal degeneration caused by MPTP, both in vitro and in vivo. Overall, our results reveal that PD-linked lysosomal impairment relies on BAX-induced LMP, and point to small molecules able to block BAX channel activity as potentially beneficial to attenuate both lysosomal defects and neurodegeneration occurring in PD.

Introduction

Mounting evidence indicates that impaired lysosomal function contributes to the pathogenesis of Parkinson disease.[1] A reduced number of intraneuronal lysosomes, decreased levels of lysosomal-associated proteins, and accumulation of undegraded autophagosomes are observed in PD patients [i.e., post-mortem brain samples,[2,3] fibroblasts,[4,5] and induced pluripotent stem cell-derived dopaminergic neurons[6]] as well as in toxic and genetic rodent models of PD [i.e., mice treated with parkinsonian neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)[3] and rats overexpressing mutant SNCA/α-synuclein[2]]. Mechanistic studies in the MPTP mouse model revealed that PD-linked lysosomal deficiency is secondary to abnormal lysosomal membrane permeabilization.[3,7] In particular, MPTP-induced LMP results in a decreased number of lysosomes and impaired AP-lysosome fusion, leading to a defective clearance and subsequent accumulation of undegraded AP within affected neurons.[3,7] In addition, LMP can directly participate in MPTP-induced dopaminergic cell death by the leakage of lysosomal

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RESEARCH ARTICLE

Lewy Body Extracts from Parkinson Disease Brains Trigger α-Synuclein Pathology and Neurodegeneration in Mice and Monkeys

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Objective: Mounting evidence suggests that α-synuclein, a major protein component of Lewy bodies (LB), may be responsible for initiating and spreading the pathological process in Parkinson disease (PD). Supporting this concept, intracerebral inoculation of synthetic recombinant α-synuclein fibrils can trigger α-synuclein pathology in mice. However, it remains uncertain whether the pathogenic effects of recombinant synthetic α-synuclein may apply to PD-linked pathological α-synuclein and occur in species closer to humans.
Methods: Nigral LB-enriched fractions containing pathological α-synuclein were purified from postmortem PD brains by sucrose gradient fractionation and subsequently inoculated into the substantia nigra or striatum of wild-type mice and macaque monkeys. Control animals received non-LB fractions containing soluble α-synuclein derived from the same nigral PD tissue.
Results: In both mice and monkeys, intranigral or intrastriatal inoculations of PD-derived LB extracts resulted in progressive nigrostriatal neurodegeneration starting at striatal dopaminergic terminals. No neurodegeneration was observed in animals receiving non-LB fractions from the same patients. In LB-injected animals, exogenous human α-synuclein was quickly internalized within host neurons and triggered the pathological conversion of endogenous α-synuclein. At the onset of LB-induced degeneration, host pathological α-synuclein diffusely accumulated within nigral neurons and anatomically interconnected regions, both anterogradely and retrogradely. LB-induced pathogenic effects required both human α-synuclein present in LB extracts and host expression of α-synuclein.
Interpretation: α-Synuclein species contained in PD-derived LB are pathogenic and have the capacity to initiate a PD-like pathological process, including intracellular and presynaptic accumulations of pathological α-synuclein in different brain areas and slowly progressive axon-initiated dopaminergic nigrostriatal neurodegeneration.

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Parkinson disease (PD) is characterized mainly by the loss of dopaminergic neurons from the substantia nigra pars compacta (SNpc) and the presence in affected brain regions of α-synuclein–containing insoluble

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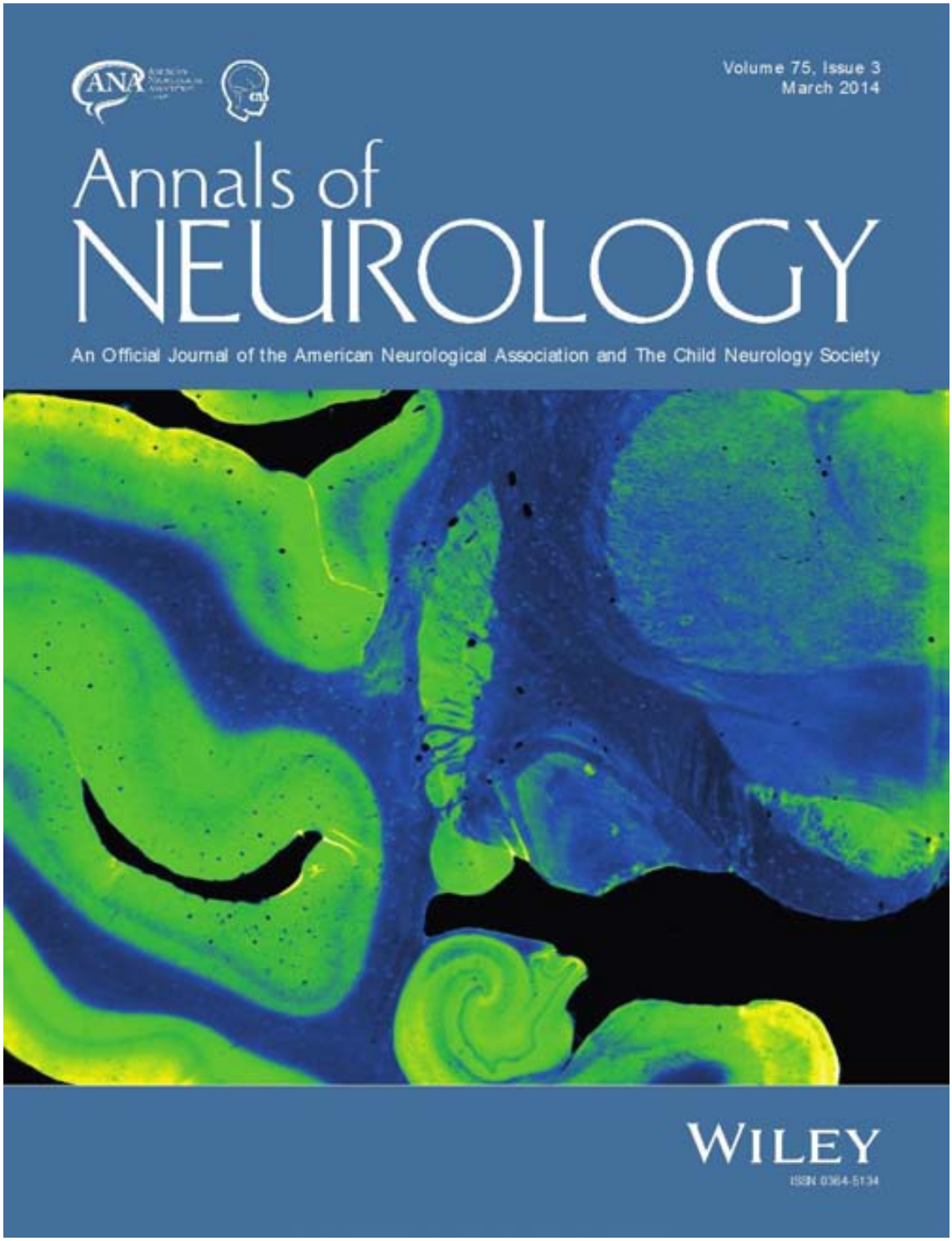
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