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THÈSE PRÉSENTÉE
POUR OBTENIR LE GRADE DE
DOCTEUR DE
L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE : Sciences de la Vie et la Santé
SPÉCIALITÉ : Neurosciences

Par Miguel LÓPEZ CUIÑA

Physiopathology and treatment of multiple system atrophy

Sous la direction de M. le Docteur Wassilios MEISSNER

Soutenue le 14 Novembre 2019

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“Science is the expression of an inherent need of the human being and, in any case, it is linked to the superior function of its intelligent nature: the capacity to create.”

Dr René Gerónimo Favaloro

Argentine cardiac surgeon, father of the coronary bypass surgery

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Résumé

Titre : *Physiopathologie et traitement de l'atrophie multisystématisée*

L'atrophie multisystematisée (AMS) est une maladie neurodégénérative rare et à évolution rapide qui affecte de nombreuses régions du système nerveux central, y compris les systèmes olivopontocérébelleux et striatonigral ainsi que divers noyaux autonomes du tronc cérébral. La caractéristique pathologique de l'AMS est la présence d'agrégats oligodendrogliaux appelés inclusions cytoplasmiques gliales dont le composant principal est la protéine α -synuclein. Le processus neurodégénératif conduit à une dysautonomie, ainsi qu'à un degré variable de syndromes parkinsoniens et cérébelleux. Il n'existe actuellement aucun traitement pour ralentir l'évolution de la maladie. Ce travail de thèse a porté, d'une part, sur des approches précliniques visant à réduire l'accumulation de l' α -synuclein dans un modèle de souris transgénique de l'AMS, et d'autre part, sur une analyse anatomo-pathologique chez des patients présentant une forme lentement progressive de la maladie. Tout au long de ces trois années de travail, nous avons évalué différents candidats thérapeutiques chez la souris transgénique de l'AMS. La rapamycine, un médicament connu pour stimuler l'autophagie et la clairance des protéines, n'a montré qu'un effet neuroprotecteur partiel contre la perte neurale dans notre modèle. Le nilotinib, un médicament qui avait démontré des propriétés neuroprotectrices dans un modèle rongeur de la maladie de Parkinson, n'a pas eu d'effet sur l'accumulation de l' α -synuclein et la neurodégénérescence. Enfin, nous avons évalué la combinaison de deux médicaments (anle 138b et belnacasan) qui ont déjà démontré leur capacité à réduire l'agrégation de l' α -synucleine et à protéger les neurones de la dégénérescence, pour déterminer si elles ont des effets synergiques.

Mots-clés : Synucleine, atrophie à systèmes multiples, inclusions cytoplasmiques gliales, étude post-mortem du cerveau humain, rongeur, approche translationnelle, troncation c-terminale, phosphorylation, autophagie, agrégation des protéines

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Abstract

Title: *Pathophysiology and treatment of multiple system atrophy*

Multiple system atrophy (MSA) is an orphan, rapidly progressive neurodegenerative disease that affects numerous regions of the central nervous system, including the olivopontocerebellar and striatonigral systems as well as various autonomous nuclei of the brainstem. The pathological hallmark of MSA is the presence of oligodendroglial aggregates called glial cytoplasmic inclusions (GCI) whose main component is the protein α -synuclein. Neuroinflammation is a known factor in all neurodegenerative diseases, including MSA. The neurodegenerative process leads to severe impairment of autonomous functions, together with a varying degree of parkinsonian and cerebellar syndromes. There is currently no disease modifying therapy available

This PhD work focused, on the one hand, on translational approaches aiming to reduce the accumulation of α -synuclein in an animal model of MSA, and on the other hand, on a pathological analysis of a rare subtype of the disease.

Throughout the three years of work, we have assessed different therapeutic candidates in an animal model of MSA. Rapamycin, a drug known to enhance autophagy and protein clearance, showed only a partial neuroprotective effect against neural loss in our model. Nilotinib, a drug that had shown neuroprotective properties in a Parkinson's disease animal model, failed to modify the disease course in our study. Finally, we evaluated the combinations of two drugs that have already proven to reduce α -synuclein aggregation and protect neurons from degeneration, to assess whether they have synergistic properties.

Keywords: Synuclein, Multiple system atrophy, glial cytoplasmic inclusions, post-mortem human brain study, rodent, translational approach, c- terminal truncation, phosphorylation, autophagy, protein aggregation.

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ABBREVIATIONS

α -synuclein	alpha-synuclein
AD	Alzheimer's disease
BBB	Blood brain barrier
cAbl	Abelson protein
CSF	Cerebrospinal fluid
DBS	Deep brain stimulation
DLB	Dementia with Lewy bodies
GCI	Glial cytoplasmic inclusions
LB	Lewy bodies
MBP	Myelin basic protein
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MSA	Multiple system atrophy
MSA-C	Multiple system atrophy cerebellar phenotype
MSA-P	Multiple system atrophy parkinsonian phenotype
OPCA	Olivopontocerebellar atrophy
PD	Parkinson's disease
PLP	Myelin proteolipid promoter
ROS	Reactive oxygen species
SN	Substantia nigra
SND	Striatonigral degeneration
SNc	Substantia nigra pars compacta
TH	Tyrosine hydroxylase
UPDRS	Unified PD Rating Scale

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INTRODUCTION

I- INTRODUCTION

Multiple System Atrophy and alpha-synuclein

Multiple System Atrophy (MSA) is a rare neurodegenerative disease that leads to early disability and death. MSA belongs to a group of disorders called synucleinopathies, characterized by an abnormal accumulation of the ubiquitous protein alpha-synuclein (α -synuclein) in different cell types in the central and peripheral nervous system (CNS and PNS). Accumulation of α -synuclein in the cytoplasm of oligodendrocytes, in the form of cytoplasmatic glial inclusions (GCI), is the defining pathological hallmark of MSA. Other synucleinopathies are Parkinson's disease (PD) and dementia with Lewy bodies (DLB). The primary trigger for α -synuclein accumulation and the underlying pathophysiology remain poorly understood. Finally, therapeutic options are restricted to symptomatic relief, while there are ongoing efforts to address the unmet need for neuroprotective and disease-modifying therapies.

Epidemiology

Previously thought to be three different disorders, namely Shy-Drager syndrome, olivopontocerebellar atrophy (OPCA) and striatonigral degeneration (SND), it was not until 1969 that their common pathology was recognized and the term MSA coined (Graham and Oppenheimer, 1969). In 1989 its defining pathological hallmark, the GCI, was identified (Papp et al., 1989), and in 1998 it was found that GCIs' major component is α -synuclein (Spillantini et al., 1998). The estimated mean incidence is 0.6 to 0.7 cases per 100,000 person-years, with a range of 0.1 to 2.4 cases per 100,000 person-years. The estimated point prevalence is 3.4 to 4.9 cases per 100,000 population, increasing to 7.8 per 100,000 among persons older than 40 years of age (Fanciulli and Wenning, 2015). Disease onset is usually in the sixth decade of life, with both sexes equally affected. The median survival from symptom onset is 6 to 10 years, although disease duration may greatly vary, from 5 years to up to two decades (Gilman et al., 2008; Jellinger, 2012; Ling et al., 2015; Lopez-Cuina et al., 2018a; Masui et al., 2012; Petrovic et al., 2012). Current consensus diagnostic criteria include two classical phenotypes, MSA with predominant parkinsonism (MSA-P) and MSA with predominant cerebellar ataxia (MSA-C)

(Gilman et al., 2008). While MSA-P is more frequent in the Western hemisphere, MSA-C is the more prevalent in Asia (Köllensperger et al., 2010; Watanabe et al., 2002).

Genetics

Contrary to what occurs in PD, unambiguous evidence for genetic causes of MSA is still lacking. Some evidence has pointed to a role of the coenzyme Q10 related *COQ2* gene in Asian cohorts, however, other studies have failed to replicate these results (Jeon et al., 2014; Lin et al., 2015; Multiple-System Atrophy Research Collaboration, 2013; Ogaki et al., 2014; Ronchi et al., 2016; Sharma et al., 2014; Sun et al., 2016). A genetic study of pathologically confirmed MSA cases of the gene linked to familial PD, leucine-rich repeat kinase 2 (LRRK2), pointed to an association between exonic variants and MSA (Heckman et al., 2014). A study of another gene known to cause familial PD, the glucocerebrosidase (GBA) gene, suggested that GBA variants are associated to MSA (Mitsui et al., 2015); notwithstanding, these were small studies and these results would need replication.

Genome-Wide Association Studies (GWAS) have failed to detect polymorphisms in the α -synuclein encoding gene (SNCA) (Sailer et al., 2016). Interestingly, two mutations of the SNCA gene, G51D and A53E, have been related to cases of atypical parkinsonian syndromes with a mixed neuropathology encompassing widespread neural and glial α -synuclein immunoreactive inclusions resembling Lewy bodies and GCIs (Kiely et al., 2015; Pasanen et al., 2014). Although speculative, this may suggest a possible link between PD and MSA, with both disorders being two extremes of the same spectrum

Finally, well characterized genetic disorders can mimic MSA clinical presentation, such as Perry syndrome, hereditary spastic paraplegias, spinocerebellar ataxias (SCA), and others. This further complicates the task of accurately diagnosing this MSA for clinicians (Kim et al., 2016; Stamelou et al., 2013).

α -Synuclein in physiology and disease

α -Synuclein is a small 14 kilo Dalton (kDa) protein, composed of 140 amino acids, which is physiologically expressed in the human brain. The name synuclein derives from its first characterization inside the nucleus and the synaptic terminals in neurons (Maroteaux et al., 1988). α -synuclein exists in an equilibrium between a soluble and a membrane-bound state, with its secondary structure depending on its state (Burré et al., 2018). Through interaction with

membranes it adopts an alpha-helix (Bartels et al., 2011; Wang et al., 2011), and given this interaction with vesicle membranes in the presynaptic terminal, several studies have pointed a putative role in regulating synaptic vesicles and neurotransmitter release (Bendor et al., 2013; Burre, 2015; Burré et al., 2018; Burre et al., 2010).

In contrast to its physiological conformations outlined above, α -synuclein adopts a β -sheet amyloid conformation under pathological conditions. This β -sheet conformation is associated with α -synuclein aggregation, fibril formation, and deposition into pathological inclusions (Burré et al., 2018).

α -Synuclein aggregation takes place in a stereotypical pattern, firstly, soluble unstructured monomeric forms coalesce into partially soluble oligomers, then oligomers aggregate into immature fibrils and, lastly, amyloid fibrillary aggregates are formed (Burré et al., 2018; Dehay et al., 2015; Zhang et al., 2019)

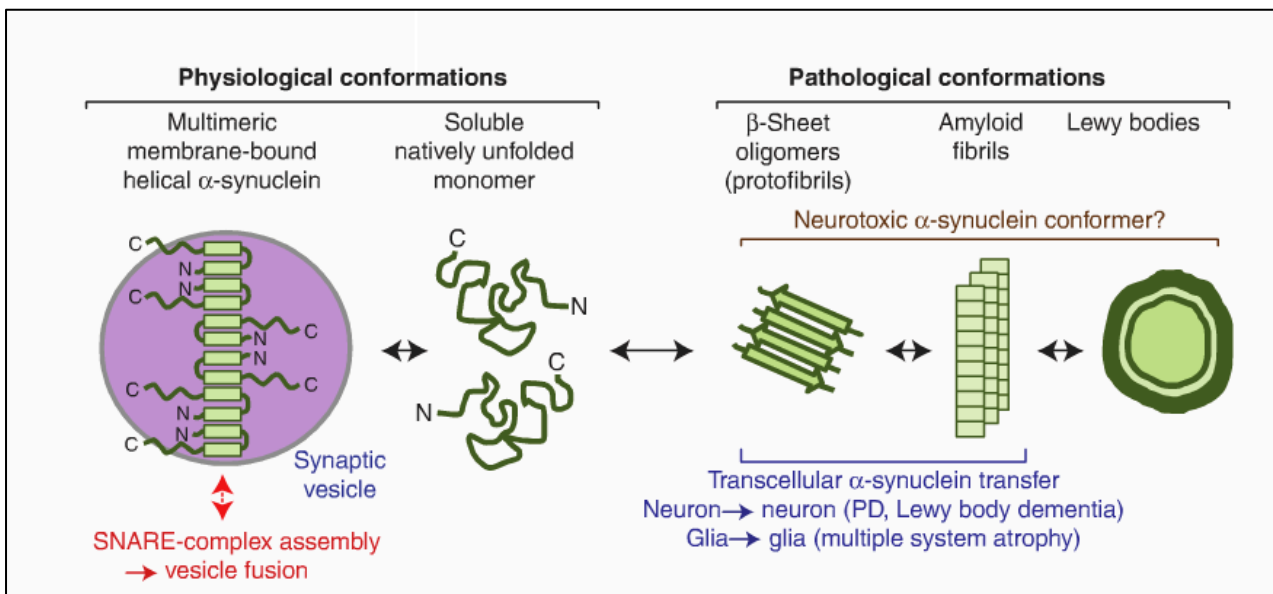


Figure 1. Schematic of α -synuclein conformations associated with its physiological function and pathological activities. Parkinson’s disease, PD; SNAP receptor, SNARE. From Burré et al. (2018)).

In vivo studies have shown that both fibrils and oligomers are toxic to dopaminergic neurons and lead to neural loss (Abdelmotilib et al., 2017; Paumier et al., 2015; Winner et al., 2011). In vivo research shows that α -synuclein exerts its toxic effect in numerous ways, oligomers show mitochondrial toxicity, they do so through inhibition of mitochondrial membrane protein

transporters, and metabolite flux controlling channels, also located in the mitochondria membrane (Di Maio et al., 2016). Oligomers induce fragmentation of mitochondrial membranes in vitro, they also disrupt mitochondrial calcium homeostasis and accelerate the release of respiration-related proteins (Luth et al., 2014; Plotegher et al., 2014). A disruption of intracellular calcium equilibrium also occurs, as the lipophilic α -synuclein interacts with the cellular membrane, and, through pore-forming mechanisms, disrupts calcium influx. It also alters, via membrane interactions, calcium signalling, further leading to calcium dysregulation. Additionally, binding of α -synuclein to components of the ubiquitin proteasome system (UPS) has been shown to inhibit its proteolytic activity, thus hampering protein degradation (Emmanouilidou et al., 2010; Lindersson et al., 2004). It has been reported that neuron released α -synuclein is an agonist of the toll-receptor 2 (TLR2), which is a microglial receptor responsible for activating its inflammatory response (Kim et al., 2013; Stirling et al., 2014). Finally, α -synuclein provokes synaptic dysfunction through several mechanisms, such as prevention of synaptic vesicle fusion, inhibition of microtubule formation, which hampers axonal transport, and reduced production of synaptic vesicles by the Golgi apparatus (Choi et al., 2013; Danzer et al., 2007; Gosavi et al., 2002; Prots et al., 2013)

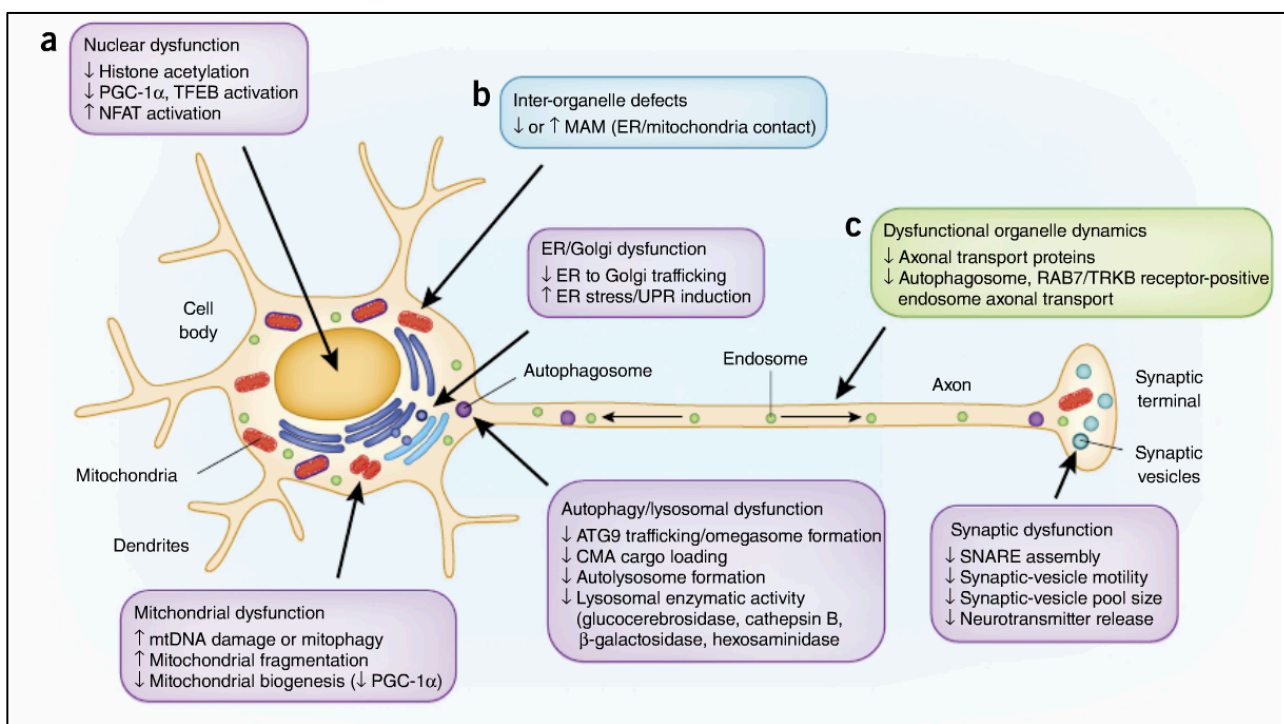


Figure 2. Pathways implicated in α -synuclein toxicity. Organelle dysfunction, defects in inter-organelle contacts and dysfunctional organelle dynamics.; endoplasmic reticulum, ER; chaperone mediated autophagy, CMA; mitochondria-associated ER membrane, MAM;

mitochondrial DNA, mtDNA; nuclear factor of activated t-cells, NFAT; peroxisome proliferator-activated receptor gamma coactivator 1-alpha, PGC-1 α ; SNAP receptor, SNARE; Transcription factor EB, TFEB; tropomyosin receptor kinase B, TRKB; unfolded protein response, UPR. From Wong and Krainc (2017)

α -Synuclein post translational modifications

α -Synuclein can undergo a variety of post-transcriptional modifications (PTM) which alter the conformation and/or biological function of proteins, and can also affect protein folding and aggregation, but the precise contribution of different posttranslational modifications to disease mechanism is still unclear.

Phosphorylation can affect protein configuration, function and fate in several ways: it may induce conformational changes, it may be required for protein folding, it might act as signalling for further modifications, it may alter protein localization and influence protein-to-protein interactions (Salazar and Höfer, 2009). In neurodegeneration, it is known that phosphorylation is involved in protein toxicity and aggregation (Tenreiro et al., 2014).

The predominant, most studied and understood α -synuclein modification is phosphorylation at serine 129 (S129). Although it is found in synucleinopathy inclusions and it has been linked to oligodendroglial α -synuclein induced toxicity in vitro, with over ten kinases targeting this site, the cross-talk between these enzymes remains to be elucidated. Phosphorylation at S129 modulates α -synuclein binding to membranes, influencing neurotransmitter uptake; it enhances α -synuclein interaction with metal ions, which promotes fibrillization. Data suggest it also modulates protein-to-protein interactions, regulates α -synuclein turnover and its subcellular localization (Beyer and Ariza, 2013; Burré et al., 2018; Kleinknecht et al., 2016; Oueslati, 2016; Tenreiro et al., 2014; Wang et al., 2012). Other phosphorylated residues have been reported, such as serine 87 and 125 (S87 and S125) and tyrosine 39, 125, 133 and 136 (Y39, Y125, Y133 and Y136), and while there is some knowledge regarding the kinases that target them and their biological consequences, further research in this regard is warranted (Brahmachari et al., 2016; Ellis et al., 2001; Feany and Bender, 2000; Hasegawa et al., 2002; Mahul-Mellier et al., 2014; Nakamura et al., 2001; Paleologou et al., 2010; Wang et al., 2012; Zhang et al., 2019). In vitro studies have suggested that Y39 is phosphorylated by the tyrosine kinase c-Abl, which also targets Y125, and that this phosphorylation prevents α -synuclein degradation by the proteasomal and lysosomal pathways (Brahmachari et al., 2016; Mahul-Mellier et al., 2014). Additionally, inhibition of this kinase

has proven neuroprotective in mouse models of PD (Brahmachari et al., 2016; Hebron et al., 2013; Imam et al., 2013; Imam et al., 2011; Karuppagounder et al., 2014).

Proteolytic cleavage of α -synuclein may occur at its carboxyl-terminus (C-terminus) and amino-terminus (N-terminus) regions. C-terminal and N-terminal truncated α -synuclein species exist in brains of healthy individuals and PD as well as MSA patients, albeit in differing quantities. It has been shown that C-terminal truncated α -synuclein can be found in aggregates, further, truncated α -synuclein acts as seed and increases aggregation propensity (Campbell et al., 2001; Duda et al., 2000; Hoyer et al., 2004; Li et al., 2005; Murray et al., 2003; Peng et al., 2018; Tofaris et al., 2003; Ulusoy et al., 2010). Although the physiological role of truncated α -synuclein is unknown, several proteolytic enzymes that cleave α -synuclein at the C-terminus have been identified: neurosin, proteasome 20S, calpain-1, cathepsin D, matrix metalloproteinases and caspase 1, the latter has been linked to α -synuclein aggregation in PD (Iwata et al., 2003; Lewis et al., 2010a; Lewis et al., 2010b; Mishizen-Eberz et al., 2003; Seveler et al., 2008; Sung et al., 2005; Wang et al., 2016). Further, inhibition of caspase-1 has shown to reduce α -synuclein aggregation and dopaminergic cell loss in a transgenic mouse model of MSA (Bassil et al., 2016).

Other PTMs include ubiquitination, SUMOylation, nitration and glycation, all have been described within GCIs, they mostly promote aggregation but the current knowledge of their roles is more limited than for phosphorylation and truncation (Bose and Beal, 2016; Danielson et al., 2009; Dorval and Fraser, 2006; Giasson et al., 2000; Gomez-Tortosa et al., 2000; Krumova and Weishaupt, 2013; Melchior et al., 2003; Popova et al., 2015; Rott et al., 2017; Rowan et al., 2018; Schapira and Jenner, 2011; Shin et al., 2005; Tetzlaff et al., 2008; Vicente Miranda et al., 2017; Videira and Castro-Caldas, 2018).

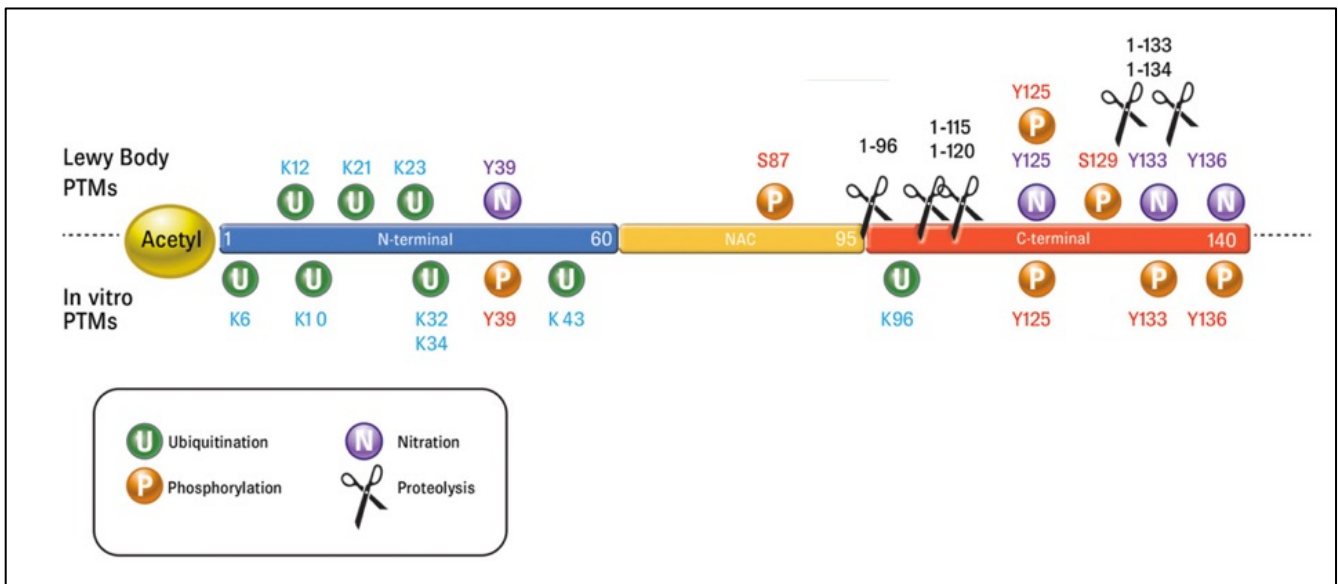


Figure 3. Location of the main α -synuclein PTMs (phosphorylation, ubiquitination, nitration, and truncation) are shown. Disease-associated PTMs identified in Lewy bodies are shown in the upper part of the scheme, and those identified from in vitro studies are shown below. PTM, post translational modification; lysine residues K6, 10, 12, 21, 23, 32, 34, 43 and 96; tyrosine residues Y39, 125, 133; serine residues S87 and S129. From Schmid et al. (2013)

α -Synuclein and protein degradation mechanisms

The ubiquitin-proteasome system (UPS) is the main mechanism for protein degradation in mammalian cells. Proteins are tagged by the attachment of ubiquitin molecules, which will be recognized by the proteasome. Proteasomes are small, barrel-like structures with a proteolytic core (called 20S proteasome), comprising three types of catalytic enzymes. Proteins are broken down into peptides that undergo further degradation (Nandi et al., 2006). Ubiquitination of α -synuclein promotes its proteasomal degradation (Liani et al., 2004; Rott et al., 2011). Proteasomal components can be found within GCIs (Gai et al., 2003), post mortem studies have further shown the lack of some proteasomal subunits in the substantia nigra (SN) of MSA patients (Bukhatwa et al., 2010), and it has been demonstrated that pharmacological inhibition of the UPS enhances α -synuclein induced degeneration in a mouse model of MSA (Stefanova et al., 2012b). In addition, in vitro evidence indicates that α -synuclein is degraded by the UPS and autophagic lysosomal pathway (ALP) (Webb et al., 2003), and that there is a complex cross-talk between these two systems (Yang et al., 2013). Also, in vivo research suggests that the UPS is the principal mechanism of degradation of α -synuclein in health, while autophagy

is involved in α -synuclein degradation only in a pathological scenario where increased α -synuclein burden exists (Ebrahimi-Fakhari et al., 2012).

Autophagy is an evolutionarily conserved cellular mechanism for degradation of proteins and organelles in lysosomes. It is an effective neuroprotective mechanism that actively contributes to the removal of pathogenic proteins. Considerable evidence suggests that α -synuclein clearance takes place in lysosomes, be it through the classical macroautophagy pathway, the chaperone-mediated pathway (CMA) or endosomal microautophagy. Dysfunction of these systems can facilitate α -synuclein aggregation, and, conversely, α -synuclein aggregates may disrupt their function (Dehay et al., 2010; Mak et al., 2010; Scrivo et al., 2018a; Tofaris et al., 2011; Vogiatzi et al., 2008).

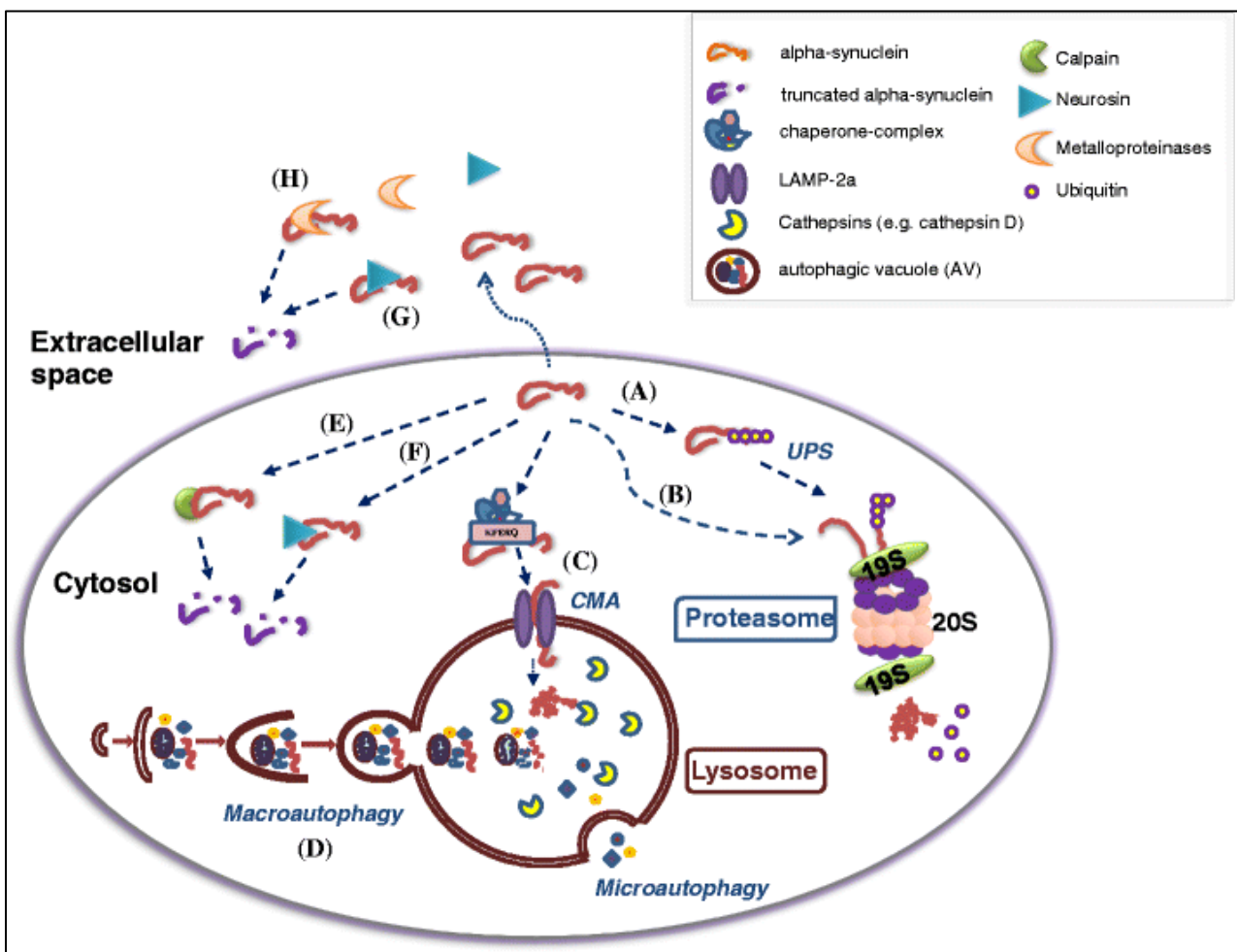


Figure 4. Proteolytic pathways implicated in α -synuclein processing. Lysosome-associated membrane protein 2a, LAMP2a; proteasome core particle 20, S20; regulatory particle, S19. From Xilouri et al. (2013)

Pathogenesis

The presence of aggregated α -synuclein containing GCIs is the defining hallmark of the disease, nonetheless, data show that α -synuclein also accumulates in neurons (Cykowski et al., 2015; Papp et al., 1989).

The origin of the α -synuclein present in GCIs is matter of an ongoing debate, with earlier studies failing to prove the expression of α -synuclein in mature oligodendrocytes and data showing that α -synuclein secreted by neurons can be uptaken by oligodendrocytes and astrocytes (Lindström et al., 2017; Miller et al., 2005; Ozawa et al., 2001; Reyes et al., 2014; Reyes et al., 2019). More recent research has shown that, albeit in small quantities, oligodendrocytes seem to express α -synuclein (Asi et al., 2014; Djelloul et al., 2015; Kaji et al., 2018). Latest data suggest that pathologically misfolded exogenous α -synuclein uptaken by oligodendrocytes promotes the misfolding and accumulation of endogenous α -synuclein forming insoluble aggregates. This process would not be due to an increase in expression of the endogenous protein, in accord with previous reports of low α -synuclein encoding messenger ribonucleic acid (mRNA) in oligodendrocytes, but, rather, to an impairment of autophagic catabolism of α -synuclein (Mavroei et al., 2019). In addition, a recent study revealed a widespread presence of α -synuclein oligomers in neurons throughout the brain, further suggesting that neural α -synuclein accumulation might be an early event in MSA (Sekiya et al., 2019)

Beyond α -synuclein, the oligodendroglial-specific phosphoprotein p25 α (tubulin polymerization promoting protein, TPPP) is a major component of GCIs and facilitates α -synuclein aggregation in vitro (Kovács et al., 2004; Lindersson et al., 2005; Song et al., 2007). In MSA, p25 α is relocated from the myelin sheath to the abnormally expanded oligodendroglial cell bodies, an event followed by a reduction of total myelin basic protein (MBP) levels, myelin fragmentation, and accumulation of abnormal α -synuclein (Ota et al., 2014; Song et al., 2007). In vitro research has shown that p25 α has a central role in the seeding of misfolded α -synuclein and the formation of pathological aggregates (Mavroei et al., 2019).

The process by which abnormally misfolded proteins serve as template for the pathological misfolding of normal proteins has been named seeding or permissive templating (Hardy, 2005). This is the molecular mechanism behind a group of disorders commonly known as prion diseases, in which abnormal proteinaceous particles (prions) with an infectious behaviour spread and propagate through the CNS; being the bovine spongiform encephalopathy, commonly referred to as “mad cow disease”, the most recognizable by the

general public (Nathanson et al., 1997; Prusiner, 1982). In recent years, the aggregative behaviour of pathological α -synuclein in synucleinopathies has been likened to that of prion disease, but, even though there is evidence that α -synuclein assemblies might adopt different conformations, called strains, with distinct structural, biochemical, biological and pathological features (Candelise et al., 2019; Gribaudo et al., 2019; Guo et al., 2013; Mamais et al., 2013; Melki, 2015; Peelaerts and Baekelandt, 2016; Peelaerts et al., 2015; Peng et al., 2018), whether this equates to prion disorders is currently matter for scientific debate (Surmeier et al., 2017; Walsh and Selkoe, 2016; Wenning et al., 2018; Woerman et al., 2018b).

Several studies have demonstrated that inoculation of synthetic aberrant α -synuclein into the brain of transgenic mouse models of synucleinopathy (PD or MSA) would accelerate the accumulation and aggregation of α -synuclein. Also, others have shown that injection of MSA brain homogenates into transgenic mice expressing mutant α -synuclein would induce neurodegeneration with α -synuclein accumulation, and the authors have characterized these α -synuclein aggregates as prions. However, even though these data suggest that α -synuclein spreads through the brain, for this pathological propagation to take place, expression of mutant α -synuclein is a requirement, meaning that healthy, non-transgenic mice do not develop the disease. This does not happen in actual prion disease, where prion particles do not require pre-existing mutant protein to spread (Krejciova et al., 2019; Prusiner et al., 2015; Watts et al., 2013; Woerman et al., 2018a). Furthermore, compelling epidemiological evidence that MSA might be a prion disease is lacking. Put together, these data do not allow, thus far, to unambiguously claim that MSA is a prion disease (Peelaerts and Baekelandt, 2016; Wenning et al., 2018).

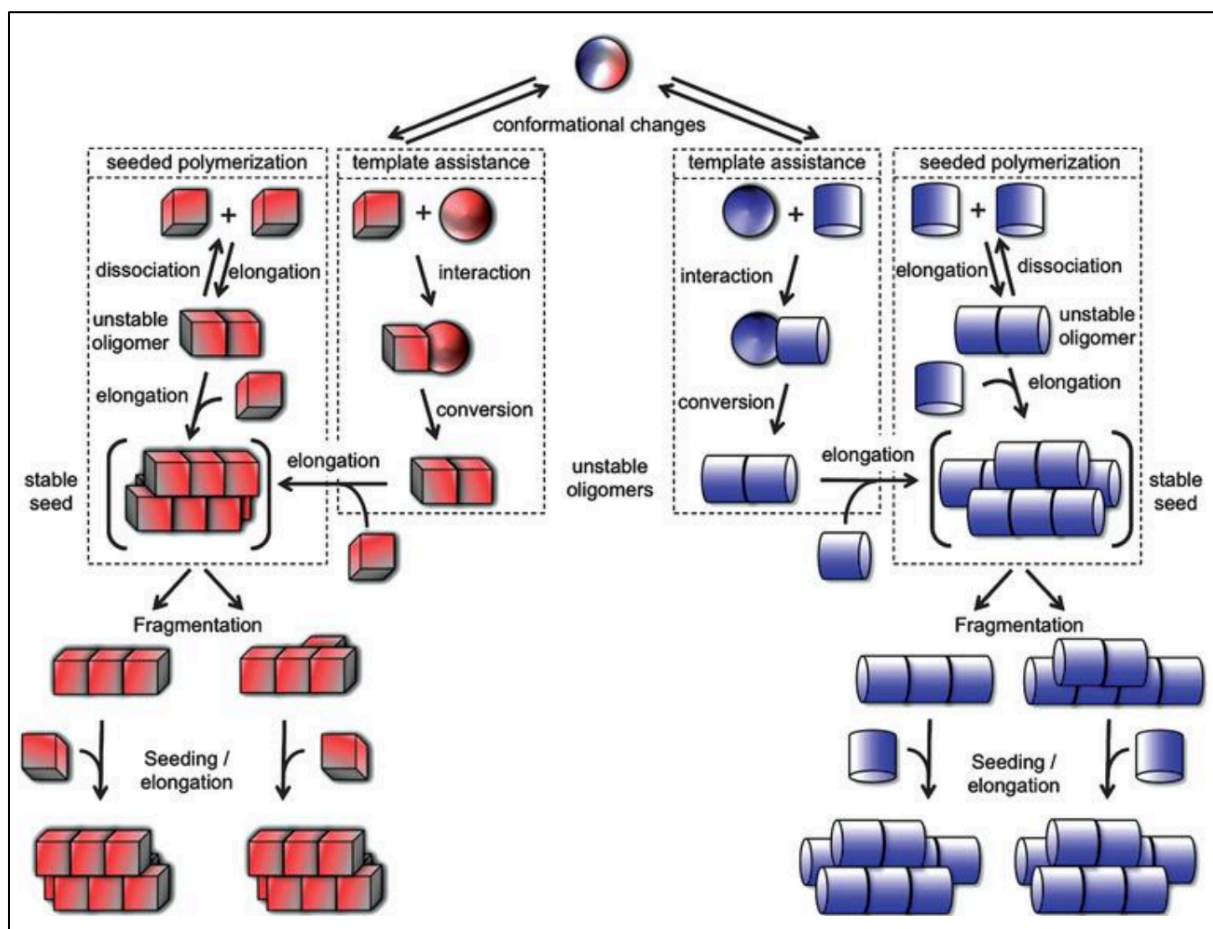


Figure 5. Protein misfolding and aggregation into infectious fibrillar assemblies. Native or natively unfolded polypeptide chains (sphere) undergo conformational changes that lead to distinct abnormal (cube or cylinder) forms. From Melki (2015)

Neuroinflammation has long been known to play a role in neurodegenerative disorders (Ransohoff, 2016), this is also the case for MSA. Microglial activation is commonly detectable in patients' brains (Ishizawa et al., 2004; Jellinger, 2014), while positron emission tomography (PET) imaging studies have reported activated microglia in MSA (Gerhard et al., 2006), and it appears microglia activation is driven by α -synuclein, and, in a vicious circle, this activation promotes further aggregation (Su et al., 2009; Su et al., 2008; Vieira et al., 2015). In a mouse model of the MSA, microglial activation correlates with neuronal loss, furthermore, inhibition of the microglia-expressed myeloperoxidase attenuates neurodegeneration, although only if treatment is initiated in very early stages of the disease (Kaindlstorfer et al., 2015; Stefanova et al., 2012a; Stefanova et al., 2007). There is evidence that microglia and astrocytes interact with α -synuclein and undergo phenotypic changes in the context of a chronic pro-inflammatory

medium (Refolo and Stefanova, 2019). Even though the activation of the inflammatory cascade may be secondary to other phenomena, it likely contributes to many of the detrimental processes which can be observed in the disease (Compagnoni and Fonzo, 2019).

Another facilitating factor of protein accumulation is the impairment of its clearance. Neuropathological studies show altered levels of autophagy markers in pathology-burdened regions of the brain, while in vitro and in vivo experimental data point to defective autophagolysosomal clearance (Compagnoni et al., 2018; Schwarz et al., 2012; Tanji et al., 2013; Valera et al., 2017b). Further, experimental inhibition of the proteasomal machinery exacerbated neurodegeneration in a mouse model of MSA, while this was not the case in non-transgenic mice, and proteasomal abnormalities have been reported in MSA brains (Bukhatwa et al., 2010; Furukawa et al., 2002; Stefanova et al., 2012b).

Finally, neuroinflammation, loss of oligodendroglial neurotrophic support, and neuronal dysfunction due to α -synuclein inclusions may synergistically promote neuronal death and subsequent reactive astrogliosis (Fanciulli and Wenning, 2015).

Neuropathology

Macroscopic changes

The clinical differences between the two MSA phenotypes, MSA-P and MSA-C, reflect their underlying diverging anatomopathological features. While in MSA-P, symptoms point to predominant degeneration of the striatonigral system, the cerebellar phenotype of MSA-C indicates degeneration of olivopontocerebellar (OPC) structures. Both phenotypes have distinct neuropathological features, but with disease evolution, substantial overlap will develop. In MSA-P, atrophy of the caudate nucleus, putamen and globus pallidus (GP) with pallor of the SN in the midbrain are usually found. Conversely, in MSA-C there is atrophy of the pons, middle cerebellar peduncle, inferior olivary nuclei of the medulla and cerebellar cortex and white matter. Frontal cortex thinning is a late anatomical feature (Ahmed et al., 2012; Ozawa et al., 2004).

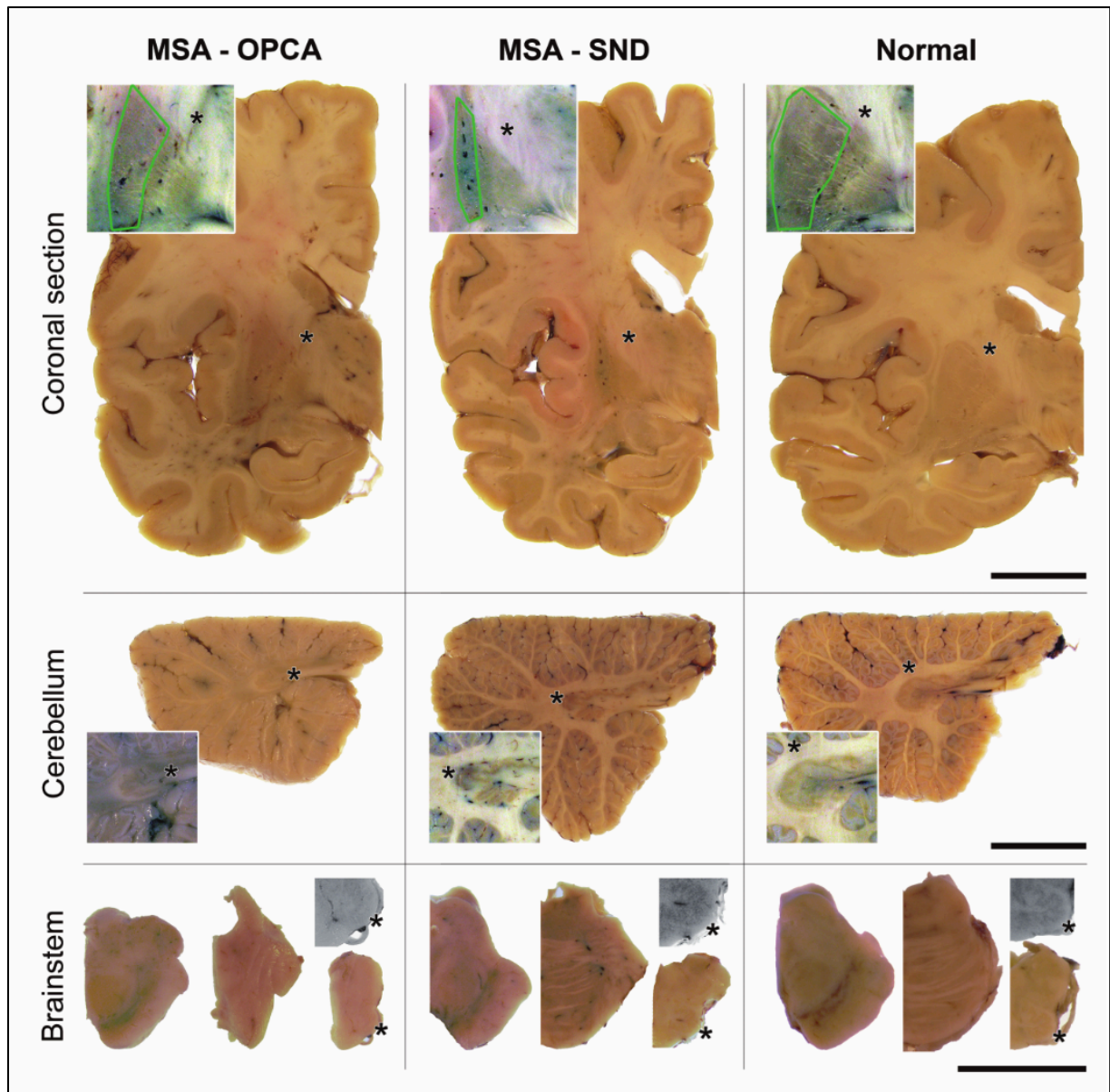


Figure 6. Main histological features of MSA phenotypes, from Ahmed et al. (2012). MSA-OPCA, multiple system atrophy-olivopontocerebellar atrophy; MSA-SND, multiple system atrophy-striatonigral degeneration.

Histopathology

Even though the pathological hallmark and requirement for post-mortem definitive diagnosis of MSA is the presence of GCIs (Trojanowski et al., 2007), other α -synuclein containing inclusions are found in brains of MSA patients. These are glial nuclear (GNI), neuronal cytoplasmic (NCI), and neuronal nuclear inclusions (NNI), as well as neuronal threads (Song

et al., 2009; Wenning and Jellinger, 2005). Neuronal α -synuclein pathology, considered ancillary, has been historically overlooked. However, growing evidence suggests that neuronal α -synuclein pathology is not simply an epiphenomenon in MSA but may play a role in the disease process, as more research reveals that accumulation of α -synuclein in neurons is more widespread than previously believed (Cykowski et al., 2015; Halliday, 2015; Homma et al., 2016; Jellinger, 2007; Rohan et al., 2015). Further, recent research has shown that α -synuclein oligomers in neurons are widely present throughout the brain, even earlier than in oligodendrocytes (Sekiya et al., 2019)

GCIIs contain full length, truncated and phosphorylated α -synuclein, though α -synuclein only accounts for a fraction of the proteinaceous content in these inclusions. Over 40 proteins have been described within GCIIs, these are normally involved in numerous cellular mechanisms, including, but not limited to, cell cycle regulation, signal transduction, metal binding, protein folding and degradation, autophagy components and regulators (Jellinger, 2018) (table 1).

Protein identified by routine immunohistochemistry or mass spectrometry (MS+)	Main function / Cellular process
α -Synuclein (MS+) (Syn 202, 205, 215 > SNL-4 > LB509 > Syn 208), (S129-P, S87-P)	Presynaptic vesicle release
β -Tubulin (MS+), α -Tubulin (MS+) ^b	Microtubule nucleation
HDAC6 (histone deacetylase 6) ^b	Tubulin degradation
20S proteasome subunits ^b	
p62/SQSTM1 (26 kDa protein/sequestosome 1) ^b	Autophagy
14-3-3 protein (in subset of GCIs)	Signal transduction
Elk1	Transcription factor
Bcl-2 (MS+)	Apoptosis
P39	CDK5-activator
Carbonic anhydrase isoenzyme IIa (MS+)	
cdk-5 (cyclin-dependent kinase 5) (MS+)	Cell cycle regulation
Midkine ^a	Neurotrophic factor
τ 2 (reversible on exposure to detergent)	Microtubule-associated
Isoform of 4-repeat tau protein (hypo-phosphorylated) (MS+)	Microtubule-associated
DARPP32	Regulation of signal transduction
Dorfin	Protein degradation
Heat shock proteins Hsc70, Hsp70b Hsp90b (MS+)	Protein folding
DJ-1	
LRRK2	
Rab5, Rabaptin-5	Endocytosis regulation
Parkin	
Mitogen-activated protein kinase (MAPK)	Signal transduction
NEDD-8 (MS+)	Protein degradation
Other microtubule-associated proteins (MAPs): MAP-1A and -1B; MAP-2 isoform 1, and isoform 4 (all MS+)	
Phosphoinositide 3-kinase (PI3K) (MS+)	
p25 α /TPPP (MS+) (tubulin polymerization-promoting protein)	
Septin-2, -3, -5, -6 and -9	
Synphilin-1	α S interaction protein (SNCAIP)
Transferrin ^a	
HtrA2/Omi	Apoptosis
Ubiquitin (MS+) SUMO-1 (small ubiquitin modifier 1)	Protein degradation
Leu-7 ^a	
p62-co-localization with α -Syn (inconsistent)	
AMBRA1	Autophagy regulation
NBR1 - autophagic adapter protein	Autophagy
Metallothionein-III (MT-III)	Metal binding
α - β -Crystallin	Protein folding
NUB-1 (negative regulator of ubiquitin-like protein 1)	Negative regulation of NEDD8
Parkin co-regulated gene (PACRG)	Regulation of cell death
Protein disulfide isomerase (PDI)	Protein folding
F-box only protein 7 (FBXO7)	Ubiquitination
XIAP (x-linked inhibitor of apoptosis protein)	regulation of apoptosis

Table 1. Proteins found within GCIs from Jellinger (2018)

GCI density is positively correlated with neural loss and disease duration and severity. GCIs are found in cerebral white and grey matter, they are present in motor cortex, putamen, GP, motor nuclei of cranial nerves, pons, cerebellar cortex, spinal cord and autonomic nerve structures. They also occur in the internal and external capsules, corpus callosum, corticospinal tracts and cerebellar white matter (Halliday et al., 2011; Inoue et al., 1997; Jellinger, 2018; Jellinger and Lantos, 2010; Ozawa et al., 2004; Wenning et al., 2008).

A number of neuroinflammatory triggers such as cytokines, reactive oxygen species (ROS) and nitric oxide are commonplace in MSA. In response to these, activated microglia and reactive astrocytes infiltrate the diseased tissue (gliosis), and their presence is believed to be an important contributor to the disease process in MSA. In general, the degree of region specific astrogliosis parallels the severity of neurodegeneration and correlates positively with α -synuclein pathology. In addition, microglial activation, also accompanying α -synuclein pathology and phagocytosing degenerating myelin, is prominent in degenerating regions (putamen, GP, SN, pons, and prefrontal cortex) (Gerhard et al., 2006; Ishizawa et al., 2008; Ishizawa et al., 2004; Kaufman et al., 2013; Kikuchi et al., 2002; Ozawa et al., 2004; Radford et al., 2015; Refolo and Stefanova, 2015; Refolo and Stefanova, 2019; Song et al., 2009).

Animal models of MSA

In order to investigate underlying mechanisms of pathogenesis and pathophysiology of MSA, animal models have been utilized for many years. These have helped further our understanding of the disease process, and have also been used to identify therapeutic targets and validate candidate drugs that might be further evaluated in clinical trials. Earliest models of MSA were produced through systemic or surgical intracerebral delivery of neurotoxins which induced robust neurodegeneration of the striatonigral system together with motor phenotypes, though lacking α -synuclein pathology. More recently, models expressing human α -synuclein in oligodendrocytes were developed through genetic manipulation, with three transgenic models using oligodendroglial promoters and one a Cre-loxP system to express inducible α -synuclein in mice (Bleasel et al., 2016; Fernagut and Tison, 2012; Kahle et al., 2002; Overk et al., 2018; Shults et al., 2005; Tanji et al., 2019; Yazawa et al., 2005). The mouse model expressing human α -synuclein through the proteolipid protein promoter (PLP-SYN) is commonly used and the one used for this PhD work. With the use of adeno-associated virus, expression of human α -synuclein has been achieved in brains of rats and primates (Bassil et al., 2017b; Mandel et al., 2017).

Beyond the use of mammals, recently reported, a fly model expressing wild-type human α -synuclein in glial cells recapitulates α -synuclein aggregation in glial cells, loss of dopaminergic neurons, impaired locomotion, autonomic dysfunction and transcriptional changes. The authors reported 30 transcripts with mammalian orthologs known to intervene in pathways relevant to synucleinopathy pathogenesis (including mitochondrial function, lysosomal function, myelin synthesis, cytoskeletal function, fatty acid metabolism, apoptosis,

and adenosine metabolism), opening the way to further understanding of these pathways and possible therapeutic approaches (Olsen and Feany, 2019)

Clinical presentation

Motor features

Parkinsonism, understood as a combination of bradykinesia (slowness of movement), rigidity and postural instability, characterizes the parkinsonian subtype of MSA. While in PD patients motor symptoms are levodopa sensitive, in MSA there is poor to no response to dopamine replacement therapy, and this lack of responsiveness to dopamine has been included in the consensus diagnostic criteria of MSA as a supportive feature (Fearnley and Lees, 1990; Gilman et al., 2008; Hughes et al., 1992).

Patients may present postural alterations, with involuntary muscular contractions, and deformities, such as bent spine, foot dystonia or disproportionate anterocollis. On the other hand, in MSA-C cerebellar signs predominate. Cerebellar features include a wide-based gait, uncoordinated limb movements, action tremor and different types of nystagmus (Köllensperger et al., 2010; Krismer and Wenning, 2017; Wenning et al., 2013b).

Non-motor features

Widespread degeneration of structures such as the brainstem, hypothalamus, the intermedio-lateral column of the dorsal spinal cord and the Onuf nucleus in the sacral spinal cord, which are implicated in autonomic functions, is responsible for the majority of the non-motor symptoms and signs of MSA (Benarroch, 2002; Benarroch, 2007; Benarroch et al., 2006; Dugger et al., 2012; Ozawa, 2007; Schmeichel et al., 2008; Yamamoto et al., 2005; Yoshida, 2007). Urogenital and cardio-vascular symptoms are the most common. Erectile dysfunction typically occurs at disease onset in male patients, while genital hyposensitivity during intercourse characterizes the sexual dysfunction in women (Köllensperger et al., 2008; Oertel et al., 2003). Urinary dysfunction includes urinary urgency and frequency, urge incontinence, nocturia, and incomplete bladder emptying. Moreover, MSA patients also suffer from orthostatic hypotension and frequently complain of gastrointestinal problems such as gastroparesis and constipation (Fanciulli and Wenning, 2015; Gilman et al., 2008; Köllensperger et al., 2007; Sakakibara et al., 2000).

Patients often suffer from depression, mood swings, anxiety, panic attacks and suicidal ideation, they also show cognitive impairment, most frequently executive dysfunction; in the course of the disease, while 10-15% of cases will develop dementia (Auzou et al., 2015; Kao

et al., 2009; Schrag et al., 2010). A fundamental aspect in MSA is respiratory dysfunction, given that it indicates the extent of neurodegeneration in the ponto-medullary respiratory system, with inspiratory stridor and sleep apnoea being frequent signs, frequently provoking sleep disturbances (Benarroch, 2003; Benarroch, 2007). Additionally, patients report other sleep-related problems, such as insomnia, sleep fragmentation, excessive daytime sleepiness and nocturnal agitation during rapid eye movement sleep (Ghorayeb et al., 2014; Iranzo et al., 2005; Palma et al., 2015).

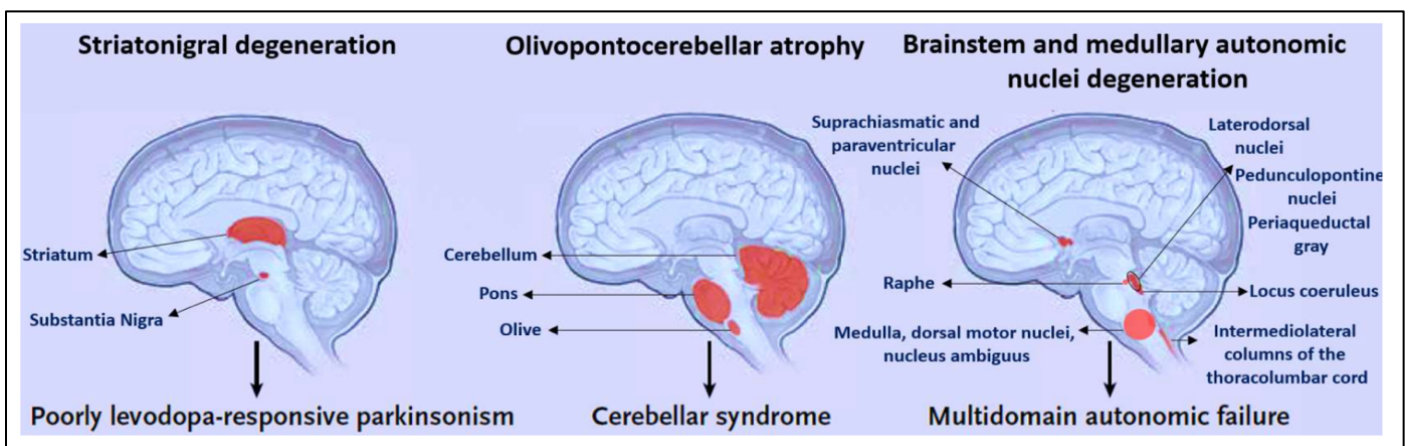


Figure 7. Summary of brain regions affected in the two MSA phenotypes and their clinical correlates. Modified from Fanciulli and Wenning (2015)

Clinical heterogeneity

Even though current consensus diagnostic criteria include two classical phenotypes, MSA-P and MSA-C, clinical heterogeneity has been repeatedly described in the literature, ranging from an asymmetrical, slowly progressive levodopa-responsive phenotype that may last up to two decades (Jellinger, 2012; Masui et al., 2012; Petrovic et al., 2012) to the very aggressive so-called ‘minimal change’ variant, which might lead to death within 5 years after having reached most of the clinical milestones by year 3 (Ling et al., 2015). Patients presenting the levodopa responsive variant might be misdiagnosed as PD, and, as such, may be subject to advanced invasive treatments, like deep brain stimulation. Unfortunately, the outcome of this intervention is very poor in these cases, underscoring the importance of recognizing this phenotype and a correct early diagnosis of MSA (Lezcano et al., 2004; Meissner et al., 2016; Thavanesan et al., 2014; Ullman et al., 2012).

Diagnosis

Diagnosis of MSA relies principally on clinical findings, but might be reinforced by ancillary tests. The latest revision on consensus diagnostic criteria was published in 2008. The only way to achieve a definite diagnosis is through post mortem analysis of the brain; before death the diagnostic criteria allow for two levels of certainty, possible and probable MSA. Probable MSA is defined as a sporadic, progressive disorder in adults, clinically characterized by severe autonomic failure (urinary incontinence or severe orthostatic hypotension) and poorly levodopa-responsive parkinsonism or cerebellar ataxia. Diagnosis of possible MSA requires a sporadic, progressive adult-onset disorder with parkinsonism or cerebellar ataxia to be accompanied by at least one feature suggesting autonomic dysfunction plus one additional clinical or neuroimaging abnormality (figure 8) (Gilman et al., 2008).

Even though these consensus criteria yield a high specificity, sensibility is still poor, and accurate diagnosis in early stages remains challenging (Koga et al., 2015; Miki et al., 2019; Osaki et al., 2009).

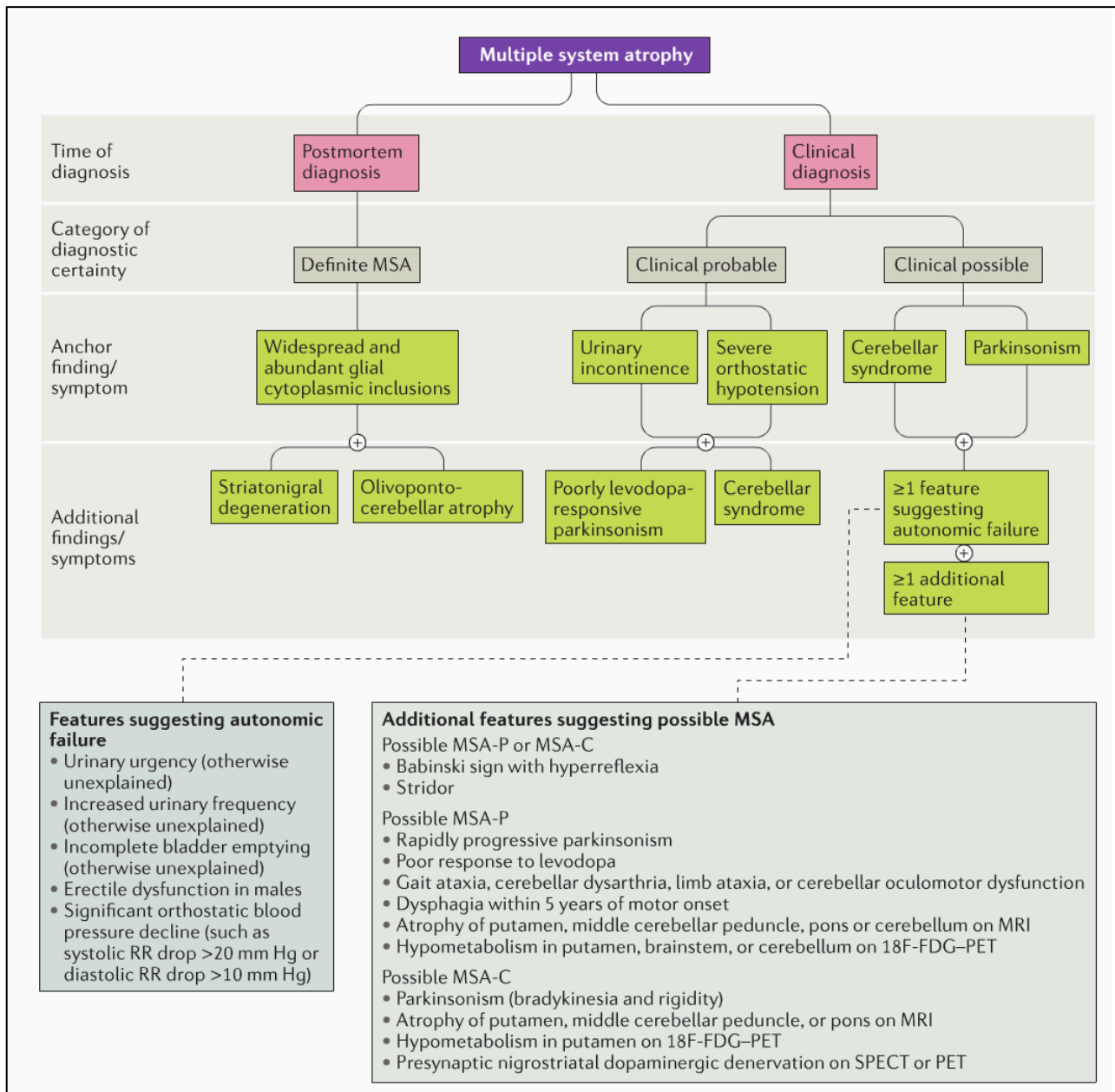


Figure 8. Summary of consensus diagnostic criteria for MSA. Magnetic resonance imaging, MRI; 18-Fludeoxyglucose positron emission tomography, 18F-FDG-PET. From Krismer and Wenning (2017)

Natural history and prognosis

MSA progresses relentlessly, and patients become soon disabled, with frequent falls, needing walking aids, in average, 3-5 years after onset; significant autonomic dysfunction, speech and swallowing difficulties. Death arrives 6 to 10 years after disease onset, and it is usually provoked by aspiration pneumonia, sepsis and sudden death (Ben-Shlomo et al., 1997; Lee and Koh, 2012; Low et al., 2015; O'Sullivan et al., 2008; Wenning et al., 2013a).

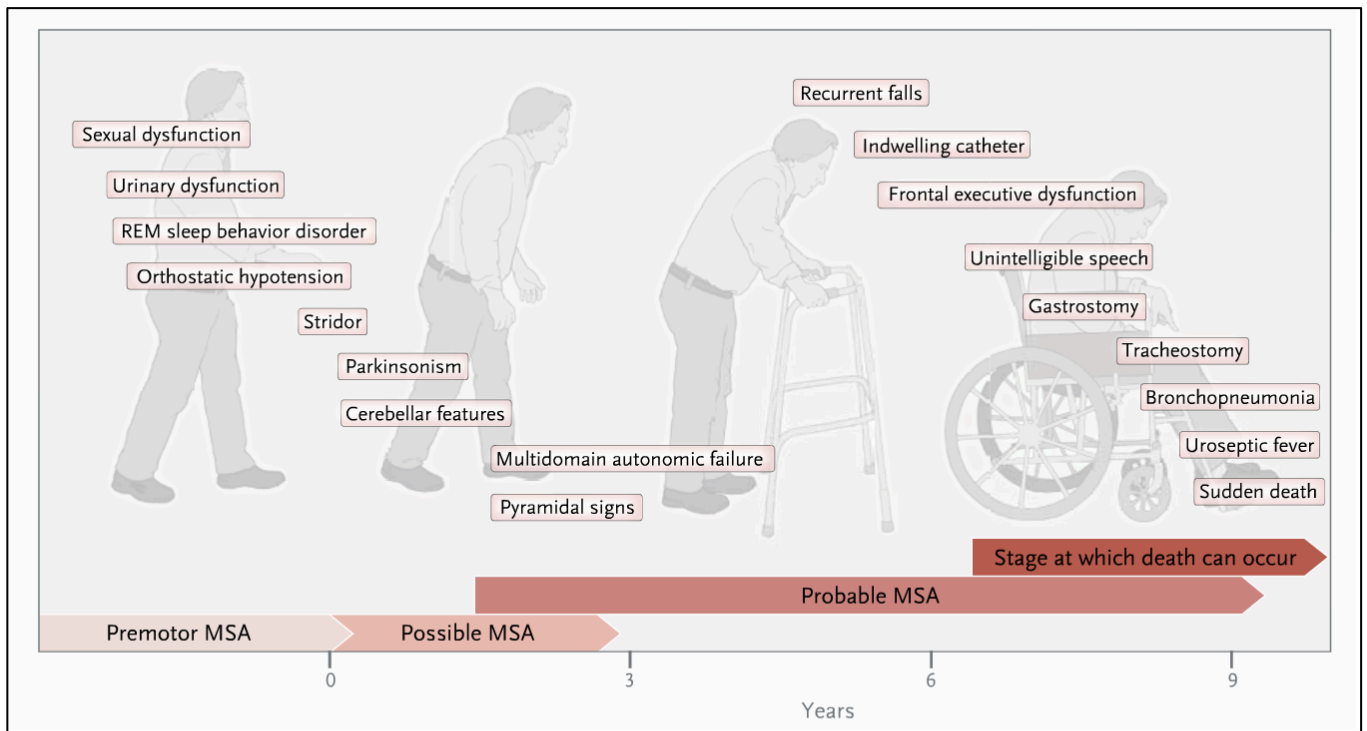


Figure 9. Summary of MSA natural history from Fanciulli and Wenning (2015)

Treatment

Since, regrettably, there is no curative therapy for MSA, current strategies are aimed at alleviating symptoms.

Symptomatic therapy

Management of MSA's most disabling features can prove challenging for clinicians. For the parkinsonian symptoms, levodopa is the first-line treatment, up to 83% of patients might show dopa responsiveness to some extent in early stages, although this effect is typically transient. First-line therapy for ataxia is physiotherapy. Orthostatic hypotension can, sometimes, be the most disabling feature, and its management consists of pharmacological and non-pharmacological measures. Neurogenic bladder might be relieved through intermittent catheterization, anti-cholinergics and botulinum toxin. Erectile dysfunction may respond to intracavernosal papaverine injections, and sildenafil might offer relief and is a less daunting alternative for patients. The best option for focal dystonia and some postural deformities is botulinum toxin injections. A summary of current expert suggestions for symptomatic management can be found in table 2 (Perez-Lloret et al., 2015).

Feature	Current First-Line Treatment	Alternative Treatments
Parkinsonism	Levodopa up to 1 g/day	DAs, amantadine (positive trend in small trial)*, paroxetine (positive trend in small trial)*, physiotherapy
Ataxia	Physiotherapy	Clonazepam, vitamin E, propranolol, baclofen, or amantadine, buspirone, gabapentin
OH	Nonpharmacological measures (custom-fitted elastic stockings, raising the head of the bed when sleeping, water drinking, small meals). Midodrine*, droxidopa*	Fludrocortisone, pyridostigmine*, desmopressin at bedtime
Neurogenic lower urinary tract dysfunction	Postvoid residual volume \geq 100 mL: clean intermittent self-catheterization. Postvoid residual volume <100 mL: anticholinergic agents for detrusor hyperactivity, (alpha-adrenergic blockers for detrusor sphincter) dyssynergia.	BoNT-A in the detrusor muscle or urethral sphincter, surgery (sphincterotomy or sphincteric wall stenting), permanent catheterization
Constipation	High fluid and fiber intake, classical laxative therapy, polycarbophil 70, macrogol	Lubiprostone
Erectile dysfunction	Intracavernosal injection of papaverine or prostaglandin E1	Sildenafil (not recommended in patients with OH)*, SC apomorphine injections
Drooling	Anticholinergic agents, injection of BoNT-A into the salivary glands	
Breathing disorders	Continuous positive air pressure	Tracheotomy (life-threatening and/or daytime stridor, abnormal vocal cord, mobility on laryngoscopy), laryngeal surgery or BoNT therapy, adaptative servoventilation
Dystonia	BoNT injection (focal dystonia)	Anticholinergics, amantadine, DAs, muscle relaxants, or tetrabenazine
Camptocormia	Physiotherapy in combination with specific orthosis, wearing a backpack	BoNT injections, proterelin
RBD	Clonazepam, melatonin	
RLS	Pregabalin, pramipexole, ropinirole, and rotigotine	Gabapentin, enacarbil, pramipexole, ropinirole, L-dopa
Depression	Psychotherapy, SSRIs	Electroconvulsive therapy, repetitive TMS

Table 2. Summary of symptomatic management in MSA patients from Perez-Lloret et al. (2015). OH, orthostatic hypotension; BoNT-A, botulinum toxin A; SC, subcutaneous; RBD, REM sleep behaviour disorder; RLS, restless legs syndrome; SSRIs, selective serotonin reuptake inhibitors; DA, dopamine agonists; TMS, transcranial magnetic stimulation.

Disease-modifying therapy

All but one randomized controlled trials (RCT) aiming at neuroprotection have failed (Bensimon et al., 2009; Dodel et al., 2010; Krismer and Wenning, 2017; Lee et al., 2012; Low et al., 2014; Poewe et al., 2015). The one study that reported positive effects for intra-venous and intra-arterial injections of mesenchymal stem cells raised safety concerns (Lee et al., 2012). An open label study of intra-thecal injections reported a good security profile, and this approach is being challenged in a phase III trial (Singer et al., 2017; Singer et al., 2019),

Therapy	Patients	Presumed mechanism of action	Phase	Design	Primary Endpoint (Secondary Endpoint)	Outcome
Growth hormone	43	Pro-survival effects	II	RCT	Total UPDRS score and autonomic tests at 6 and 12 months (total UMSARS score at 6 and 12 months)	Ineffective, high dropout rate
Minocycline	63	Inhibition of microglial activation	III	RCT	Change in UMSARS II score at 48 weeks (change in UMSARS I, III, UPDRS III, SF-12 and EQ-5D at 48 weeks; [11C]-PK11195-PET was assessed in small ancillary study, n=8)	Ineffective (reduced microglial activation on PET in ancillary study)
Riluzole	404	Anti-excitotoxic activity, free-radical scavenging	III	RCT	Survival (rate of decline in motor function)	Ineffective
MSC delivery (intra-arterial and intravenous)	33	Neurotrophic effects, neural cell differentiation	II	RCT	Change in total UMSARS score at 12 months (change in UMSARS II score and imaging outcomes at 12 months)	Smaller increase in total UMSARS score (smaller increase in UMSARS II score and positive effects on imaging outcomes), safety concerns (ischemic MRI brain lesions in one third)
Rifampicin	100	Inhibition of formation of α -synuclein fibrils	III	RCT	Change in UMSARS I score (slope) at 12 months (change in UMSARS I, II and total, COMPASS-select)	Study prematurely terminated, ineffective
Rasagiline	174	MAO-B inhibitor	II	RCT	Change in total UMSARS score at 48 weeks (change in GCI-I, COMPASS-select and MSA-QoL at 48 weeks, change in total UMSARS II at 24 weeks; Putaminal diffusivity was assessed in ancillary imaging study)	Ineffective
Epigallocatechin gallate (EGCG)	92	Inhibition of α -synuclein aggregation	III	RCT	Change in UMSARS II score (exploratory assessment of MRI parameters)	Ineffective
BHV-3241	58	Myeloperoxidase inhibition	II	RCT	Safety and tolerability, striatal change in [11C]-PBR28 binding as indicator of microglial activation	Safe and well tolerated, no change in [11C]PBR28 binding
Fluoxetine	81	Serotonin reuptake inhibition, increased neurotropic support	II	RCT	Change in total UMSARS at 3 month (change in SCOPA-Aut, BDI, MSA-QoL, SF-36, change in total UMSARS at 6 month)	Ineffective (preliminary results only reported for primary outcome)
Intrathecal MSC delivery	24	Neurotrophic mediation, neural cell differentiation	I/II	OL	Frequency and type of adverse events (change in total UMSARS, indices of autonomic failure)	Safe and well tolerated, except for low back/leg pain, associated with thickening of lumbar nerve roots in highest-dose group, slower rate of UMSARS progression compared to historical control group
α -synuclein targeted active immunization	30	Induction of immune response against toxic α -synuclein forms	I	Patient + examiner blind	Safety and tolerability, immunogenicity against α -synuclein	Safe and well tolerated

Table 3. Completed clinical trials in MSA modified from Lopez-Cuina et al. (2018b). BDI, Beck Depression Inventory; COMPASS, Composite Autonomic Symptom Score; MRI, Magnetic Resonance Imaging; MSA-QoL, Multiple System Atrophy Health-related Quality of Life; MSC, Mesenchymal Stem Cells; OL, Open label; PET, Positron Emission Tomography; PD, Parkinson Disease; RCT, Randomized Placebo-Controlled Trial; SCOPA-Aut, Scales for Outcomes in Parkinson's disease-Autonomic; SF-12/36, Short-Form Survey 12/36; UPDRS, Unified Parkinson's Disease Rating Scale; UMSARS, Unified Multiple System Atrophy Rating Scale.

Significant research on preclinical models of MSA is underway and recent studies show some molecules with promising neuroprotective potential that warrant further investigation, and might be assessed in future RCT. Classically, the main target of these therapies is α -synuclein, either aggregation inhibition or immunization, other drugs aim to improve cell survival via α -synuclein independent pathways (see Table 4 on page 109 for a summary of the latest research in this regard and suggests approaches deemed interesting for assessment in MSA mice).

We recently published a review describing the latest knowledge obtained from RCT on disease modifying therapies. This will serve as a closing piece to the introduction of this PhD work.

Present and future of disease-modifying therapies in multiple system atrophy (Review).



Review

Present and future of disease-modifying therapies in multiple system atrophy

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ABSTRACT

Through the last decade seven clinical trials on Multiple System Atrophy have been published, virtually all of them reported negative results. Patients and family remain hopeful while facing this devastating disease, but as doctors we still cannot offer them disease-modifying therapies. The field has seen many advances regarding pathophysiology, translational research, diagnostic accuracy, natural history and imaging, but successful treatment remains elusive. This review provides an overview of the available tools for designing clinical trials, critically analyzes the past studies and describes the knowledge obtained from them, and finally gives some orientation for future trials that could meet the current needs of patients and clinicians, overcoming the hurdles met by previous studies.

1. Introduction

Multiple System Atrophy (MSA) is a rapidly progressing and fatal neurodegenerative disease characterized by a variable combination of parkinsonism, ataxia and autonomic failure (Fanciulli and Wenning, 2015; Gilman et al., 2008). The pathological hallmark is the presence of α -synuclein bearing glial cytoplasmic inclusions (GCI) (Jellinger, 2012; Papp and Lantos, 1994).

MSA is an orphan disease with an estimated prevalence ranging from 1.9–4.9/100,000 (Krismer and Wenning, 2017). Treatment is available for some symptoms, in particular autonomic dysfunction, while disease modification remains an urgent unmet need.

This review focuses on the seven randomized clinical trials (RCT) that have been completed in the last decade, six of which reported negative results. We begin by outlining the current tools available for designing a robust clinical trial fitting the characteristics of a rare disease; later we critically analyze the above-mentioned trials, inferring the possible causes of their failures to show significant results, while appraising the knowledge that can be garnered from them. Finally, we give some orientation for conceiving future RCT that could meet the current needs of patients and clinicians, overcoming the hurdles met by previous studies.

2. Diagnosis and outcomes for clinical trials in MSA

2.1. Diagnostic criteria

A first consensus statement was published in 1999 (Gilman et al., 1999), which was the standard in clinical setup and in research until the revised consensus criteria were released in 2008. The most relevant changes being related to the criteria for possible MSA with the addition of the results of paraclinical investigations, such as atrophy of the putamen, middle cerebellar peduncle (MCP), pons, or cerebellum on magnetic resonance imaging (MRI); hypometabolism on 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) and pre-synaptic nigrostriatal denervation on single-photon emission computed tomography (SPECT) or PET (Gilman et al., 2008).

These criteria allow three levels of certainty in diagnosing MSA, possible, probable and definite. While for the diagnosis of probable MSA unequivocal signs of autonomic failure are required, the diagnosis of possible MSA allows for the use of the above-mentioned paraclinical investigations. The diagnosis of definite MSA requires post-mortem confirmation.

A study compared the performance of both consensus in a neuropathologically confirmed cohort of 59 patients with MSA (Osaki et al., 2009). In later stages of the disease, the old and new consensus for possible and probable MSA performed similarly, with sensitivities

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above 90%. However, differences became more apparent in earlier stages, where the new consensus criteria for possible MSA achieved a better sensitivity, albeit still remaining low (41% compared to 28%). In contrast, both consensus showed good positive predictive values in both early and late visits (ranging from 86 to 100). This illustrates that consensus diagnosis criteria are reliable but also underscores the challenge in recruiting patients in the early phase, when clinical signs remain non-specific and interventions are believed to be more likely to modify the disease course, given that once the diagnosis becomes more obvious (often meeting criteria for probable MSA) the neurodegenerative process is in a relatively advanced stage (Gilman et al., 1999; Gilman et al., 2008). A further revision of current diagnostic criteria is warranted to improve the above-mentioned low sensibility for early-stage disease, and also to account for clinical symptoms that are increasingly being recognized. In this regard, cognitive impairment is frequent but currently considered as non-supportive feature according to consensus diagnosis criteria for MSA (Stankovic et al., 2014).

2.2. Clinical rating scales

The most frequently used outcome measure in MSA clinical trials is the Unified MSA Rating Scale (UMSARS), which has been thoroughly validated, is disease-specific, and its progression rates have been established in large natural history studies (Low et al., 2015; Wenning et al., 2013). By analyzing data from the MSA rasagiline trial, a minimally clinically meaningful decline in MSA-P was estimated to be 1.5 points on the UMSARS ADL subscale, 1.5 points on the UMSARS motor examination subscale, and 3.5 points on the UMSARS total scale, although the trial's failure to show improvement in outcome measures did not allow establishing a minimal relevant improvement, which would prove more useful at determining accurate sampling size (Krismer et al., 2016).

A prospective assessment of autonomic symptoms performed in MSA patients through the Scales for Outcomes in Parkinson's Disease–Autonomic questionnaire (SCOPA-Aut) questionnaire reported slow progression of total and subdomain scores over time, thus, not being a useful endpoint for disease-modification or neuroprotection trials (Damon-Perrière et al., 2012).

MSA health-related quality of life (Hr-QoL) has been appraised by the MSA-QoL questionnaire, the Short Form 36 Health Survey Questionnaire, the EuroQoL instrument and the Parkinson's Disease Questionnaire-39 (Köllensperger et al., 2007; May et al., 2007; Miyashita et al., 2011; Schrag et al., 2006; Schrag et al., 2007; Winter et al., 2011).

Prospective studies of Hr-QoL have estimated sample sizes larger than those required by UMSARS sum scores, thus making the latter the most sensitive to change over time (Geser et al., 2006; May et al., 2007; Meissner et al., 2012). Regarding Hr-QoL in MSA, some items did not change over time probably due to the scores at baseline being already too high (e.g. difficulty with handwriting), or because some symptoms might appear in later stages of the disease (e.g. difficulty with swallowing). Given the fact that Hr-QoL measures are more and more considered as very relevant outcomes, it could prove worthwhile to revise or develop specific tools that are sensitive to change for clinical trials in MSA.

A recent study prospectively compared several clinical assessment tools (i.e. MSA-QoL questionnaire, UMSARS, Scale for the Assessment and Rating of Ataxia, Berg Balance Scale and SCOPA-Aut) during 12 months, reporting that UMSARS was the most responsive to change over time. Putting together the best responding items from all scales, they suggested a brief, 8-item scale which would require a sample size of 98 patients per group in order to detect a 30% effect with a statistical power of 90%. A limitation to the interpretation and generalizability of the results from this study is the low representation of MSA-P, given that it is less common in Japan (Matsushima et al., 2016).

It is also worth mentioning that clinical milestones are well known

markers of disease progression in MSA; namely frequent falling, urinary catheterization, wheelchair dependency, dysphagia, and cognitive disability (Lee and Koh, 2012; O'Sullivan et al., 2008; Wenning et al., 2013). These could prove useful in assessing the efficacy of therapies to delay progression.

2.3. Biomarkers

A biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention (Biomarkers Definitions Working Group, 2001). Biomarkers, especially neuroimaging, have clear potential to serve as objective endpoints for future RCT in MSA.

2.3.1. Neuroimaging

There have been interesting advances in the last decade in this field; nonetheless, it should be noted that studies have been largely performed in small cohorts, and technologies and techniques utilized vary, making it difficult to compare results and draw firm conclusions from them. Accordingly, there is a need for a joint, multicenter force in order to recruit a sufficient number of patients and follow them for, at least, one year. This would provide invaluable insights into the variability and natural history of image biomarkers in this rare disease. Beyond disease progression, imaging biomarkers for the diagnosis of MSA may be useful for patient stratification in clinical trials.

2.3.1.1. MRI. There has been an increasing interest in the use of MRI to improve the diagnostic accuracy of MSA (Brooks et al., 2009). A recent review illustrates the contribution of high field MRI and advanced MRI modalities, and their role in the diagnosis of MSA (Kim et al., 2017).

It is equally relevant to develop MRI biomarkers of disease progression to understand the underlying pathophysiology and improve patient management, as well as to monitor changes over time in RCT. Although none of the currently available MRI studies has provided a useful imaging biomarker for disease progression, numerous cross-sectional studies have shown a relation between disease severity and duration and MRI findings (Hara et al., 2016; Minnerop et al., 2007; Pellicchia et al., 2009; Tha et al., 2010; Watanabe et al., 2002). For a better understanding of the natural history of MSA, large longitudinal follow-up studies need to be conducted to provide more robust information on the correlation between MRI changes and disease progression.

Earlier studies reported a mean annual cerebral brain atrophy rate of 2.5% (3% for MSA-C and 1.9% for MSA-P) and 1.7% (Horimoto et al., 2000; Konagaya et al., 2002). Later, a whole-brain atrophy rate of 1% was reported, while regional measurements yielded annual atrophy rates of 4.5% and 3.2% in pons and cerebellum, with a correlation between infratentorial atrophy and Unified Parkinson's Disease Rating Scale (UPDRS) motor scores (MSA-P, $n = 11$) (Paviour et al., 2006). A more recent study reported a whole-brain annual atrophy rate of 1.65%, but without correlation with UPDRS motor scores (MSA-P, $n = 8$), and in a later retrospective study in 35 MSA-P and 6 MSA-C patients, this same group reported a similar annual whole-brain atrophy rate of 1.65% (Guevara et al., 2016; Guevara et al., 2017).

Utilizing voxel-based morphometry (VBM), no correlation was found between UPDRS and atrophy rates in any brain region including the striatum, mesencephalon, cerebellum, and cortex (MSA-P, $n = 14$) (Brenneis et al., 2007).

Another study reported increased atrophy rates of the cerebellum and putamen in MSA-C patients compared to healthy controls (14 MSA-C, 6 controls) (Hauser et al., 2006). Progression of atrophy has also been reported in corpus callosum and MCP (32 MSA-C, 16 MSA-P) (Minnerop et al., 2007). More recently, a retrospective study described the relation between the annual cerebellar volume atrophy rate and the increase in International Cooperative Ataxia Rating Scale (ICARS)

scores in MSA-C. This study reported a mean annual loss of cerebellar volume of 7.8 ml ($n = 17$) (Tanaka et al., 2016).

A study compared rates of atrophy in four subcortical regions and reported a greater rate of atrophy in the putamen and caudate in MSA-P than in MSA-C. Moreover, by quantifying iron deposition with R2*, they showed progression in putaminal iron deposition in MSA-P, but not in MSA-C (8 MSA-P, 9 MSA-C) (Lee et al., 2015).

Diffusion-weighted imaging (DWI) has been used to detect and quantify early neurodegenerative changes in patients with MSA (Barbagallo et al., 2016; Ito et al., 2007; Nicoletti et al., 2006; Nicoletti et al., 2013; Paviour et al., 2007b; Schocke et al., 2002; Seppi et al., 2006a; Seppi et al., 2003). One longitudinal study reported an increase in diffusivity in the putamen, which correlated with UPDRS motor scores in 10 MSA-P cases (the large time range between scans being its main caveat, while the mean was 1.2 years [range 5–22 months]) (Seppi et al., 2006b). In contrast, another study reported progression in diffusivity in putamen, frontal white matter, pons and cerebellum, albeit without correlation to UPDRS, perhaps due to analyzing a mixed cohort of MSA cases (MSA-C $n = 4$, MSA-P $n = 7$, mean interval between MRI scans 0.7 years [range 9–13 months]) (Pellecchia et al., 2011). A third study of 12 MSA-P cases with a longer follow-up described a positive correlation between the increase in diffusivity in the MCP and UPDRS motor scores (mean interval between scans 2.25 years) (Reginold et al., 2014).

Two recent RCT on MSA have used MRI as outcome measure. The MSA rasagiline trial reported rates of progression of diffusivity in the putamen, caudate and MCP that were slower than those described in the above mentioned studies (MSA-P $n = 40$, time between scans 0.83 years) (Poewe et al., 2015). The trial with mesenchymal stem cells (MSC) assessed prospectively gray matter density for the first time (Lee et al., 2012).

2.3.1.2. Functional imaging. Most studies in MSA performing functional neuroimaging are cross-sectional. One longitudinal single ROI-driven β -CIT SPECT study included five MSA-P cases followed for 1.8 years. This study reported a mean yearly reduction in striatal β -CIT binding of 9.6%, which proved to be faster than PD (this study did not use the first consensus statement criteria for the diagnosis of MSA) (Pirker et al., 2002). A second study assessed progression in 11 MSA-P patients using β -CIT SPECT with a voxel-based analysis approach. It reported a faster decline in dopamine transporter uptake in anterior putamen and caudate in 11 MSA-P compared to PD, consistent with a faster disease progression in MSA-P, but did not find a correlation with UPDRS motor scores (Nocker et al., 2012).

In the MEMSA trial, a subgroup of MSA patients underwent [11C] (R)-PK11195-PET, a marker of activated microglia. In contrast to the placebo group, two of the three assessed patients who received minocycline showed decreased binding at follow-up. However, this observation did not translate into a better clinical outcome (Dodel et al., 2010; Gerhard et al., 2006).

In the clinical trial assessing the efficacy of MSC, patients underwent FDG-PET scans at baseline and after 12 months of follow-up. A reduction in cerebral glucose metabolism in cerebellum and several cerebral regions was observed, and in the treatment group, it was found to have decreased less in the cerebellum (Lee and Koh, 2012).

2.3.2. Fluid biomarkers

Despite recent research efforts, reliable fluid biomarkers for a sensitive and specific diagnosis of MSA are currently lacking. A combination of several cerebrospinal fluid (CSF) biomarkers, for instance neurofilament light chain (NFL), metabolites of the catecholamine pathway and proteins such as α -synuclein, DJ-1 and t-tau may prove useful in the future for the diagnosis of MSA (Laurens et al., 2015).

Longitudinal studies of fluid biomarkers would provide valuable insight not only into the pathophysiology of the disease, but also help establishing objective outcomes for disease modification trials. In this

regard, one study did not find changes in cerebrospinal NFL and neurofilament heavy chain (NFH) levels over a 12 month period (Petzold et al., 2009); a later study assessed NFL and glial acidic protein (GFAP) levels in MSA and other atypical parkinsonisms, and reported no significant change over a mean period of 11 months for MSA (Constantinescu et al., 2010).

2.4. Collaborating networks

Recruitment of sufficient numbers of patients to perform well-powered RCT is hampered by the low prevalence and its geographical spread. Thus, no single study center is able to recruit enough patients to perform large clinical trials. A coordinated effort at an international level is key in advancing MSA research.

The European MSA Study Group published reports of the natural history of the disease (Geser et al., 2006; Wenning et al., 2013), as did the North American MSA Study Group (NAMSA) (Low et al., 2015). The Asian counterparts are The Japan Multiple System Atrophy Research Consortium (JAMSAC) and the Chinese MSA Study Group (CNMSA-SG). Lastly, the International Movement Disorders Society-sponsored MSA Study Group seeks to develop a common data-set, establish a global patient registry, a decentralized biomaterial bank, and recommendations for interventional trials, among others. It also enables joining forces between the different regional study groups.

2.5. Power analysis for clinical trial design

Sample size estimations have been reported for various clinical and neuroimaging outcomes (Guevara et al., 2016; Meissner et al., 2012; Nocker et al., 2012; Paviour et al., 2007a; Singer and Low, 2015; Wenning et al., 2013). Table 1 is adapted from (Fernagut et al., 2014) and summarizes these figures. The results of preliminary brain imaging studies suggest smaller numbers to demonstrate significant effects compared to clinical ratings scales. This remains to be tested in future RCT.

3. Completed disease-modifying trials in MSA

With the exception of one trial randomizing patients to intra-arterial and intravenous delivery of MSC, all published clinical trials in MSA have failed to show disease modification (Table 2). This small trial of 33 patients reported a statistically significant slowing in the UMSARS progression rate, as well as a less extensive decline in glucose metabolism and gray matter density after 1 year. The main limitations of this study were the single-center design and safety issues related to the intra-arterial procedure, namely subclinical acute ischemic cerebral DWI lesions that were found in 30% of patients (more frequently in the placebo group). Moreover, the included patient population was very homogeneous compared to other studies increasing the likelihood to demonstrate significant differences between treatment groups despite the small number of recruited patients. The exact mechanism of action remains uncertain, although it is hypothesized that stem cells offer neurotrophic support and modulate neuroinflammation (Lee et al., 2012). Secondary endpoints included differences in FDG-PET uptake between groups and gray-matter density, their decline proved to be slower in the MSC group. Despite identifying a treatment effect on the UMSARS total and motor scores, no correlation was found between the decline in glucose metabolism and clinical progression. PET scans were performed in 27/33 patients, suggesting that including functional imaging as endpoint is feasible. Additional longitudinal FDG-PET studies should provide data to better understand how to use this technology in RCT. A yet unpublished open-label phase I/II study reported that intrathecal administration of MSC was well tolerated at low and medium dose, and an apparent dose-dependent slowing of disease progression (Singer et al., 2017).

Growth hormone (GH) is known to be involved in neural

Table 1
Sample size estimates.

Outcome	Number of patients that served for the calculation	Mean disease duration at inclusion (years)	Improvement of outcome under active treatment						Reference	
			20%		30%		50%			
			Power							
			80%	90%	80%	90%	80%	90%		
Survival										
1 year follow-up	398	4.4	1761	2357	727	972	219	293	Bensimon et al. (2009)	
2-year follow-up			808	1082	342	457	109	146		
3-year follow-up			470	629	204	273	69	92		
UMSARS I (ADL)										
1 year follow-up	67	4.6	–	–	–	–	162	216	May et al. (2007)	
1 year follow-up	100		395	520	190	250	47	50	Singer and Low (2015)	
1 year follow-up	87	5.5	556	745	248	331	89	120	Wenning et al. (2013)	
2-year follow-up	49	5.5	206	275	92	123	33	44	Wenning et al. (2013)	
UMSARS II (motor examination)										
1 year follow-up	57	4.6	455	609	203	271	73	98	May et al. (2007)	
1 year follow-up	87	5.5	329	441	147	196	53	74	Wenning et al. (2013)	
2-year follow-up	49	5.5	212	284	95	126	34	46	Wenning et al. (2013)	
UMSARS I + II (total)										
1 year follow-up	57	4.6	–	–	–	–	83	111	May et al. (2007)	
1 year follow-up	100	4.8	655	877	292	390	105	141	Meissner et al. (2012)	
1 year follow-up	85	5.5	389	520	173	231	63	84	Wenning et al. (2013)	
2-year follow-up	49	5.5	175	234	78	104	28	38	Wenning et al. (2013)	
MSA-QoL motor subscores										
1 year follow-up	100	4.8	2003	2681	890	1192	321	429	Meissner et al. (2012)	
Putaminal trace D imaging										
1 year follow-up	11	3.5	1111	1486	494	661	179	239	Pellecchia et al. (2011)	
Relative DAT decline										
1 year follow-up (caudate)	11	2.4	219	–	79	–	35	–	Nocker et al. (2012)	
1 year follow-up (putamen)			414	–	151	–	67	–		
1 year follow-up (striatum)			211	–	75	–	34	–		
Brain atrophy										
1 year follow-up (pons)	9	5.4	222	295	99	131	37	49	Paviour et al. (2007a)	
1 year follow-up (cerebellum)			176	232	79	103	29	39		
1 year follow-up (whole brain)	8	3.8	–	234	–	–	–	38	Guevara et al. (2016)	

Required group sizes below 100 are highlighted in bold.

development acting through insulin/insulin like growth factor 1 (IGF-1) and has several neurotrophic and pro-survival actions (Ajo et al., 2003). A randomized, double-blind, placebo-controlled trial that enrolled an arbitrary number of patients - deemed to be low - due to the difficulties of reliably estimating sample sizes at the time this RCT was conducted. It showed a mean difference of 6.3 points at 12 months in UMSARS scores that failed to reach statistical significance, very likely as a result of being underpowered and the very high discontinuation rate of 37% (45.5% in the GH and 28.6% in the placebo group). The high discontinuation rate in this trial points further to possible challenges when using the Last Observation Carried Forward (LOCF) strategy to impute for missing values. Retrospectively, a sample size of 90 patients per group was calculated to be necessary. Accordingly, the conduction of a better-powered trial is achievable and may provide positive results in the future; and since the current dose proved to be well tolerated and

showed a trend for improving the outcome, perhaps it would suffice to maintain it, instead of increasing the dose as has been suggested (Holmberg et al., 2007).

Microglia and inflammation play a pivotal role in neurodegeneration (Perry and Holmes, 2014). In this regard, minocycline's potential as disease-modifying therapy had faced conflicting evidence in preclinical studies (Bonelli et al., 2004; Diguët et al., 2004a; Diguët et al., 2004b; Stefanova et al., 2004; Stefanova et al., 2007; Wu et al., 2002). As mentioned before, the MEMSA trial randomized 63 patients to either minocycline or placebo and a subgroup of 8 patients underwent [¹¹C] (R)-PK11195-PET to assess the effects of the drug on microglial activation. The study lost 22% of patients and failed to show a significant effect on clinical motor outcomes (UMSARS and UPDRS motor scores). Patients receiving minocycline showed decreased microglial activation, but the subgroup was too small to draw meaningful conclusions and

Table 2
Completed clinical trials.

Therapy	Number of patients	Presumed mechanism of action	Preclinical data	Phase	Primary endpoint (Secondary Endpoint)	Outcome (Reference)
Growth hormone	43	Pro-survival effects	n. a	II	UPDRS total, autonomic tests (UMSARS)	Ineffective, high dropout rate (Holmberg et al., 2007)
Minocycline	63	Inhibition of microglial activation	No effect on motor behavior and survival of dopamine neurons in double lesion rat model (Stefanova et al., 2004)	III	Change of UMSARS motor score	No change on clinical measures, reduced microglial activation on neuroimaging in subgroup (Dodel et al., 2010)
Riluzole	404	Anti-excitotoxic activity, free-radical scavenging, anti-apoptotic and neurotrophic effects and inhibition of protein aggregation	Neuroprotection in double lesion rat model and toxic mouse model of MSA (Diguët et al., 2005; Scherfler et al., 2005)	III	Survival	Ineffective (Bensimon et al., 2009)
MSC delivery (Intra-arterial and intravenous)	33	Neurotrophic mediation, neural cell differentiation	Immunomodulation and neuroprotection in PLP-SYN mice (Stemberger et al., 2011)	II	Change of UMSARS total score	Significantly lower increase in disease severity, safety concerns (Lee et al., 2012)
Rifampicin	100	Inhibition of formation of α -synuclein fibrils	Reduced astrogliosis, α -synuclein load and neurodegeneration in MBP-SYN mice (Ubhi et al., 2008)	III	UMSARS I (UMSARS II, UMSARS I + II, COMPASS-Select)	Study prematurely terminated, ineffective (Low et al., 2014)
Rasagiline	174	MAO-B inhibitor	Neuroprotection in PLP-SYN mice (Stefanova et al., 2008)	II	Change in UMSARS total score (CGI-I scale, COMPASS-select, MSA-QoL, putaminal mean diffusivity)	Ineffective (Poewe et al., 2015)

COMPASS, Composite Autonomic Assessment Score; CGI-I, Clinical Global Impression-Global Improvement; MAO-B, Monoamine Oxidase B; MBP, Myelin Basic Protein; MSA, Multiple System Atrophy; MSA-QoL, MSA Quality of Life; MSC, mesenchymal cells; PLP, Proteolipid Promoter; UMSARS, Unified Multiple System Atrophy Rating Scale; UPDRS, Unified Parkinson's Disease Rating Scale.

there was no difference in clinical outcomes. The study demonstrated that using ancillary tests as objective outcome measures is feasible (Dodel et al., 2010).

The NNIPPS trial randomized patients to receive either riluzole or placebo for three years. The primary outcome, based on survival, was negative. This study demonstrated (i) that survival rates can be used as outcome measure in disease-modification trials in MSA and (ii) that recruiting up to 400 patients in this rare disease is a feasible feat (Bensimon et al., 2009).

Despite positive preclinical evidence (Stefanova et al., 2008), a randomized, double-blind, placebo-controlled trial assigning 174 MSA-P patients to either rasagiline or placebo was negative. The primary outcome measure was the change from baseline to week 48 in total UMSARS scores (Poewe et al., 2015). Despite positive preclinical data, a randomized, double-blind, placebo-controlled trial assessing the efficacy of rifampicin in 100 MSA patients was also negative (Low et al., 2014; Ubhi et al., 2008). The results of these trials underscore not only the importance of translatable preclinical studies but also a preclinical framework designed for optimal validation of therapies (e.g. patients received doses of rasagiline more than a hundred times lower than those used in the mouse model, while a dose sixty times higher than those used in humans was ineffective in MSA mice). In the rasagiline trial, MRI was performed to assess putaminal diffusivity in a subgroup of 40 patients. As previously mentioned (Section 2.3.1.1), the progression in putaminal diffusivity was slower than previously reported and rasagiline failed to modify it (Poewe et al., 2015). This study confirmed that the use of this technique is a promising approach to objectively judge disease progression in RCT assessing compounds with putative disease-modifying effects. Despite the fact that only a small subgroup of patients was studied, it should be noted that it included more cases than prior longitudinal imaging studies.

4. Looking forward in clinical trials in MSA

Through the last decade, the quality of RCT has greatly improved. In spite of this fact, virtually all trials have reported negative results, while the field is ready for conducting large RCT. Regardless of the result, future trials should allow us to learn as much as possible, including secondary or exploratory outcomes based on fluid (e.g. blood or CSF) and image biomarkers; by collecting data that could help understand the disease process and progression of biomarkers, and, at the same time, help developing objective outcomes for better designing future studies.

Regarding the near future, a phase I trial assessing the safety and tolerability of active immunization with AFFITOPE PD01 and PD03 in 30 MSA patients was recently completed (NCT02270489), its final study report is expected in early 2018. The rationale behind this trial is that immunization may lead to a reduction of the α -synuclein load through upregulated clearance by activated microglia, reducing demyelination and neurodegeneration (Mandler et al., 2015). A phase II trial evaluating the effect of the myeloperoxidase inhibitor AZD3241 on microglial activation as assessed by PET imaging was also recently completed (NCT02388295). Preclinical data suggest the drug suppresses microglial activation in MSA, albeit highly dependent on the time of introduction of the treatment (Kaindlstorfer et al., 2015; Stefanova et al., 2012). A PET study in Parkinson's disease (PD) with AZD3241 showed a statistically significant reduction in the microglial activation marker, further supporting its effect on neuroinflammation (Jucaite et al., 2015).

Regarding translational therapies, molecules targeting distinct disease mechanisms have shown encouraging effects in preclinical models of MSA. The European ARTEMIS consortium is currently testing, individually and in combination, the efficacy of several complementary strategies targeting α -synuclein in vitro and in transgenic MSA mice (<http://www.erare.eu/financed-projects/artemis>). As a strategy to reduce seeding of aggregation by preventing its cleavage, the caspase-1

inhibitor prodrug VX-765 showed an attenuation in neurodegeneration and decreased α -synuclein burden in PLP-SYN transgenic mice (Bassil et al., 2016). Anle138b is a small molecule that has been shown to directly modulate α -synuclein aggregation at the oligomer level, blocking the formation of toxic pore-forming oligomers. It proved neuroprotective in preclinical models of PD (Wagner et al., 2013) while it failed to show neuroprotection in very advanced-stage PLP-SYN transgenic mice that received an additional toxic lesion with 3-nitropropionic acid (Fellner et al., 2016; Levin et al., 2014). A novel drug candidate, CLR01, that targets α -synuclein oligomer formation and promotes clearance of already formed oligomers, has been shown to reduce α -synuclein pathology in mouse models of PD and is currently being tested in the PLP-SYN mouse model of MSA (Acharya et al., 2014; Attar and Bitan, 2014; Prabhudesai et al., 2012; Richter et al., 2017). The muscarinic acetylcholine receptor antagonist bentsropine addresses MSA myelin dysfunction linked pathology through enhancement of remyelination (it promotes myelin gene expression in premyelinating oligodendrocytes and directly enhances myelin formation in mature oligodendrocytes). In the MBP-SYN transgenic mouse model of MSA, this drug promoted a recovery of myelination and a reduction in motor cortical neuronal death (Ettle et al., 2016). A different approach to reducing dopaminergic neural death is improving insulin and IGF-1 signaling. It has been shown that neurons and oligodendrocytes are insulin-resistant in animal models of MSA and PD, as well as in patient post-mortem brain tissue of both disorders, and that treatment with exendin-4, a glucagon-like peptide 1 agonist, reduced cell death and α -synuclein burden in transgenic MSA mice. Exendin-4 has also shown promise in PD patients in an open label trial and, more recently, a single-center RCT (Athauda et al., 2017; Aviles-Olmos et al., 2013a; Aviles-Olmos et al., 2014; Aviles-Olmos et al., 2013b; Bassil et al., 2017a; Bassil et al., 2017b). The mounting evidence supports the design of a clinical trial in MSA patients for these drugs.

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HYPOTHESES AND THESIS OBJECTIVES

II- Hypotheses and thesis objectives

This work was part of a translational approach on MSA. It consists of a neuropathological study of the rare slowly progressive, dopa responsive form of MSA. These patients had been diagnosed as having PD for many years and underwent deep brain stimulation surgery, with poor outcomes; we aimed to study potential pathological differences between this phenotype and classical MSA-P. A second approach consisted of three preclinical proof of concept studies in view of disease modification in MSA. The first two projects aimed to assess the effects of drugs that have shown neuroprotective properties in models of PD on the neurodegenerative process in MSA. At the same time we try to identify underlying similarities and differences between the two entities, namely the roles of autophagy and α -synuclein aggregation-enhancing modifications. In the last study, we compared two drugs with previously reported neuroprotective effects in transgenic MSA mice and determined whether there is a synergistic effect when combining them.

1. Our first project focused on MSA patients who had received deep brain stimulation and had shown poor outcome and survival in the following years. Our hypothesis being that DBS surgery might have induced inflammatory changes thereby accelerating the neurodegenerative process, and also that neuropathological differences between the MSA-DBS cohort and classical MSA-P patients may explain the more benign disease course in the PD-resembling MSA-P phenotype.
2. Secondly, we aimed to test the efficacy of rapamycin following the hypothesis that its inhibition of the mammalian target of rapamycin (mTOR) pathway would enhance autophagic clearance of α -synuclein in PLP-SYN mice, which in turn would protect nigral dopaminergic neurons from death. Previous research has shown that rapamycin protects dopaminergic neurons in toxin-based and transgenic mouse models of PD and reduces α -synuclein accumulation, mostly by restoring autophagy. We determined whether daily administration of rapamycin has beneficial effects on the aggregation of α -synuclein and the neurodegenerative process in the PLP-SYN mouse model of MSA.
3. The third project was proposed following the fact that the c-Abl tyrosine kinase phosphorylates α -synuclein at the tyrosine residue 39, thereby promoting and enhancing its aggregation; and that its inhibition would prevent α -synuclein accumulation. Nilotinib is a c-Abl tyrosine kinase inhibitor, with market approval for treating a form of human leukaemia. c-Abl inhibition by nilotinib and other c-Abl inhibitors has been

reported to alleviate α -synuclein accumulation and protect nigral dopaminergic neurons from death in toxin-based and transgenic models of PD. We aimed to determine whether daily administration of nilotinib has beneficial effects on the aggregation of α -synuclein and the neurodegenerative process in the PLP-SYN mouse model of MSA.

- Our fourth and ongoing project aimed to assess the combined effects of two drugs that have shown to reduce α -synuclein accumulation and to exert neuroprotective effects in transgenic MSA mice. Belnacasan inhibits the C-terminal cleavage of α -synuclein by the proteolytic enzyme caspase-1. This post-translational modification is known to enhance α -synuclein aggregation, and it was reported that inhibiting caspase-1 significantly reduces α -synuclein polymerization and rescues dopaminergic neurons from death (Bassil et al., 2016). In addition, Anle138b is a small molecule that binds physically to α -synuclein, disassembles its oligomers and hampers its aggregation. Anle138b has been shown to also reduce α -synuclein accumulation and alleviate dopaminergic neurodegeneration in PLP-SYN mice (Heras-Garvin et al., 2018). We aimed to evaluate if there is a synergistic effect between these compounds in terms of α -synuclein aggregation and determine whether these drugs act upon different species of α -synuclein.

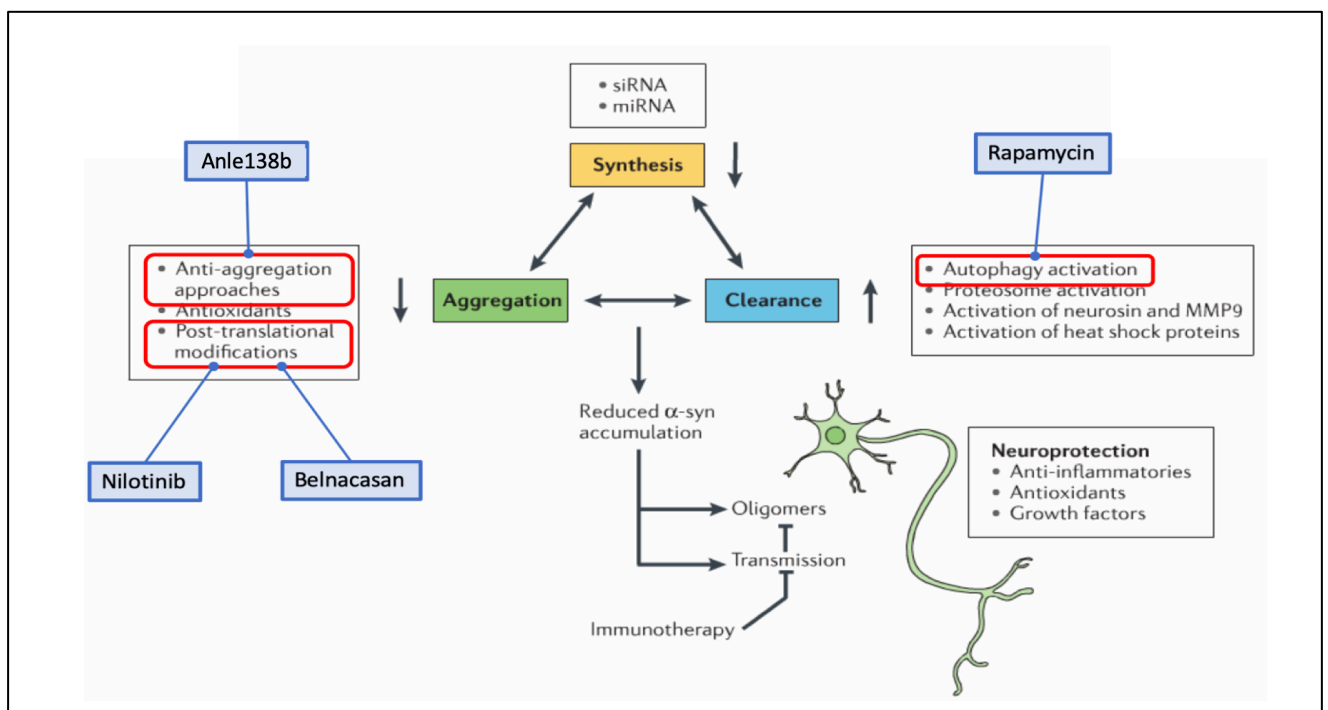


Figure 10. Summary of therapeutic approaches to combat α -synuclein accumulation, circled in red strategies used in this PhD; adapted from Lashuel et al. (2013). Matrix metalloproteinase 9, MMP9; microRNA, miRNA; small interfering RNA, siRNA.

RESULTS

III- Results

Article 1:

Deep brain stimulation does not enhance neuroinflammation in multiple system atrophy

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(Neurobiology of Disease)

Contexte : Un phénotype lentement progressive et sensible à la lévodopa de l'atrophie multisystématisée (AMS) peut être diagnostiquée à tort comme étant une maladie de Parkinson. La stimulation cérébrale profonde est généralement inefficace chez les patients AMS et peut même aggraver l'évolution clinique. Nous avons évalué ici si les différences neuropathologiques entre les patients atteints d'AMS qui ont été traités par stimulation cérébrale profonde en raison d'une présentation clinique trompeuse et de cas typiques d'AMS-P peuvent expliquer l'évolution plus bénigne de la première maladie et le déclin clinique rapide après la chirurgie.

Méthodes : Évaluation post-mortem basée sur l'immunohistochimie du noyau sous-thalamique, du globus pallidus, du thalamus et du putamen chez cinq patients atteints d'AMS ayant reçu une stimulation cérébrale profonde et neuf cas types de d'AMS-P typiques.

Résultats : Il n'y avait aucune preuve de profils neuroinflammatoires distincts entre les deux groupes qui pourraient être reliés à l'intervention chirurgicale ou qui pourraient expliquer la progression clinique rapide suite à l'initiation de la stimulation cérébrale profonde. Les patients ayant reçu une stimulation cérébrale profonde présentaient une proportion plus élevée d'inclusions cytoplasmiques neuronales portant l' α -synucléine dans le putamen par rapport aux cas typiques, tandis que le nombre de neurones préservés n'était pas différent entre les groupes.

Conclusions : Nos résultats suggèrent que la stimulation cérébrale profonde n'induit pas de changements neuropathologiques chez les patients atteints d'AMS, au moins plusieurs années après la chirurgie. Nous émettons en outre l'hypothèse que des patrons distincts d'accumulation de l' α -synucléine contribuent à des différences dans le phénotype clinique, une plus grande proportion d'inclusions neuronales dans le putamen étant associée à un phénotype atténué ressemblant à une "maladie de Parkinson", avec réponse soutenue à la lévodopa et progression plus lente.



Deep brain stimulation does not enhance neuroinflammation in multiple system atrophy



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ABSTRACT

Slowly progressive, levodopa-responsive multiple system atrophy (MSA) may be misdiagnosed as Parkinson's disease (PD). Deep brain stimulation (DBS) is mostly ineffective in these patients and may even worsen the clinical course. Here we assessed whether neuropathological differences between patients with MSA who were treated with DBS of the subthalamic nucleus because of a misleading clinical presentation and typical disease cases may explain the more benign disease course of the former, and also the rapid clinical decline after surgery. The post-mortem assessment included the subthalamic nucleus, the globus pallidus, the thalamus and the putamen in five patients with MSA who received DBS and nine typical disease cases. There was no evidence for distinct neuroinflammatory profiles between both groups that could be related to the surgical procedure or that could explain the rapid clinical progression during DBS. Patients who received deep brain stimulation displayed a higher proportion of α -synuclein bearing neuronal cytoplasmic inclusions in the putamen compared with typical cases, while the number of surviving neurons was not different between groups. Our findings suggest that DBS does not induce neuroinflammatory changes in patients with MSA, at least several years after the surgery. We further hypothesize that the peculiar pattern of α -synuclein pathology may contribute to differences in the clinical phenotype, with a greater proportion of neuronal inclusions in the putamen being associated to a milder, "PD-like" phenotype with sustained levodopa response and slower disease progression.

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1. Introduction

Multiple system atrophy (MSA) is a fatal orphan neurodegenerative disorder that manifests in a variable combination with autonomic, parkinsonian, cerebellar, and pyramidal features (Fanciulli and Wenning, 2015; Gilman et al., 2008). The pathological hallmark is the accumulation of misfolded alpha-synuclein (α -syn) in oligodendrocytes, forming glial cytoplasmic inclusions (GCI), and to a lesser extent in neurons, typically as neuronal cytoplasmic inclusions (NCI) (Inoue et al., 1997; Kato and Nakamura, 1990; Lin et al., 2004; Nishie et al., 2004; Papp et al., 1989; Papp and Lantos, 1992). The median disease duration is 6–10 years (Gilman et al., 2008).

Current consensus diagnostic criteria include two classical phenotypes, MSA with predominant parkinsonism (MSA-P) and MSA with predominant cerebellar ataxia (MSA-C). Clinical heterogeneity has been repeatedly described in the literature, ranging from an asymmetrical, slowly progressive levodopa-responsive phenotype that may last up to two decades (Jellinger, 2012; Masui et al., 2012; Petrovic et al., 2012), to the very aggressive so-called 'minimal change' variant, which may lead to death within 5 years after having reached most of the clinical milestones by year 3 (Ling et al., 2015). Factors that have been associated with poor survival in MSA are older age at onset, female gender, MSA-P subtype, shorter period from onset to first clinical milestone, stridor within the first three years after symptom onset and early autonomic failure (Giannini et al., 2016; Low et al., 2015; O'Sullivan et al., 2008; Wenning et al., 2013).

In MSA, the neurodegenerative process is most pronounced in the substantia nigra (SN), putamen, globus pallidus (GP, particularly in MSA-P), vermis, cerebellar hemispheres and inferior olivary nucleus (particularly in MSA-C) (Fearnley and Lees, 1990; Wenning et al., 1997), while significant involvement of the thalamus, subthalamic nucleus (STN), cerebellar dentate nucleus and anterior horn cells is rare (Ozawa et al., 2004).

The relevance of recognizing the more benign slowly progressive, levodopa-responsive variant, lies in the fact that these patients can be misdiagnosed as having Parkinson's disease (PD) with significant levodopa-related motor complications and undergo deep brain stimulation (DBS) surgery. In this regard, we recently reported the poor clinical outcome of STN-DBS in a series of five post-mortem confirmed MSA patients. These patients were initially considered as having PD with motor fluctuations and underwent DBS surgery, followed soon after the operation by a rapid deterioration and the appearance of clinical features suggestive of MSA (Meissner et al., 2016).

The present study investigated whether neuropathological differences between these MSA-DBS cases and a cohort of typical MSA-P patients might account for the more benign disease course of the MSA-DBS cases, and also for their rapid clinical decline after DBS.

2. Methods

2.1. Human brain samples

Formalin-fixed and paraffin-embedded material of 5 MSA-DBS and 9 typical MSA-P cases was assessed. Written informed consent was obtained prior to autopsy for the collection of the brain and the use of clinical and post-mortem data from all subjects or their legal representatives. Human brain samples were obtained from the brain banks in Marseille, Lyon, Strasbourg and Bordeaux (DC-2014-2164).

2.2. Immunohistochemistry

Brain regions included for comparative analysis were the putamen and GP (studied within sections demonstrating the lentiform nucleus), since these regions are severely affected in MSA; the thalamus, because it is classically spared by the degenerative process; and the STN, being the target of the DBS surgery. Dewaxed 4 μ m thick coronal paraffin

sections were either stained with hematoxylin eosin and Cresyl violet for the semiquantitative assessment of neuronal loss or processed for immunohistochemistry as follows.

Immunostaining was performed with antibodies against α -syn (mouse monoclonal antibody, clone LB509, 1:100; Invitrogen by life technologies), glial fibrillary acidic protein (GFAP, polyclonal rabbit, 1:4000; Dako), CD68 (monoclonal mouse anti-Human, clone PG-M1, ref. M 0876 DAKO) to label microglia and CD3 (polyclonal rabbit anti-Human, ref. A 0452 DAKO), a marker of mature T lymphocytes.

After antigen retrieval and blocking with 5% normal goat serum containing 0.05% Tween in phosphate-buffered saline (PBS) for 30 min at room temperature, sections were incubated overnight with the primary antibody. Immunoreactions were revealed with the appropriate secondary antibody, a ready-to-use goat anti-rabbit or goat anti-mouse EnVision-HRP enzyme conjugate (Dako) followed by the highly sensitive diaminobenzidine plus (Dako) as substrate chromogen for GFAP or a high sensitivity AEC (3-amino-9-ethylcarbazole) substrate-chromogen system (Dako) for α -synuclein, CD3 and CD68. Finally, sections were counterstained with Mayer's hemalum and mounted in a suitable mounting medium.

The number of immunopositive cells was obtained using a computerized image analysis system (Mercator V6.50, Explora Nova) linked to a Leica microscope type DM-6000B. Quantitative evaluation was carried out on one section per structure and boundaries on each structure were first delineated at low magnification ($\times 2.5$). Quantification was performed at $\times 20$ magnification on the whole thalamus, STN and GP except for the putamen where, from a random start position, the computer-generated sampling grid placed the counting frames. Within each frame, cells were counted only if the totality of the cell was inside the frame taken into account. Results were expressed as an average of immunopositive cells per mm². To minimize the inherent variability in the immunohistochemical procedures, all sections from all patients were processed simultaneously for a given antibody and structure. Putaminal degeneration was assessed by counting the number of surviving neurons in the putamen.

2.3. Statistics

Comparisons between groups were done using a Student's *t*-test whenever the variable had a normal distribution, while a Mann-Whitney *U* test was used otherwise. The proportion of the total α -syn inclusion load represented by NCI was compared between groups by using a Fisher exact test (cut-off value of 10% of the total burden). For the comparison between brain structures (NCI, GCI, total α -syn inclusion load, CD3, CD68 and GFAP positive cells), a one-way ANOVA or, if appropriate, a one-way ANOVA on ranks was performed including all 14 subjects. Post-hoc Tukey or Dunn tests were performed whenever appropriate. A *p*-value < 0.05 was considered significant. If not indicated otherwise, results are presented as mean \pm standard deviation. Statistical analyses were performed with GraphPad Prism Software, version 6.

3. Results

3.1. Patient characteristics

A detailed clinical description of the MSA-DBS cases is reported elsewhere (Meissner et al., 2016). Mean age at disease onset was lower in the MSA-DBS group (50.4 ± 6.3 years) compared to the MSA-P control group (56.9 ± 9.5 years, *p* < 0.05), while mean age at death was similar between groups with 61.6 ± 7.1 and 62.3 ± 8.5 years respectively. Accordingly, mean disease duration was higher in the MSA-DBS group (11.2 ± 3.0 years) compared to the MSA-P control group (5.4 ± 2.5 years, *p* < 0.005, Table 1).

The response during the pre-surgical levodopa challenge was excellent in all five MSA-DBS patients, ranging from 49 to 88%

improvement on the UPDRS part III subscale. Following DBS, all patients showed progressive clinical worsening. Patient one presented with daily falls and urinary incontinence two years after surgery. For patient two, clinical symptoms worsened one year after DBS with the onset of severe end-of-dose dystonic and dyskinetic episodes and postural instability. The status of patient three worsened 18 months after surgery with the onset of gait instability and falls. Patient four presented a few weeks after the procedure ataxia with falls, dysarthria and dysphagia. The parkinsonism of patient five progressed severely and ataxia with falls appeared during the first year after DBS.

3.2. Neuronal loss in the putamen and α -syn pathology in the putamen, the GP, the thalamus and the STN

Neuronal cell counts revealed no significant differences in the putamen between MSA-DBS (178.9 ± 94.6 neurons/mm²) and MSA-P control patients (211.1 ± 118.2 neurons/mm²).

NCI and GCI were found in all brain regions. A comparison of NCI and GCI load between groups for each region did not show significant differences (Fig. 1), while an overall comparison between regions after pooling the data of all 14 subjects revealed significant differences in the number of NCI ($H = 14.3$, $df = 3$, $p < 0.05$), GCI ($H = 21.5$, $df = 3$, $p < 0.0001$) and total α -syn burden ($H = 14.1$, $df = 3$, $p < 0.005$). A post-hoc analysis showed a higher number of NCI in the thalamus compared to the GP and the STN, a lower number of GCI in the thalamus compared to all other regions, and a higher total α -syn inclusion burden in the putamen compared to the thalamus. NCI accounted for > 10% of the total number of α -syn inclusions in the putamen in four of the five MSA-DBS cases, while this was only the case in one of the nine MSA-P controls ($p < 0.005$, Table 1 and Fig. 2).

Three out of five MSA-DBS cases showed a few Lewy bodies (LB). LB pathology was restricted to the SN in patient 2, while patient 4 showed some LB in the putamen, the caudate nucleus, the GP, the thalamus, the STN, the midbrain, the pons and the medulla, as well as Lewy neurites in the internal capsule, the subcortical and the cerebellar white matter. Patient 5 had a few LB in the locus coeruleus, raphe nucleus and SN. Conversely, only two out of nine MSA-P controls showed some LB pathology. MSA-control 2 had rare LB in the midbrain, the pons and the thalamus, and moderately in the caudate nucleus and the putamen. MSA-P control 3 displayed some LB in the thalamus.

3.3. Neuroinflammation in the putamen, the GP, the thalamus and the STN

Numerical densities of microglia (CD68), CD3 positive T lymphocytes and GFAP positive astrocytes are presented in Table 2. There was

a trend for a higher number of CD68-positive microglia in the putamen in MSA-P controls compared to MSA-DBS cases (Table 2). There was also a trend for a higher number of CD3 positive T lymphocytes in the GP in MSA-DBS patients, while the number of GFAP-positive astrocytes was not different between MSA-DBS and MSA-P controls in any of the investigated regions. Collectively, the quantification of these markers revealed no evidence for a distinct inflammatory profile in the brains of patients who underwent DBS surgery.

When pooling data of all 14 MSA cases, an overall comparison between regions showed a significant difference for CD3 positive T lymphocytes ($F_{(3,50)} = 23.4$, $p < 0.0001$). Post-hoc testing further revealed a higher number of CD3 positive cells in the putamen compared to thalamus and STN. An overall comparison for CD68 between regions yielded also a significant difference ($F_{(3,50)} = 3.8$, $p < 0.05$), with the presence of more CD68 positive cells in the putamen compared to the thalamus. A comparison for GFAP expression between regions did not reveal significant differences ($F_{(3,50)} = 0.9$).

4. Discussion

This is the largest study comparing histological findings in post-mortem confirmed MSA-DBS patients with slow disease progression and sustained levodopa response (the so-called MSA-DBS group) and a control group of typical MSA-P cases. No differences were found between groups when comparing neuronal cell loss in the putamen, or markers of neuroinflammation. The brain structure with the highest total α -syn inclusion burden was the putamen, while no differences were found regarding NCI or GCI load between groups for the four assessed regions. However, a detailed analysis showed that the proportion of NCI in the putamen was higher in the MSA-DBS group. Moreover, three out of five MSA-DBS cases presented a few LB, while only two out of nine MSA controls showed some LB.

The degree of putaminal degeneration is associated with the level of levodopa responsiveness in MSA (Fearnley and Lees, 1990; Hughes et al., 1992). In this series, MSA-DBS cases and MSA-P controls showed no difference in putaminal neural loss although MSA-DBS cases had responded to levodopa for many years. This may be explained by the fact that both groups had reached the end stage of the condition at death, with a slower rate of putaminal degeneration in the MSA-DBS group who had a mean disease duration twice as long compared to the typical MSA cases.

Classically, MSA is considered to be a primary oligodendroglialopathy, with GCI being the neuropathological hallmark of the disease (Jellinger, 2003; Papp and Lantos, 1994; Wenning et al., 2008). In PD, the opposite is observed, where α -syn inclusions develop typically in

Table 1
Demographics.

	Gender	Age at disease onset	Age at surgery	Age at death	Disease duration*	Tissue available for IHC	Putamen NCI/total inclusions (%)**
MSA-DBS 1	F	54	62	69	15	GP, P	14.6
MSA-DBS 2	F	41	50	54	13	GP, P, STN, T	96
MSA-DBS 3	M	47	52	54	7	GP, P, STN, T	100
MSA-DBS 4	M	54	58	65	11	GP, P, STN, T	0
MSA-DBS 5	M	56	64	66	10	GP, P, STN, T	16.0
MSA-control 1	M	51	–	59	8	P, STN, T	0.9
MSA-control 2	M	52	–	60	8	GP, P, STN, T	2.6
MSA-control 3	M	46	–	54	8	GP, P, STN, T	0
MSA-control 4	F	68	–	74	6	GP, P, STN, T	0
MSA-control 5	F	55	–	57	2	GP, P, STN, T	22.2
MSA-control 6	F	71	–	74	3	GP, P, STN, T	4.5
MSA-control 7	M	48	–	52	4	GP, P, STN, T	1.2
MSA-control 8	F	68	–	71	3	GP, P, STN, T	0.6
MSA-control 9	M	53	–	60	7	GP, P, STN, T	1.7

IHC: Immunohistochemistry, NCI: Neuronal cytoplasmic inclusion, P: Putamen, T: Thalamus, STN: Subthalamic nucleus, GP: Globus pallidus.

* $P = 0.002$.

** $P = 0.004$.

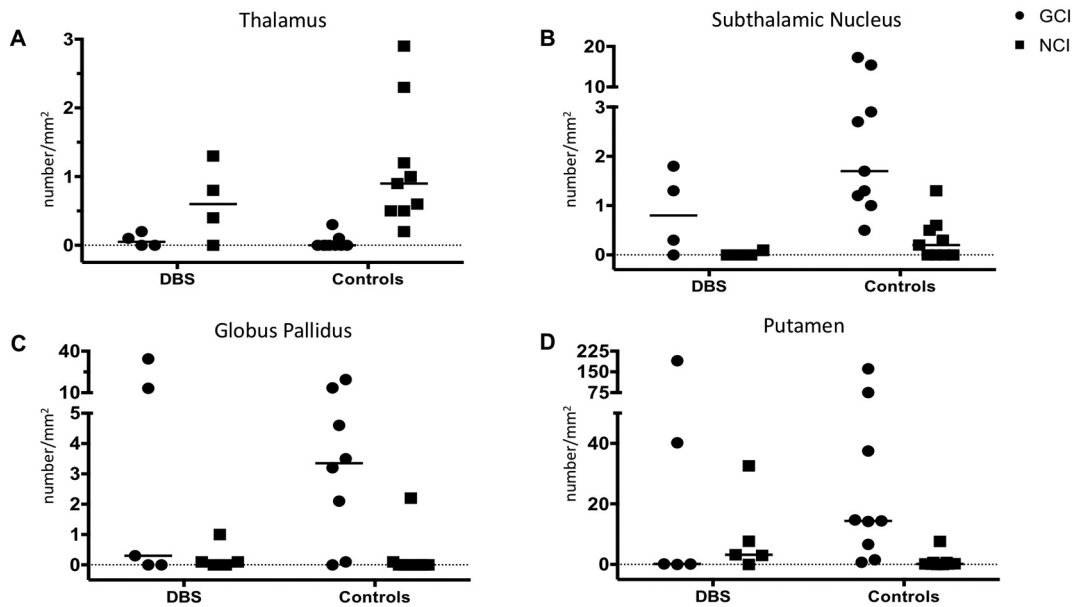


Fig. 1. Alpha-synuclein bearing inclusions in the four assessed regions. (A) thalamus, (B) subthalamic nucleus, (C) globus pallidus, and (D) putamen. GCI: glial cytoplasmic inclusions, NCI: neural cytoplasmic inclusions. Horizontal lines in panels A-D indicate the median.

neurons (e.g. LB, neuronal perikaryal structures, Lewy neurites, axonal spheroids and dot-like structures), while α -syn inclusions in glial cells are rare (Dickson et al., 2009; Houlden and Singleton, 2012; Jellinger, 2003).

Neuronal α -syn pathology, considered ancillary, has been historically overlooked. However, growing evidence suggests that neuronal α -syn pathology is not simply an epiphenomenon in MSA but may play a role in the disease process (Cykowski et al., 2015; Halliday, 2015; Homma et al., 2016; Jellinger, 2007; Rohan et al., 2015). Accordingly, Lewy body-like inclusions in the substantia nigra pars compacta were observed in 10 out of 44 MSA patients and neuronal α -syn pathology was abundantly found throughout the brain in a series of 35 MSA patients (Cykowski et al., 2015; Jellinger, 2007). In the latter study, 29/35 MSA patients showed neuronal α -syn pathology in the putamen, while the extent of overall neuronal α -syn pathology as determined by a semiquantitative assessment was not correlated with age at onset or disease duration (Cykowski et al., 2015). Additionally, two cases with an exceptionally long disease duration and a relatively slow disease progression showed abundant α -syn NCI and neurites in the striatum and limbic cortices (Yoshida, 2007). Based on these findings, it was suggested that inclusions of surviving neurons in the striatum or limbic areas may play a role in cell repair and/or protection (Yoshida, 2007).

In our series, MSA-DBS cases had a significantly larger proportion of NCI in the putamen compared to MSA-P controls. It could be argued that this difference is simply due to the fact that MSA-DBS patients lived longer, thus allowing more propagation of α -syn pathology, be it from

Table 2

Immunohistochemistry findings.

		MSA-DBS	MSA-Controls	P value
CD68 (/mm ²)	Thalamus	18.2 (0.03–54.9)	2.2 (0.5–3.6)	0.63
	STN	26.3 (1.6–78.3)	6.6 (0.0–22.7)	0.50
	GP	4.8 (0–10.7)	19.9 (0.6–59.8)	0.20
	Putamen	14.3 (3.3–24.5)	43.2 (1.8–103.3)	0.06
CD3 (/mm ²)	Thalamus	4.9 (0.9–9.9)	4 (0.1–13.5)	0.60
	STN	4.2 (0.3–10)	12.4 (0.0–40.7)	0.48
	GP	45.1 (9.6–81.8)	15.9 (0.4–59.1)	0.09
	Putamen	112.6 (3.1–263.6)	119 (10.2–263.5)	0.91
GFAP astrocytes (/mm ²)	Thalamus	129.7 (95.2–159.8)	117.6 (55–187.7)	0.61
	STN	167.1 (52.8–258.3)	140.3 (72.5–200)	0.47
	GP	155.5 (122.2–188.9)	124.6 (72.5–231.8)	0.25
	Putamen	148.2 (19.1–292.8)	182.3 (22.1–513.5)	0.65

Values expressed as mean and range.

glia to neurons or from neurons to neurons. However, if that were the case, one would expect an increase in the overall α -syn load as well (including GCI), as described in some studies but not found by us and others (Cykowski et al., 2015; Ishizawa et al., 2004). On the other hand, perhaps, neurons may be more prone to the accumulation of aggregated

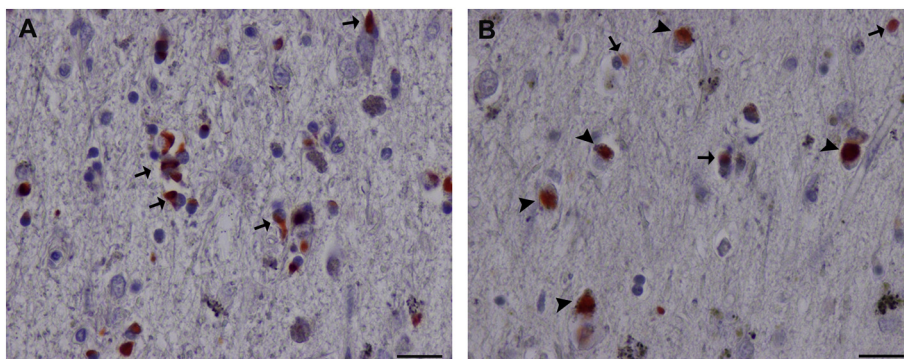


Fig. 2. Alpha-synuclein bearing inclusions (stained in red) in the putamen of a MSA-P control (A) and a MSA-DBS case (B). Arrow heads and arrows indicate neuronal and glial cytoplasmic inclusions, respectively. In contrast to panel A, panel B shows several neuronal cytoplasmic inclusions. Scale bar: 20 μ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

α -syn as a consequence of an unknown process in this more benign phenotype of MSA. Because of the oligodendroglial aggregation of α -syn and distinct clinical features (early dysautonomia and levodopa unresponsive parkinsonism), MSA is often presented as standing apart within the family of synucleinopathies. Our finding of a higher proportion of neuronal α -syn pathology in slowly progressive levodopa-responsive MSA-P may suggest a possible continuum between PD and MSA.

Recent post-mortem studies in patients with G51D and A53E SNCA gene point mutations have described neuropathological profiles that share features of both PD and MSA. These mutation carriers show widespread neural and glial inclusions resembling NCI, LB and GCI, pointing to a possible link between PD and MSA. Disease duration, ranging from 6 to 30 years, and neural involvement were not related, i.e. a greater neural α -syn load was not explained by a longer life-span (Kiely et al., 2013; Kiely et al., 2015; Pasanen et al., 2014).

One may also hypothesize that the differences in the clinical phenotype could be related to different strains of α -syn with varying seeding and spreading properties, as suggested for tauopathies (Kaufman et al., 2016; Sanders et al., 2014). Moreover, the recent study by Peng et al. (2018) describes how cellular milieu imparts distinct pathological α -syn strains in the different synucleinopathies. Perhaps in this rare subset of patients, different strains of α -syn co-exist, where some strains may first involve structures and cells (e.g. neurons) affected more commonly in PD - explaining a syndrome resembling PD with sustained response to levodopa - and in a later phase trigger a more aggressive phenotype, with spreading to other regions and involvement of oligodendrocytes. This may further explain the distinct combination of α -syn inclusions (NCI, LB and GCI) and clinical evolution found in our cohort. This remains highly speculative since current knowledge of α -syn spreading in human brains is limited, and further research on the behavior of protein aggregates in vivo is needed (Walsh and Selkoe, 2016).

Our quantitative analysis showed a higher number of T lymphocytes (CD3) in the putamen, a region with severe neurodegeneration in MSA, compared to other regions, and a larger amount of activated microglia (CD68) in the GP compared to thalamus (classically less affected in MSA). To our knowledge, lymphocytic infiltration in brains of MSA patients through CD3 staining has not been previously reported and adds to our understanding of the neuroinflammatory process that occurs in the brains of MSA patients.

Even though patients of the MSA-DBS group showed a rapid clinical decline within two years after the intervention, there were no signs of the surgical procedure or DBS inducing morphological, inflammatory, or gliotic changes in the STN. Furthermore, we did not identify any changes in the brain regions analyzed that could appear to be related to the intervention. However, since the autopsy was only performed two to seven years after the DBS surgery, the ongoing neurodegenerative process may have concealed acute and direct influences on disease progression. On the other hand, DBS-related mechanisms may explain the clinical worsening in the MSA-DBS group. In PD, STN-DBS is effective in treating dopamine dependent symptoms such as tremor, bradykinesia, and rigidity through the disruption of the abnormal activity in the STN and connected structures of the motor loop (Krack et al., 2003; Meissner et al., 2007; Meissner et al., 2005). In MSA and other forms of atypical parkinsonism, the response to STN-DBS is limited because of the degeneration of basal ganglia nuclei and connected structures. Nevertheless, electrical disruption of complex neural compensatory circuits different from PD may explain the pronounced post-surgical decline in the MSA-DBS group. However, this interpretation is unlikely since the worsening was mostly due to the progression of dopamine independent symptoms (e.g. ataxia, postural instability and autonomic failure) that are related to dysfunction of unconnected structures outside the basal ganglia.

The main weakness of this study is the small number of patients included, which is a limitation to the drawing of conclusions due to low

statistical power, bearing in mind that these are very rare patients, making it virtually impossible to muster a larger cohort. Additionally, since the primary objective was to describe histological findings that would help elucidate the causes of the clinical worsening subsequent to the surgical intervention, the assessment was limited to four brain regions. Further studies addressing these rare subtypes of MSA are warranted to comprehensively assess the disease process and the possible neuropathological continuum between PD and the slowly progressive levodopa-responsive MSA phenotype.

In conclusion, in this study comparing post-mortem brains of MSA-P and slowly progressive MSA patients who underwent DBS, no pathological differences were found that could be related to the surgical procedure or that could explain the rapid decline of these patients after DBS. Differences were observed regarding α -syn pathology, with a greater proportion of NCI in the putamen of MSA-DBS patients. We hypothesize that this observation may be related to the differences in the clinical presentation, with a greater proportion of NCI being associated to a milder, "PD-like" phenotype with sustained levodopa response for many years and slower disease progression.

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Article 2:

Inhibition of c-Abl by nilotinib does not prevent alpha-synuclein aggregation and neurodegeneration in a mouse model of multiple system atrophy

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(Soumis)

Contexte : L'atrophie multisystématisée (AMS) est une maladie neurodégénérative rare sans traitement efficace. Elle est caractérisée par l'accumulation de la protéine α -synucléine dans les inclusions oligodendrogiales. À ce titre, l'AMS forme le groupe des synucléinopathies avec la maladie de Parkinson (MP) et la démence à corps de Lewy (DCL). L'activation de la tyrosine kinase c-Abl conduit à la phosphorylation de l' α -synucléine en tyrosine 39, favorisant ainsi son agrégation et la neurodégénérescence ultérieure. Le nilotinib, un inhibiteur de l'enzyme c-Abl utilisé pour le traitement de la leucémie myéloïde chronique, a montré des effets bénéfiques dans des modèles précliniques de la maladie de Parkinson, ce qui a motivé son évaluation dans un essai clinique ouvert de petite taille chez des patients atteints de MP et DCL.

Objectifs : Évaluer l'efficacité du nilotinib dans le modèle préclinique de l'AMS.

Méthodes : Des souris exprimant l' α -synucléine de type sauvage humaine dans les oligodendrocytes ont reçu une injection quotidienne de nilotinib (1 ou 12 mg/kg) pendant 12 semaines. L'analyse post-mortem comprenait l'évaluation de l'activation de c-Abl, de la charge en α -synucléine et de la neurodégénérescence dopaminergique.

Résultats : L' α -synucléine phosphorylée à la tyrosine 39 a été détectée dans des inclusions cytoplasmiques gliales chez des patients atteints d'AMS. Une activation accrue de la phosphorylation c-Abl et de l' α -synucléine à la tyrosine 39 a été observée chez des souris transgéniques. Malgré l'inhibition de c-Abl et la réduction concomitante de la phosphorylation de l' α -synucléine, le nilotinib n'a pas permis de réduire la charge globale de l' α -synucléine dans le striatum ou à diminuer la neurodégénérescence dans la substance noire.

Conclusions : Cette étude préclinique suggère que l'inhibition de la c-Abl et la réduction de la phosphorylation de la tyrosine 39 de l' α -synucléine à ne semblent pas être une cible pertinente pour l'AMS.

Inhibition of c-Abl by nilotinib does not prevent alpha-synuclein aggregation and neurodegeneration in a mouse model of multiple system atrophy

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Abstract

Background: Multiple system atrophy (MSA) is a rare untreatable neurodegenerative disorder, characterized by accumulation of the protein alpha-synuclein in oligodendroglial inclusions. As such, MSA is a synucleinopathy along with Parkinson's disease (PD) and Dementia with Lewy bodies (DLB). Activation of the tyrosine kinase c-Abl leads to phosphorylation of alpha-synuclein at tyrosine 39, thereby promoting its aggregation and subsequent neurodegeneration. The c-Abl inhibitor nilotinib used for the treatment of chronic myeloid leukemia was found in preclinical models of PD to interfere with pathogenic mechanisms that are relevant to PD and DLB, which motivated its assessment in an open label unblinded clinical trial in PD and DLB patients.

Objectives: To assess the preclinical efficacy of nilotinib in the specific context of MSA

Methods: Mice expressing human wild-type alpha-synuclein in oligodendrocytes received daily injection of nilotinib (1 or 12 mg/kg) during 12 weeks. Post-mortem analysis included the assessment of c-Abl activation, intracellular alpha-synuclein burden and dopaminergic neurodegeneration.

Results: Alpha-synuclein phosphorylated at tyrosine 39 was detected in glial cytoplasmic inclusions in MSA patients. Increased activation of c-Abl and alpha-synuclein phosphorylation at tyrosine 39 were found in transgenic mice. Despite adequate inhibition of c-Abl and associated reduction of alpha-synuclein phosphorylation at Y39, nilotinib failed to reduce alpha-synuclein aggregates burden in the striatum or lessen neurodegeneration in the substantia nigra.

Conclusions: This preclinical study suggests that inhibition of c-Abl and reduction of alpha-synuclein phosphorylation at tyrosine 39 may not be a relevant target for MSA.

INTRODUCTION

Multiple system atrophy (MSA) is an adult-onset rare neurodegenerative disorder characterized by a combination of parkinsonism, cerebellar impairment, and autonomic dysfunction (Gilman et al., 2008). The cytopathological hallmark of MSA is the accumulation of α -synuclein aggregates in oligodendrocytes, forming glial cytoplasmic inclusions (GCIs). As such, MSA is a synucleinopathy, a group of disorders characterized by α -synuclein accumulation, which also includes Parkinson's disease (PD) and dementia with Lewy bodies. Currently, only symptomatic treatments are available to these patients who face a median life expectancy of 6 to 10 years after symptom onset (Fanciulli and Wenning, 2015)

Oxidative stress is a prominent feature in neurodegenerative disorders, including MSA (Stefanova et al., 2009). The non-receptor Abelson tyrosine kinase (c-Abl) is activated by oxidative and cellular stress (Hantschel and Superti-Furga, 2004). Its levels are increased in the substantia nigra and striatum of PD patients (Imam et al., 2011; Ko et al., 2010), and phosphorylated c-Abl is present in neuronal cell bodies in Alzheimer's disease and several other neurodegenerative disorders (Derkinderen et al., 2005; Jing et al., 2009; Schlatterer et al., 2011; Tremblay et al., 2010). It has been shown that c-Abl phosphorylates α -synuclein at tyrosine 39 (pY39) and serine 125, inhibiting its clearance via the autophagic and the proteasomal pathways (Brahmachari et al., 2016; Mahul-Mellier et al., 2014).

Nilotinib is a c-Abl tyrosine kinase inhibitor used to treat Philadelphia chromosome-positive chronic myeloid leukemia (Hantschel, 2012). Neuroprotective effects of c-Abl inhibition by nilotinib and other c-Abl inhibitors have been observed in toxin-based and transgenic models of PD (Brahmachari et al., 2016; Hebron et al., 2013; Imam et al., 2013; Imam et al., 2011; Karuppagounder et al., 2014).

While this growing body of preclinical studies suggests potentially disease-modifying effects of c-Abl in PD, it is unknown if nilotinib may have similar beneficial effects in other

synucleinopathies such as MSA. In the current study, we determined whether administration of nilotinib for 12 weeks could attenuate the aggregation of α -synuclein and preserve nigral tyrosine hydroxylase (TH)-positive dopamine neurons in a transgenic mouse model of MSA.

MATERIAL AND METHODS

Human brain samples

Formalin-fixed and paraffin-embedded material of 4 MSA cases with predominant parkinsonism (MSA-P) was used to assess the presence of pY39 α -synuclein. Written informed consent was obtained prior to autopsy for the collection of the brain and the use of clinical and post-mortem data from all subjects or their legal representatives. Human brain samples were obtained from the brain bank in Bordeaux (DC-2014-2164).

Histopathological analysis and immunofluorescent labelling

To assess the presence of glial inclusions positive for pY39 α -synuclein, sequential immunofluorescence labelling was performed on 4- μ m thick paraffin-embedded putamen sections from MSA-P patients using the established MJFR1 (Abcam) α -synuclein antibody as a reference to label GCIs. Following antigen retrieval using TintoRetriever Pressure Cooker at 114°C-120°C during 10 minutes in citrate buffer pH6 (Dako) and then 5 minutes in 80% formic acid, sections were washed in TBS-Tween 0.05%, blocked with goat serum and incubated with anti pY39 α -synuclein (mouse monoclonal antibody, clone A15119B, 1/100, BioLegend) overnight at 4°C. After transfer in 3% H₂O₂/H₂O to block endogenous peroxidases, sections were treated with biotinylated anti-mouse IgG (diluted 1:200) and incubated with Tyramide Signal Amplification (TSA) reagent (Perkin Elmer). Streptavidine-Alexa fluor 488 (1/1000, Invitrogen) was used for revelation. Thereafter, sections were probed for MJFR1 α -synuclein (1:1000, Abcam) and revealed with goat anti-rabbit Alexa 568 (1:400, Invitrogen).

Immunohistochemical negative controls were performed by omission of one of the two primary antibodies. Slides were then incubated with 0.1% Sudan Black B (Sigma-Aldrich) in 70% ethanol to lower the intensity of lipofuscin autofluorescence. Nuclei were counterstained with DAPI (Sigma-Aldrich). To minimize the inherent variability in the immunofluorescence procedure, sections from all cases were processed simultaneously for a given marker.

Quantitative analysis of human brain samples

Analysis of the proportion of pY39 α -synuclein immunopositive cells over the total MJFR1 immunopositive cells, was performed using a computerized image analysis system (Morphostrider, Explora Nova) linked to a Zeiss fluorescence microscope Imager M2. For co-localization analysis, nine images were taken randomly from the putamen at x40 magnification, and image analysis was undertaken using ImageJ v1.50b. Oligodendrocytes containing GCIs were first identified based on MJFR1 α -synuclein and DAPI staining. Cell counts were performed on MJFR1 - pY39 α -synuclein overlay to discriminate between pY39- α -synuclein-positive and negative oligodendrocytes. Both total numbers and the proportion of pY39- α -synuclein-positive relative to MJFR-positive inclusions were calculated.

Animals and treatment

All experiments involving mice were performed in accordance with French guidelines (87-848, Ministère de l'Agriculture et de la Forêt) and the European Community Council Directive (2010/63/EU) for the care of laboratory animals. Animal experiments were approved by the Institutional Animal Care and Use Committee of Bordeaux (CE50, approval #2016120215297254). Mice were maintained in a temperature and humidity - controlled room on a 12:12 light-dark cycle with food and water ad libitum.

Mice expressing human α -synuclein in oligodendrocytes under the control of the proteolipid promoter (PLP-SYN) were previously generated on a C57BL/6J background (Kahle et al., 2002). To compare the c-Abl phosphorylation state between adult PLP-SYN in mice and wild type (WT) mice, 6 PLP-SYN and 9 WT mice were used at 6 months of age. Treatment with nilotinib 10mg/kg has been reported to protect TH-positive neurons in the substantia nigra compacta (SNc) from α -synuclein toxicity (Hebron et al., 2013). To confirm brain penetration of the compound by this regimen, a group of PLP-SYN mice aged 6 months (n=12) was first allocated to receive either nilotinib (10mg/kg) (Sigma Aldrich, CDS023093) or vehicle (dimethyl sulfoxide, DMSO) during five days and then terminated. Nilotinib was diluted in a solution consisting of 90% DMSO and 10% distilled water at the desired concentrations.

In a second step, PLP-SYN mice aged 6 weeks (n=23) were allocated to receive either nilotinib high dose (10mg/kg) (n=8), nilotinib low dose (1mg/kg) (n=8), or DMSO (n=7); while WT littermates (n=9) were treated with DMSO (volume of 50 μ l per mouse, doses were adjusted according to the average weight of each group each week). Mice received daily i.p. injections of nilotinib for 12 weeks until termination.

Tissue extraction

For the first part of the study, mice were sedated with pentobarbital (100mg/kg i.p) and sacrificed by cervical dislocation 4h after the last nilotinib injection. Brains were quickly removed and cut in half between the two hemispheres to later be used for biochemical analysis and Mass Spectrometry. The rest of the animals were anaesthetized with pentobarbital (100mg/kg i.p.). They were then intracardially perfused with 25ml of 0.9% saline. After quick removal of the brain, the right hemisphere was frozen directly for biochemical analysis, while the left hemisphere was post-fixed for 5 days in 5ml of 4% (wt/vol) paraformaldehyde (PFA),

followed by cryoprotection in 10ml of 20% (wt/vol) sucrose in 0.1 M PBS, frozen in isopentane and stored at -80 °C.

Mass Spectrometry analysis of brain nilotinib levels

Fresh frozen hemispheres were used to quantify nilotinib through HPLC-MS/MS analysis (APTUIT, Verona, Italy), by a process previously described (Del Bello et al., 2018). Briefly, after defrosting, brains were weighed, diluted 1:5 with HEPES 0.1 N and homogenized with Precellys 24 homogenizer (Bertin Instruments), three homogenizations at 6100 rpm for 30" with a 15" pause. Calibration curves were prepared the same day of the study using blank matrix from naïve rats. The working solution, obtained by serial dilution in 50% (v/v) acetonitrile in water from a stock solution in DMSO, and spiking in matrix was performed by Hamilton Microlab STARlet. The calibration curves were stored at -20 °C until to the day of analysis. Samples were prepared by protein precipitation (2 volumes of acetonitrile with 20 ng/mL of rolipram used as internal standard (IS)) and vortex-mixed for 5 minutes. After centrifugation (3000 g, 10 min, 4°C), supernatants were transferred and diluted (1:6) with water using Hamilton Microlab STARlet. Chromatography was performed with HPLC (Agilent 1100 Series HPLC) on a phenomenex Synergi RPmax (50x2.1 mm) using a fast chromatography gradient (2 min; phase A 0.1% of formic acid in water, phase B 0.1% of formic acid in acetonitrile; gradient from 95% to 5% of A). The mass spectrometer was an AB Sciex triple quadrupole, API4000. Analytes were monitored in MRM using as quantifier channels 530.1/259.1 and 276.1/208.2 for nilotinib and IS, respectively.

Immunoblotting

For biochemical analysis, mouse striatal tissue was homogenized in lysis buffer (10mM Tris-HCl, 500mM NaCl, 0.5mM 1,4-dithiothreitol, 0.5mM ethylenediaminetetraacetic acid

[EDTA], 1% N-lauroylsarcosine [sarkosyl]) plus protease and phosphatase inhibitor cocktail (Halt, ThermoFisher), then centrifuged at 14 000 rpm for 15 minutes at 4°C. Supernatants were collected and stored at -80°C. Protein concentration was determined by BCA assay (ThermoFisher). Samples containing 10-30µg of protein were prepared in loading buffer (Tris-HCl 25mM pH=6.8, glycerol 7.5%, SDS 1%, DTT 250mM and bromophenol blue 0.05%) and loaded onto 8% SDS-PAGE gels, then transferred to nitrocellulose membranes (Millipore). Membranes were incubated in blocking buffer for 30 minutes (4% bovine serum albumin, BSA in TBS), rabbit anti phospho-c-Abl T245 (1:1000, Cell Signaling #2861) or rabbit anti c-Abl (1:1000, ThermoFisher, PA1-46467). After rinsing with TBS 0.1% Tween (TBS-T) and TBS, membranes were incubated in the corresponding Horseradish Peroxidase (HRP)-conjugated secondary antibody (1:2000, Jackson laboratories). Membranes were revealed through chemiluminescence (Clarity Max, BioRad), images were acquired with the ChemiDoc+XRS gel imaging system (Bio-Rad), and optical density was analyzed with Image Lab (Biorad). Actin was used as loading control (1:10000, Sigma-Aldrich).

For assessment of pY39 α -synuclein, proteins were loaded into 4-20% precast gels, transferred onto nitrocellulose membranes, which were then incubated in 4% v/v PFA in PBS for 30 minutes and later blocked with 2% BSA-TBS-T for one hour. After incubation with a rabbit anti pY39 α -synuclein polyclonal antibody (gift from Dr. T Dawson, 1/1000), membranes were revealed with an anti-rabbit secondary antibody. Next, membranes were reincubated with the mouse monoclonal anti-human α -synuclein antibody (ThermoFisher clone 4B12, MA1-90346), and later revealed with an anti-mouse secondary antibody as described above.

Histopathological analysis in mice

After washing with PBS 0.1M (PBS), 40µm sections of the SNc were incubated in a citrate solution (pH=6, Dako Target Retrieval Solution, diluted in distilled water) and heated at 80°C

for 30 minutes for antigen retrieval. Sections were then washed with PBS and incubated in a 0.3% hydrogen peroxide solution (PBS dilution) for 10 min to quench endogenous peroxidases. After rinsing, sections were incubated in PBS with 2% BSA, 0.3% Triton X-100 solution for one hour at room temperature (RT). Then, they were incubated with the primary anti-TH mAB 318 antibody (Millipore, 1:4000, PBS dilution, 1% BSA, 0.3% Triton X-100) overnight at RT. After rinsing, sections were incubated in goat anti-mouse EnVision-HRP enzyme conjugate (Dako) anti-mouse polymer for 30 minutes, rinsed again and then revealed with the Dako DAB kit following the supplier's instructions. Finally, sections were mounted and dried on gelatinized slides, immersed overnight in a solution composed of 50% absolute ethanol and 50% chloroform, and stained with a cresyl-violet solution. A series of SNc (1/4, allowing the counting of 5 sections) was used per animal for the stereological counting of TH-positive and Nissl-positive neurons. Systematic random sampling was performed with the Mercator Pro V6.5 (Explora Nova) software coupled with a Leica DM-6000B microscope. After delineation of the SNc with x5 objective, counting was done with x40 objective (Fernagut et al., 2014a).

Striatal sections of PLP-SYN mice were treated to eliminate proteinase-K sensitive forms of α -synuclein. The number of α -synuclein positive cells was counted by fluorescence microscopy. For the detection of oligodendroglial α -synuclein aggregates, striatum sections were first washed three times in PBS, and incubated for 10 minutes at RT in a 10mg/mL solution of proteinase-K (Sigma, diluted in PBS). Sections were then rinsed twice in distilled water and twice in PBS for 10 minutes each, at RT to wash proteinase K. Later they were immersed in the blocking solution (30 min at RT) followed by the primary rabbit anti-human α -synuclein MJFR1 antibody (1:10 000, overnight at RT). After rinsing, sections were incubated in secondary goat anti-rabbit 568 antibody (1:400, Alexa Fluor) for two hours at RT, rinsed in PBS and mounted on slides with a Vectashield + DAPI mounting medium. For immunofluorescent labelling of α -synuclein, one striatum slice per animal was used. Six

captures per mouse striatum in specific areas were taken with a x20 objective and the number of α -synuclein positive cells was counted using ImageJ.

Statistics

Statistical analysis was performed using GraphPad Prism (version 8; GraphPad Software Inc., La Jolla, CA). Mean \pm standard error of the mean (SEM) was used to present the results. For comparisons between two groups, a *t*-test was used if data were normally distributed. If not, a Mann Whitney test was used instead. For the comparison of several groups, a one-way ANOVA was applied followed, if appropriate, by a Bonferroni post hoc test in case of a normal distribution and a Kruskal-Wallis test followed, where appropriate, by a Dunn multiple comparison in case of a non-Gaussian distribution. For a two-factor comparison, a two-way ANOVA was used followed, if appropriate, by Tukey's post hoc test. A *p* value <0.05 was considered statistically significant.

RESULTS

pY39 α -synuclein is present in GCIs in MSA

Double immunofluorescence for α -synuclein and pY39 α -synuclein revealed that most α -synuclein-positive GCIs also contained pY39 α -synuclein (Fig.1 A-C). On average, $91.7 \pm 2.9\%$ of α -synuclein-positive GCIs also stained positive for pY39 α -synuclein (Fig. 1D).

c-Abl activation and pY39 α -synuclein levels are increased in PLP-SYN in mice

We first assessed the phosphorylation state of c-Abl in PLP-SYN mice, and found that the phosphorylated c-Abl at tyrosine 245 (pY245) to total c-Abl ratio was significantly increased in adult PLP-SYN compared to age matched WT mice ($p < 0.05$) (Fig.1 E-F). Levels of pY39

α -synuclein were similarly significantly elevated in PLP-SYN mice compared to non-transgenic animals ($p=0.001$) (Fig.1 G-H).

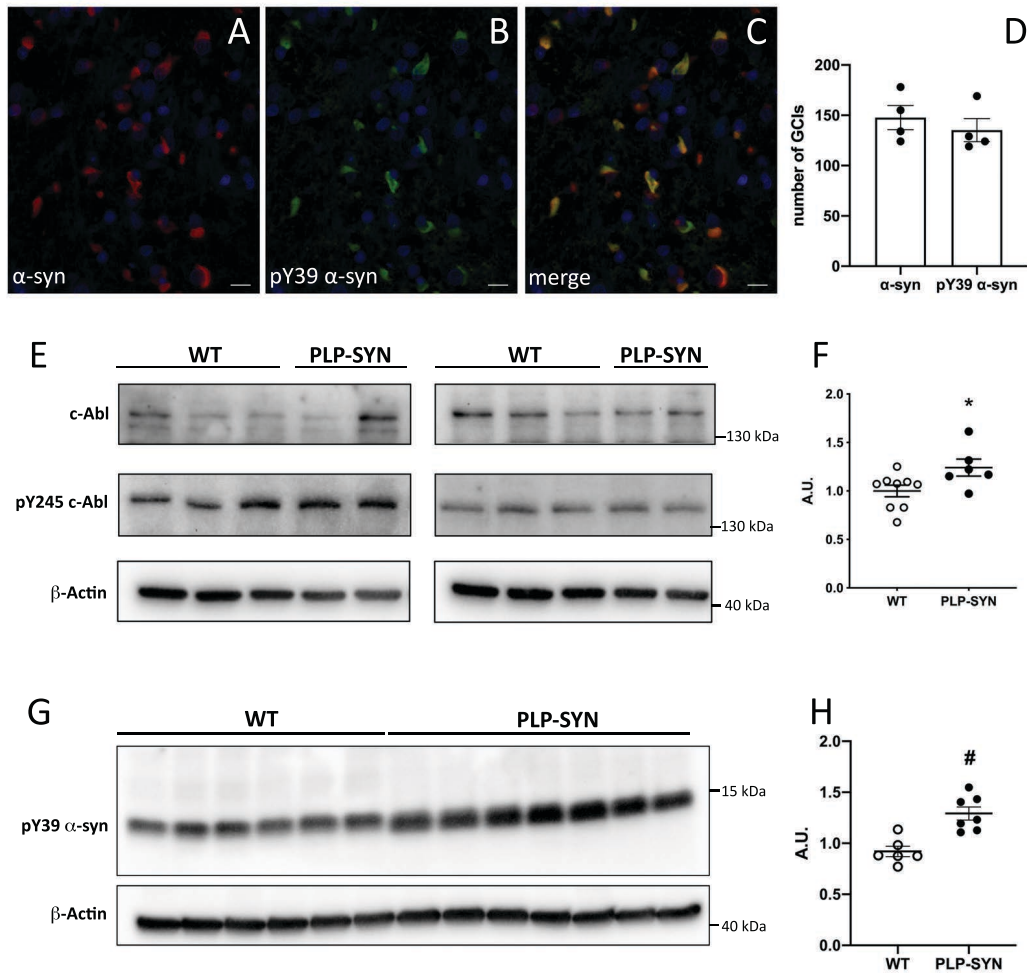


Figure 1: α -synuclein phosphorylated at tyrosine 39 (pY39) in MSA and PLP-SYN mice, and c-Abl activation in the PLP-SYN mouse model.

Immunofluorescence for α -synuclein (A) and pY39 α -synuclein (B) in the putamen of MSA patients. Over 90% of α -synuclein-positive GCIs are also positive for pY39 α -synuclein (C, D). Western blot analysis of total and phosphorylated c-Abl (pY245 c-Abl) demonstrates increased activation of c-Abl in PLP-SYN in mice, as shown by an increased ratio of phosphorylated over total c-Abl compared with WT mice (E, F). Western blot analysis of pY39 α -synuclein PLP-SYN mice demonstrated elevated ratio of pY39 α -synuclein over total human alpha-synuclein compared to WT mice (G, H). *, $p<0.05$; #, $p=0.001$. Scale bar = 10 μ m

Nilotinib reduces activation of c-Abl in PLP-SYN in mice

To confirm brain penetration of nilotinib, a dedicated group of mice received daily i.p. injections of 10mg/kg nilotinib during five days. Nilotinib was detected in brain homogenates by Mass Spectrometry, indicating that the dose used in this study results in significant increase levels of nilotinib in the brain (Fig. 2A). To confirm target engagement with the dose of 10mg/kg, we observed a significant reduction of the pY245 to total c-Abl ratio by Western blot in nilotinib-treated animals compared to those that received vehicle (Fig.2 B, $p < 0.0001$).

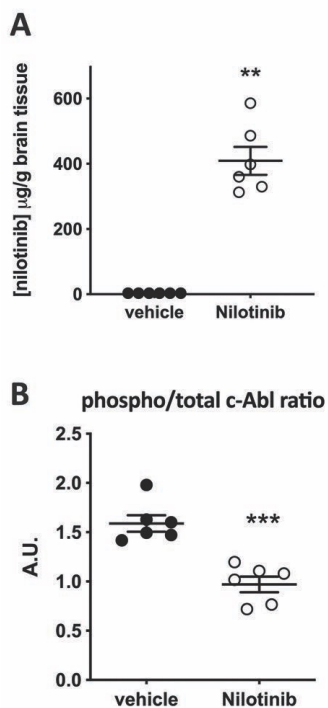


Figure 2: *Brain bioavailability of nilotinib and target engagement following nilotinib administration.*

Mass spectrometry (HPLC MS/MS) analysis of PLP-SYN in brains demonstrates that systemic administration of 10mg/kg leads to significant brain levels of nilotinib (A). Western blot analysis of total and phosphorylated c-Abl demonstrates that chronic administration of nilotinib (10mg/kg) reverses the hyperactivation of c-Abl in PLP-SYN in mice, as shown by a decreased ratio of phosphorylated over total c-Abl compared with vehicle treated animals (B). **, $p < 0.01$; ***, $p < 0.001$.

Nilotinib does not modify the number of α -synuclein aggregates in the striatum of PLP-SYN mice despite reducing levels of pY39 α -synuclein

Counts of alpha-synuclein positive inclusions in the striatum of placebo-treated mice (Fig.3 A), PLP-SYN mice given a daily dose of 1mg/kg (Fig.3 B) or 10mg/kg (Fig.3 C) revealed no effect of nilotinib on α -synuclein inclusions burden in the striatum ($F(2,22) = 2.3$ and $p > 0.1$, Fig.3 D). Biochemical analysis of striatal levels of pY39 α -synuclein over total human α -synuclein revealed a significant decrease following nilotinib treatment ($F(2,19) = 21.29$, $p < 0.0001$, Fig.3 E-F). Given these results, we then assessed whether nilotinib would nevertheless lead to a preservation of nigral dopaminergic neurons.

Nilotinib has no effect on degeneration of dopaminergic neurons in the SNc

PLP-SYN mice classically show about 30-40% loss of TH-positive neurons in the SNc, (Fernagut et al., 2014b; Refolo et al., 2018; Stefanova et al., 2005). We evaluated the therapeutic efficacy of nilotinib on TH-positive neuron survival within the SNc (Fig. 4). A one-way ANOVA revealed differences between groups in dopaminergic cell counts in the SNc [$F(3,30) = 7$, $p < 0.005$]. A post-hoc analysis found the expected 25% loss of TH-positive neurons in placebo PLP-SYN compared to placebo WT mice ($p < 0.05$). It also revealed a lack of protection of dopaminergic neurons for the low and high dose of nilotinib, as cell counts were also significantly reduced in these groups compared to WT mice ($p < 0.05$ and $p < 0.001$ respectively, Fig.4) and did not differ from PLP-SYN placebo mice. An overall analysis of Nissl-positive neurons showed a significant difference between groups [$F(3,30) = 5$, $p < 0.005$], and the post-hoc analysis confirmed that the low and high dose of nilotinib did not preserve PLP-SYN in mice from SNc neural loss ($p < 0.05$) (Fig.4).

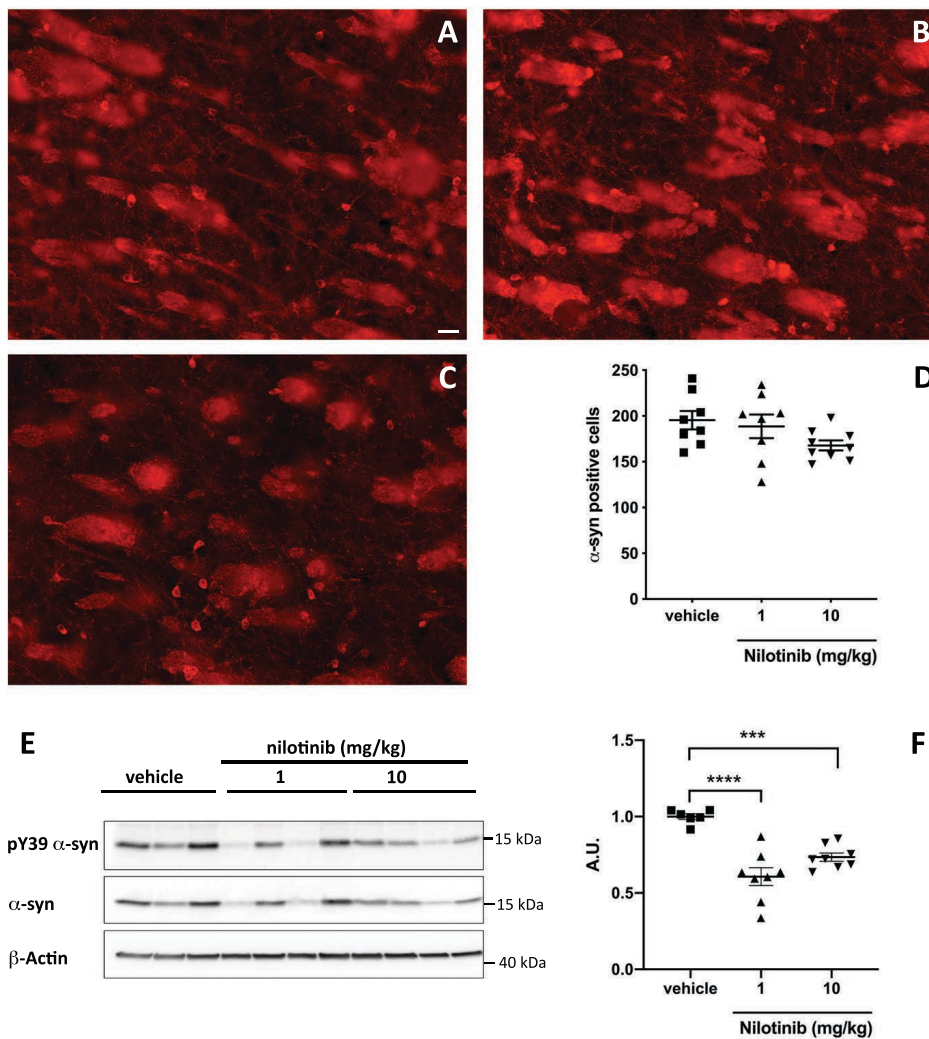


Figure 3: *Effects of chronic administration of nilotinib on pY39 α-synuclein and α-synuclein inclusion burden.*

Representative images of α-synuclein immunofluorescence in the striatum of PLP-SYN mice treated with vehicle (A), 1mg/kg (B) and 10mg/kg nilotinib (C). Cell counts of α-synuclein immunopositive oligodendrocytes revealed no effect of nilotinib on striatal α-synuclein burden (D). Western blot analysis revealed a significant effect of both doses of nilotinib on pY39 α-synuclein levels over total human alpha-synuclein (E, F). ***, $p < 0.001$; ****, $p < 0.0001$. Scale bar = 20 μm

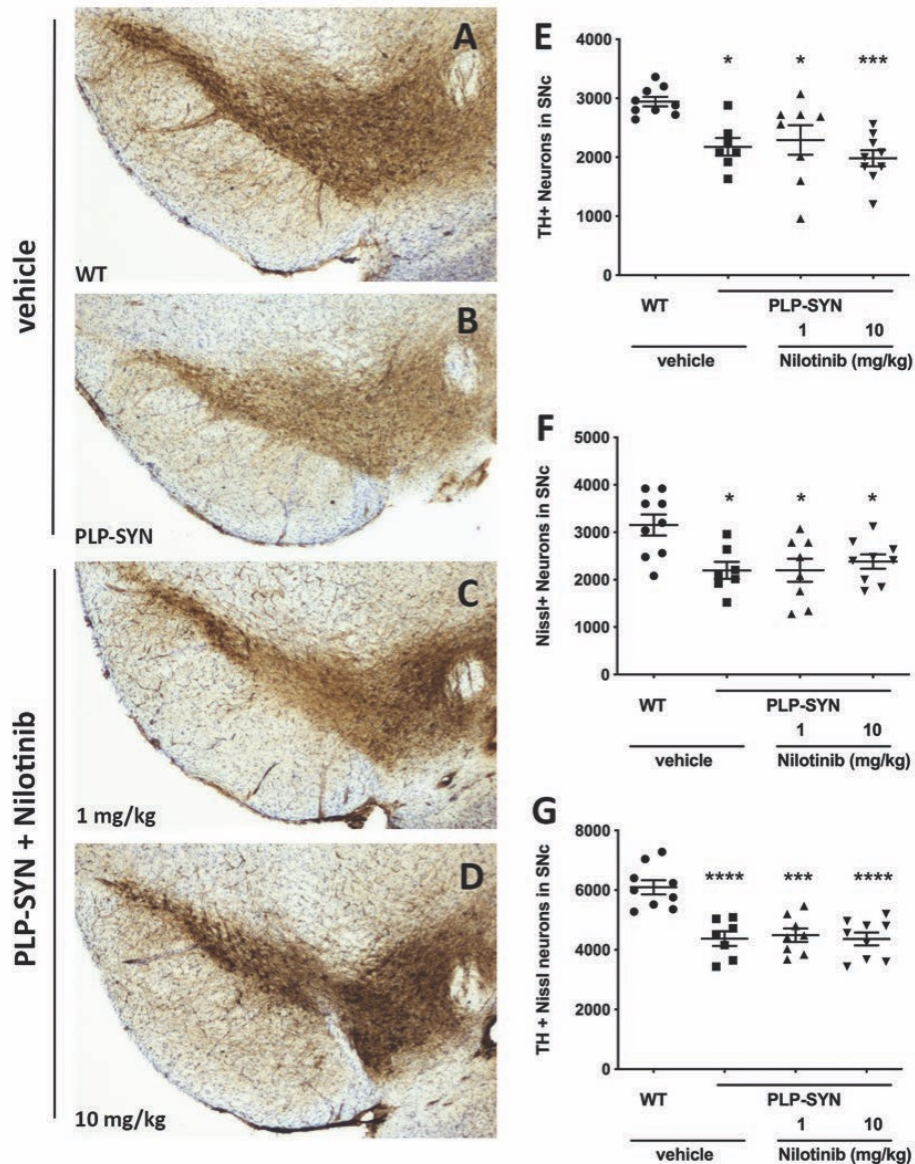


Figure 4: *Effects of chronic administration of nilotinib on dopaminergic neurodegeneration.*

Representative images of tyrosine hydroxylase (TH) immunoreactivity in WT mice (A), PLP-SYN in mice treated with vehicle (B), 1mg/kg (C) and 10mg/kg nilotinib (D). Stereological counts of TH-positive neurons demonstrate that the loss of neurons in PLP-SYN in mice is not mitigated following chronic administration of nilotinib (E). Stereological counts showing a loss Nissl-stained and TH+Nissl stained neurons in PLP-SYN mice (F, G) indicate that the observed loss of TH-positive neurons is not attributable to a loss of the TH-phenotype. *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

DISCUSSION

Nilotinib has emerged as a putative therapeutic option for synucleinopathies based on preclinical data showing its neuroprotective potential owing to inhibition of α -synuclein phosphorylation at tyrosine 39 and on an open label trial in 12 patients with PD or DLB (Pagan et al., 2016). Whether these encouraging findings may translate to all synucleinopathies is currently unknown.

This is the first report of the effects of nilotinib in a preclinical model of MSA. Here, we show that, in contrast to previous reports in PD models and despite adequate target engagement, nilotinib failed to reduce α -synuclein burden in the striatum and to rescue dopamine neurons in the SNc from cell death.

The absence of neuroprotection encountered cannot be explained by a lack of brain bioavailability or activity of nilotinib. Nilotinib was present in brains 4hs after i.p. injection as revealed by Mass Spectrometry, and Western blotting demonstrated that the treatment regimen used in our study significantly reduced the c-Abl phosphorylation ratio in the striatum, thereby confirming target engagement.

pY39 α -synuclein has been reported to be present in normal and PD human brains. We here confirm that pY39 α -synuclein is also present in GCI, indicating that this post-translational modification of α -synuclein occurs in MSA. Increased levels of pY39 α -synuclein were also detected in the striatum of the PL-SYN mouse model of MSA, indicating that oligodendroglial overexpression of human wild-type α -synuclein leads to an increased occurrence of this phosphorylation. We also demonstrate that inhibition of c-Abl with nilotinib results in reduction of pY39 α -synuclein levels, but fails to impact α -synuclein inclusions burden and nigral neuronal death. As previously mentioned, phosphorylation of this residue promotes α -synuclein aggregation and inhibits autophagic clearance of α -synuclein in animal models of PD

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differences that impede directly extrapolating pathogenic hypothesis between different synucleinopathies. While it is evident that α -synuclein in PD inclusions originates and is metabolically processed in neurons, how α -synuclein becomes part of GCI is more complex. Latest data suggest that oligodendrocytes uptake abnormal exogenous neuron-secreted α -synuclein, which would, in turn, recruit the endogenous oligodendrocyte-expressed α -synuclein (Mavroei et al., 2019; Reyes et al., 2014). Differences in the time, place and type of the post-translational modifications α -synuclein can undergo, may dictate the already known distinct features of α -synuclein conformers in MSA and PD (Peng et al., 2018; Yamasaki et al., 2019). In this context, it is unknown where and when phosphorylation at Y39 takes place in MSA and PD and its impact on the distinct protein seeds. As our results demonstrate a lack of preclinical efficacy of nilotinib, unlike in PD models, they suggest that pY39 α -synuclein might not be equally relevant to the neurodegenerative process in MSA and could, together with the differences in parkin involvement aforementioned, explain the lack of neuroprotective effect of nilotinib in the PLP-SYN model of MSA.

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Article 3:

Inhibition of the mammalian target of rapamycin alleviates alpha-synuclein aggregation and does partly protect dopamine neurons in a mouse model of multiple system atrophy

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(Soumis)

Contexte : L'atrophie multisystematisée (AMS) est une maladie neurodégénérative rare sans traitement efficace. Elle est caractérisée par l'accumulation de la protéine α -synucléine dans les inclusions oligodendrogiales. À ce titre, l'AMS forme le groupe des synucléinopathies avec la maladie de Parkinson (MP) et la démence à corps de Lewy (DCL). Des études post-mortem suggèrent une altération des mécanismes de dégradation des protéines dans l'AMS, dont l'autophagie. Il est connu que l'inhibition de mTOR par la rapamycine augmente l'activité autophagique, et il a été démontré qu'elle atténue l'agrégation des protéines dans des modèles de MP et d'autres protéinopathies.

Objectifs : Évaluer l'efficacité de la rapamycine dans le modèle préclinique de l'AMS.

Méthodes: Les souris exprimant l' α -synucléine de type sauvage humaine dans les oligodendrocytes ont reçu des aliments enrichis en rapamycine pendant 16 semaines. L'analyse post-mortem comprenait l'évaluation des marqueurs d'autophagie, la quantité intracellulaire de α -synucléine et de la neurodégénérescence dopaminergique. Une analyse biochimique des tissus humains a été effectuée pour évaluer l'activation de la voie mTOR.

Résultats : Une activation accrue de mTOR et de ses effecteurs, ainsi qu'une altération de l'autophagie ont été détectées chez les patients atteints d'ASM. L'inhibition de mTOR a permis une réduction de l'agrégation de l'alpha-synucléine et un effet protecteur partiel des neurones dopaminergiques, sans pourtant rétablir l'autophagie chez la souris transgénique d'AMS.

Conclusion : Nos résultats suggèrent que l'inhibition de mTOR par la rapamycine est insuffisante pour restaurer l'autophagie et atténuer le processus neurodégénératif chez les souris transgéniques d'AMS.

Inhibition of the mammalian target of rapamycin alleviates alpha-synuclein aggregation but only partly protects dopamine neurons in a mouse model of multiple system atrophy

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Keywords: α -synuclein, pathophysiology, treatment

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Abstract

Background: Multiple system atrophy (MSA) is a rare untreatable neurodegenerative disorder, characterized by accumulation of the protein alpha-synuclein in oligodendroglial inclusions. As such, MSA is a synucleinopathy along with Parkinson's disease (PD) and Dementia with Lewy bodies. Post-mortem studies suggest an impairment of the protein degradation machinery in MSA, including autophagy. Inhibition of mTOR by rapamycin is known to increase autophagic activity, and has been shown to reduce protein aggregation in models of PD and other proteinopathies.

Objectives: To assess the efficacy of rapamycin in a transgenic mouse model of MSA.

Methods: Mice expressing human wild-type alpha-synuclein in oligodendrocytes received rapamycin-enriched food during 16 weeks. Post-mortem analysis included assessment of autophagy markers, intracellular alpha-synuclein burden and loss of nigral dopamine neurons. Biochemical analysis of human brain tissue was performed to assess the status of the mTOR pathway.

Results: Increased activation of mTOR and downstream effectors, and alteration of autophagy were detected in MSA patients. Inhibition of mTOR with rapamycin did not restore markers of autophagy in the transgenic MSA mice, while it reduced alpha-synuclein aggregation and partially protected dopaminergic neurons from death.

Conclusion: Our results suggest that inhibition of mTOR is insufficient to restore autophagy and mitigate the neurodegenerative process in transgenic MSA mice.

INTRODUCTION

Synucleinopathies are a group of disorders characterized by alpha-synuclein (α -syn) accumulation in intracytoplasmic inclusions in the central and peripheral nervous system (CNS and PNS). These include Parkinson's disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA), and affect millions of patients worldwide (Spillantini and Goedert, 2000).

MSA is a rare, sporadic adult-onset neurodegenerative disorder clinically characterized by a variable combination of parkinsonism, cerebellar impairment, and autonomic dysfunction (Gilman et al., 2008). The cytopathological hallmark of MSA is the accumulation of α -syn aggregates in oligodendrocytes, forming glial cytoplasmic inclusions (GCIs). Currently, only symptomatic treatments with transient and limited efficacy are available to these patients who face a median life expectancy of 6 to 10 years after symptom onset, with rare cases surviving 15 years or more (Fanciulli and Wenning, 2015).

Degradation of α -syn is achieved by both the ubiquitin-proteasome system and the autosomal-lysosomal pathways (Rivero-Ríos et al., 2016; Snyder et al., 2005). There is genetic and pathological evidence indicating that the different autophagic pathways, macroautophagy (MA) or chaperone-mediated autophagy (CMA), are impaired in synucleinopathies (Arotcarena et al., 2019b). A growing number of autophagy enhancing approaches are being proposed to fight neurodegeneration or have already been tested in animal models of synucleinopathies (Arotcarena et al., 2019a; Decressac et al., 2013; Moors et al., 2017; Scrivo et al., 2018b; Torra et al., 2018).

Mammalian target of rapamycin (mTOR) is a 289-kDa ubiquitously expressed serine/threonine kinase. mTOR forms the catalytic subunit of two distinct complexes, namely mTOR Complex 1 (mTORC1) and 2 (mTORC2), which lies at the center of an intricate signaling pathway that is activated in response to several stress conditions and growth factor signals, regulating a large

number of cellular functions, including autophagy (Zoncu et al., 2011). When active, mTORC1 suppresses autophagy and, conversely, its inhibition induces autophagy (Thoreen et al., 2009). Rapamycin and other mTOR inhibitors have been used for years in clinical practice to treat disorders ranging from tuberous sclerosis, to cancer, or to prevent rejection of organ transplants (Baroja-Mazo et al., 2016; Curatolo and Moavero, 2012; Palavra et al., 2017; Tian et al., 2019). Rapamycin has been reported to have a neuroprotective effect against loss of dopaminergic neurons in toxin-based and transgenic mouse models of PD, as well as to reduce α -syn accumulation, mostly by restoring autophagy (Bové et al., 2011; Crews et al., 2010; Dehay et al., 2010; Pan et al., 2008; Zhang et al., 2017). While there is a growing body of preclinical evidence pointing to inhibition of mTOR as a possible disease-modifying option in PD, no such advances have been made in other synucleinopathies, namely MSA. In the current study, we determined whether daily administration of rapamycin for 16 weeks has beneficial effects on the aggregation of α -syn and the neurodegenerative process in a transgenic mouse model of MSA.

MATERIAL AND METHODS

Study approval

All experiments involving mice were performed in accordance with French guidelines (87-848, Ministère de l'Agriculture et de la Forêt) and the European Community Council Directive (2010/63/EU) for the care of laboratory animals. Animal experiments were approved by the Institutional Animal Care and Use Committee of Bordeaux (CE50, approval #7371-2016101216326975). Mice were maintained in a temperature and humidity - controlled room on a 12:12 light-dark cycle with food and water ad libitum.

Human brain samples

Human brain samples were obtained from the French Brain Bank (GIE-Neuro CEB, BB-0033-00011), Paris, France. Written informed consent was obtained prior to autopsy for the collection of the brain and the use of clinical and post-mortem data from all subjects or their legal representatives. Samples from 7 MSA patients with predominant parkinsonism (MSA-P) and 6 age-matched controls were processed for biochemical analysis.

Animals and treatment

Mice expressing human α -syn in oligodendrocytes under the control of the proteolipid promoter (PLP-SYN) were previously generated on a C57BL/6J background. Overexpression of α -syn in oligodendrocytes promotes accumulation of α -syn and neurodegeneration resembling MSA (Kahle et al., 2002). Rapamycin (Rapamycin Holding, USA) was microencapsulated in Eudragit and incorporated in standard mouse chow (Envigo) at a dose of 14mg/kg of food, chosen based on previously reported successful dosages (Fok et al., 2014; Harrison et al., 2009). Treatment began at age 6 weeks, with one group of transgenic and non-transgenic control mice receiving standard food and a third group rapamycin-enriched food.

Tissue extraction

Animals were anaesthetized with pentobarbital (100 mg/kg i.p.). They were then intracardially perfused with 25ml of 0.9% saline. After quick removal of the brain, the right hemisphere was directly frozen in isopentane for biochemical analysis, while the left hemisphere was post-fixed for 5 days in 5ml of 4% (wt/vol) paraformaldehyde (PFA), followed by cryoprotection in 10ml of 20% (wt/vol) sucrose in 0.1 M PBS, frozen in isopentane and stored at -80 °C

Antibodies

Primary antibodies used for biochemistry and histology experiments were: rabbit monoclonal antibody anti-human α -syn, purchased from Abcam (MJFR1, 1:10 000); the rabbit polyclonal antibodies anti-mTOR, anti-phospho-mTOR (Ser2448), anti-p70S6 Kinase and anti-phospho-p70S6 Kinase (Thr389) were purchased from Cell Signaling Technology (297S, 2971S, 9202S and 9205S, 1:1000); rabbit polyclonal antibodies against beclin-1 and LC3, purchased from Novus Biologicals (NB100 and NB500, 1:1000); the mouse monoclonal antibody anti-Tyrosine Hydroxylase was obtained from Millipore (mAb318, 1:4000); the guinea pig polyclonal anti-p62 antibody was purchased from ProGen (GP62-C-WBC, 1:1000) and the mouse anti- β -actin antibody was purchased from Sigma-Aldrich (1:10 000).

Immunoblotting

For biochemical analysis, tissue samples were homogenized in RIPA lysis buffer (Sigma) plus protease and phosphatase inhibitor cocktail (Halt, ThermoFisher), then centrifuged at 14 000 rpm for 15 minutes at 4°C. Supernatants were collected and stored at -80 °C. Protein concentration was determined by BCA assay (ThermoFisher).

Samples containing 10-40ug of protein were prepared in loading buffer (Tris-HCl 25mM pH=6.8, Glycerol 7.5%, SDS 1%, DTT 250mM and Bromophenol Blue 0.05%) and loaded onto SDS-PAGE, proteins then were transferred to 0.2 μ m pore nitrocellulose membranes (Millipore). When assessing phosphorylated proteins, membranes were blocked during 60 minutes in 4% Bovine Serum Albumin (BSA) in TBS; for non-phosphorylated proteins, 3% non-fat dry milk in PBS was used instead. Membranes were incubated overnight at 4°C with the primary antibody in 2% BSA in TBS. After rinsing with TBS 0.1% Tween (TBS-T) and TBS, membranes were incubated in the corresponding Horseradish Peroxidase (HRP)-conjugated secondary antibody. Membranes were revealed through chemiluminescence

(Clarity ECL or Clarity Max ECL substrates, BioRad), images were acquired with the ChemiDoc+XRS gel imaging system (Bio-Rad).

Histopathological analysis in mice

After washing with PBS 0.1M (PBS), 40 μ m sections of the substantia pars compacta (SNpc) were incubated in a solution of citrate x1 (pH=6, Dako Target Retrieval Solution, diluted in distilled water) and heated at 80°C for 30 minutes for antigen retrieval. Sections were then washed with PBS and incubated in a 3% hydrogen peroxide solution (PBS dilution) for 10 min to quench endogenous peroxidases. After rinsing, sections were incubated in PBS with 2% BSA, 0.3% Triton X-100 solution for one hour at room temperature (RT). Then, they were incubated with the primary anti TH antibody overnight at RT. After rinsing, sections were incubated in Dako Envision anti-mouse polymer for 30 minutes, rinsed again and then revealed with the Dako DAB kit following the supplier's instructions. Finally, sections were mounted and dried on gelatinized slides, immersed overnight in a solution composed of 50% absolute ethanol and 50% chloroform, and stained with a cresyl-violet solution. A series of SNpc (1/4, allowing the counting of 5 sections) was used per animal for the stereological counting of tyrosine hydroxylase (TH)-positive and Nissl-positive neurons. Systematic random sampling was performed with the Mercator Pro V6.5 (Explora Nova) software coupled with a Leica DM-6000B microscope. After delineation of the SNpc with x5 objective, counting was done with x40 objective (Fernagut et al., 2014a).

Striatal sections of PLP-SYN mice were treated to eliminate proteinase K sensitive forms of α -syn. The number of α -syn positive cells was counted by fluorescence microscopy. For the detection of oligodendroglial α -syn aggregates, striatum sections were first washed three times in PBS, and incubated for 10 minutes at RT in a 10mg/mL solution of proteinase K (Sigma,

PBS dilution). Sections were then rinsed twice in distilled water and twice in PBS for 10 minutes each, at RT to wash proteinase K. Later they were immersed in the blocking solution (30min at RT) followed by the primary rabbit anti-human α -syn antibody overnight at RT. After rinsing, sections were incubated in secondary goat anti-rabbit 568 antibody (1:400, Alexa Fluor) for two hours at RT, rinsed in PBS and mounted on slides with a Vectashield + dapi mounting medium. For immunofluorescent labelling of α -syn, one striatum slice per animal was used. Six captures per mouse striatum in specific areas were taken with a x20 objective and the number of α -syn positive cells was counted using ImageJ.

Statistics

Statistical analysis was performed using the software Graphpad Prism (version 8; GraphPad Software Inc., La Jolla, CA). Mean \pm standard error of the mean (SEM) was used to present the results. For comparisons between two groups, a *t*-test was used if data were normally distributed. If not, a Mann Whitney test was used instead. For the comparison of several groups, a one-way ANOVA was applied followed, if appropriate, by Tukey's post hoc test in case of a normal distribution; in case of a non-Gaussian distribution a Kruskal-Wallis test was performed, followed by a two-stage step-up method to correct for multiple comparisons. For a two-factor comparison, a two-way ANOVA was used followed, if appropriate, by Tukey's post hoc test. A p value <0.05 was considered statistically significant.

RESULTS

Alteration of the mTOR pathway and autophagy in the putamen of MSA-P patients

The demographic characteristics of MSA-P patients and control subjects are shown in Table 1. We measured levels of phosphorylated mTOR and total mTOR in the putamen of MSA-P patients and age-matched controls by immunoblotting experiments. We found a significant

reduction in the quantity of total mTOR in the putamen of MSA-P patients compared to controls (t-test, $p < 0.01$), while the ratio of phosphorylated-to-total mTOR was significantly increased in MSA (t-test, $p < 0.05$) (Figure 1). Then, we assessed the downstream effector of mTOR, p70S6K kinase, in its total and phosphorylated states. In line with the mTOR results, we found an increased phosphorylated-total p70S6K ratio in MSA-P patients (Mann Whitney test, $p < 0.05$), while the amount of non-phosphorylated p70S6K was no different between MSA-P and controls (Figure 1).

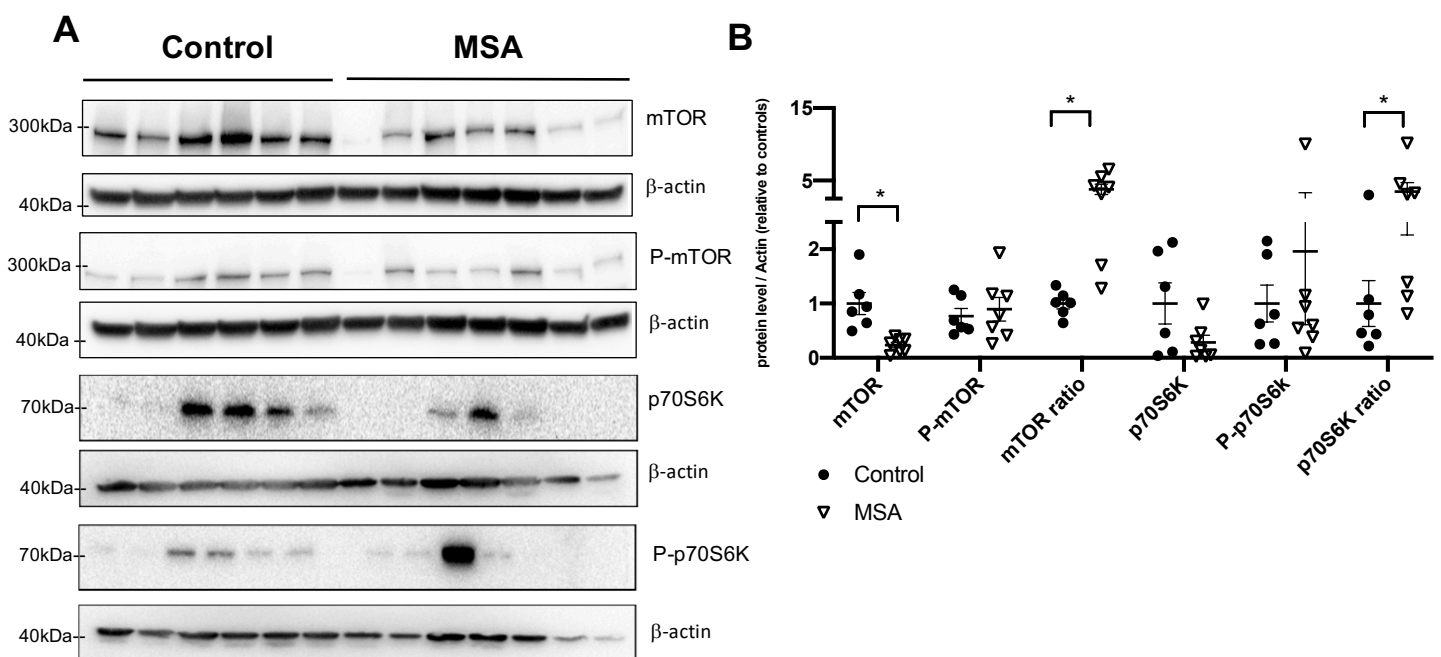


Figure 1: Alteration of the mTOR pathway in the striatum of MSA patients.

Western blot analysis of total and phosphorylated mTOR and its downstream effector p70S6K (A) revealed reduced levels of total mTOR, while the ratio of phosphorylated over total mTOR and p70S6K is increased in MSA compared to healthy controls (B). *, $p < 0.05$

We further compared expression levels of autophagy-related markers. We observed a significant decrease of beclin-1 levels in MSA-P patients compared to controls (Mann Whitney, $p < 0.005$), while there was no significant difference in levels of the p62 protein. In contrast, we

found a higher amount of both forms of LC3, LC3-I and LC3-II, in the putamen of MSA-P patients (t-test, $p < 0.05$ and Mann Whitney, $p < 0.05$, respectively) (Figure 2).

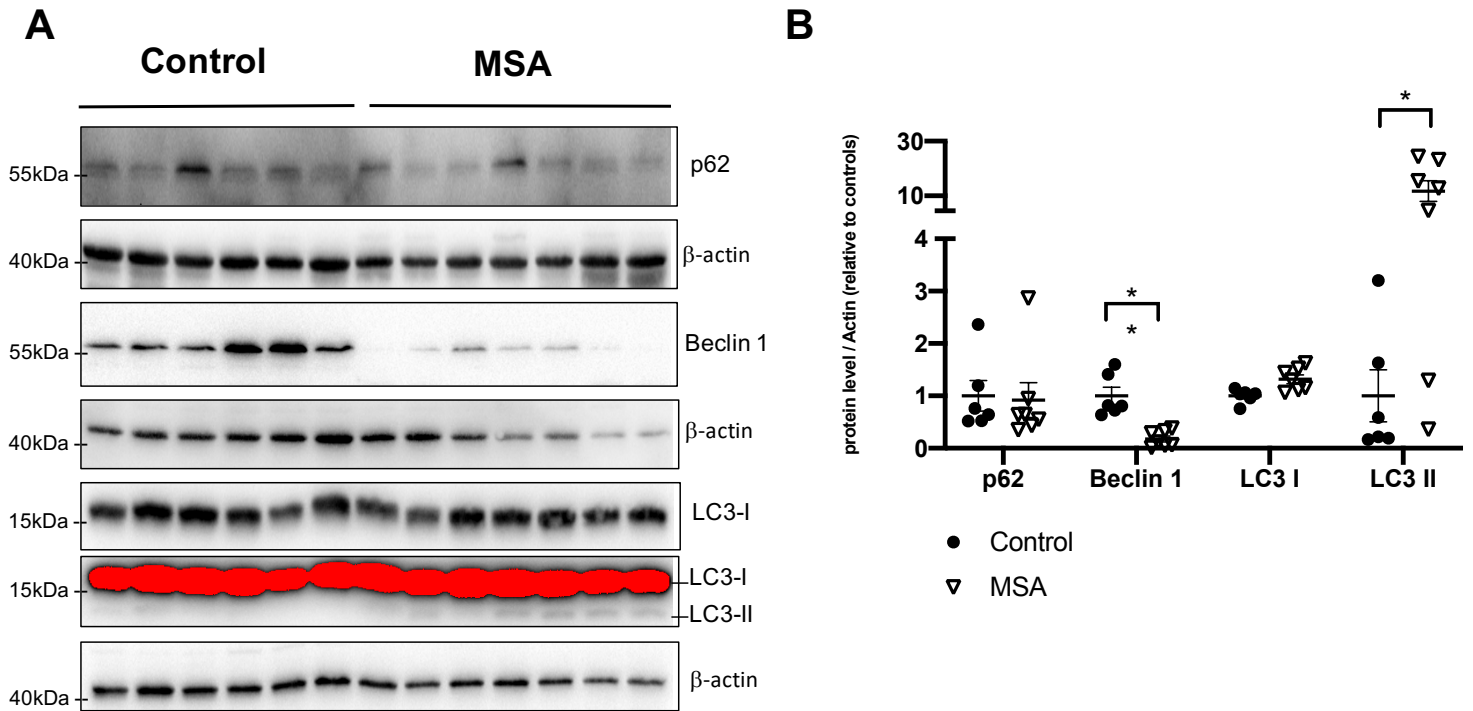


Figure 2: *Alteration of autophagy in the striatum of MSA patients.*

Western blot analysis of markers beclin 1, p62 and LC3 I and LC3 II (A). In MSA patients, levels of the autophagy-initiating protein beclin 1 are reduced, while the membrane-bound form of LC3 (LC3 II) is increased compared to healthy controls (B). *, $p < 0.05$

Autophagy is impaired in the striatum of PLP-SYN mice, while rapamycin fails to restore autophagy

Next, we aimed to determine whether PLP-SYN mice show impairment of autophagy and whether chronic treatment with rapamycin has beneficial effect on autophagy. To this end, we compared autophagy markers in WT and PLP-SYN placebo and rapamycin-treated mice. An overall analysis of beclin-1 levels in the striatum found a difference between groups [F(2, 16)=

20, $p < 0.0001$] and the post-hoc analysis revealed a significant reduction of beclin-1 levels in placebo-treated and rapamycin-treated transgenic mice compared to the non-transgenic group ($p < 0.001$ for both comparisons), while there was no difference between treated- and untreated-PLP-SYN mice (Figure 3). Next, an analysis of p62 levels found a significant difference between groups [$F(2, 16) = 9, p < 0.005$], and a post-hoc comparison confirmed reduction of p62 levels in transgenic mice compared to non-transgenic mice, independent of the treatment received ($p < 0.05$ and $p < 0.005$ for untreated and treated mice respectively) (Figure 3). Finally, we assessed LC3-I and LC3-II levels, and found, again, a significant differences between groups [$F(2, 16) = 6, p < 0.05$], and the post-hoc test revealed a significant reduction of both forms of LC3 in PLP-SYN mice compared to WT mice independently of treatment ($p < 0.05$ for both comparisons) (Figure 3).

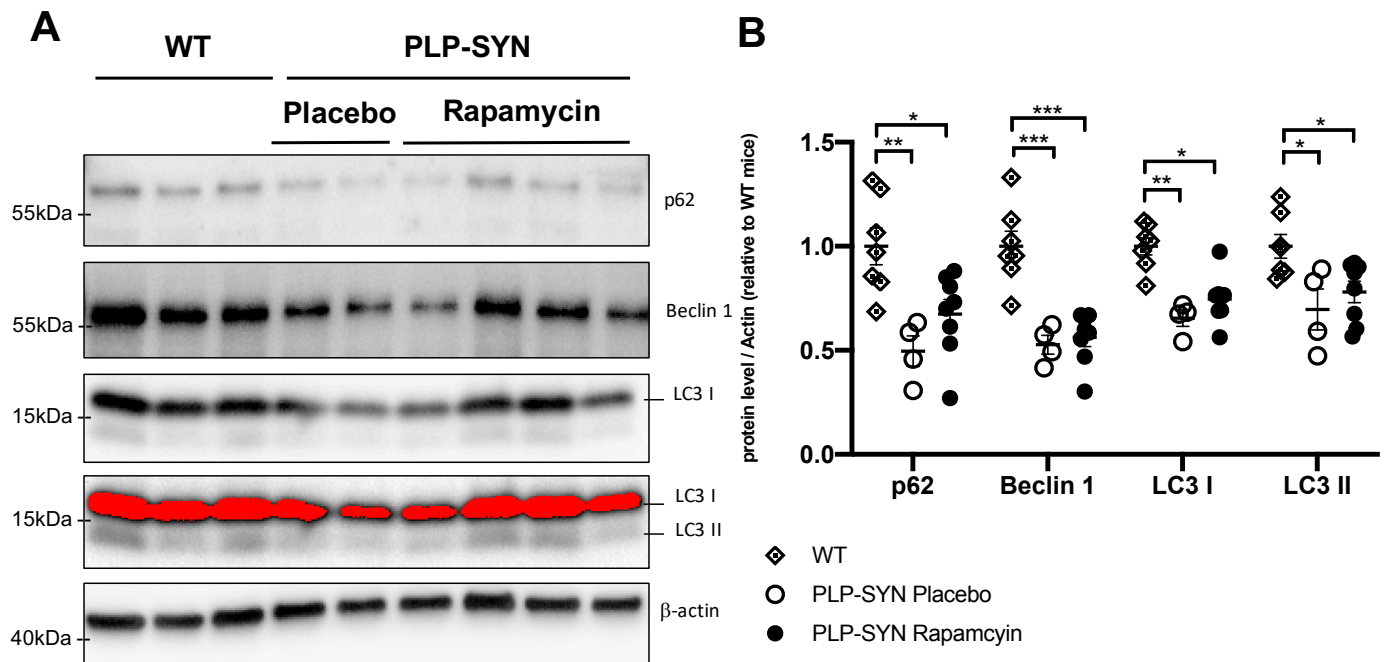


Figure 3: *Autophagy is impaired in PLP-SYN mice, while rapamycin does not restore autophagy*

Western blot analysis of markers of autophagy beclin 1, p62 and LC3 I and LC3 II (A), demonstrates that autophagy is altered in PLP-SYN mice, with reduced levels of all markers of autophagy compared to non-

transgenic mice. Treatment with rapamycin did not restore their levels to normal (**B**). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Rapamycin reduced α -syn accumulation in the striatum of PLP-SYN mice

We found that rapamycin-treated mice had, in average, a 32% lower amount α -syn-positive cells in the striatum compared to placebo treated PLP-SYN mice ($p < 0.05$, Figure 4 A-C).

Rapamycin partially protected dopaminergic neurons in the SNpc from death of PLP-SYN mice

PLP-SYN mice classically show about 25-30% loss of TH-positive neurons in the SNpc, (Fernagut et al., 2014b; Refolo et al., 2018; Stefanova et al., 2005). We assessed the effect of rapamycin on TH-positive neuron survival within the SNpc. The overall analysis revealed differences between groups in dopaminergic cell counts in the SNpc ($H = 12.9$, $df = 2$, $p < 0.005$). The post-hoc test found an expected 30% loss of TH-positive neurons in placebo-treated PLP-SYN mice compared to WT mice ($p < 0.0005$). This analysis also revealed a lack of protection of TH expressing dopaminergic neurons following rapamycin treatment, as cell counts were also significantly reduced in this group compared to WT mice ($p < 0.01$ Figure 4 D-G), and did not differ from the placebo-treated PLP-SYN group. A comparative analysis of Nissl-positive neurons showed a significant difference between groups [$F(2,21) = 5$, $p < 0.05$], and a post-hoc test found that placebo-treated mice had a lower number of Nissl-positive neurons than the WT and rapamycin-treated mice ($p < 0.05$ for both comparisons), while there was no difference between the latter two (Figure 4 H).

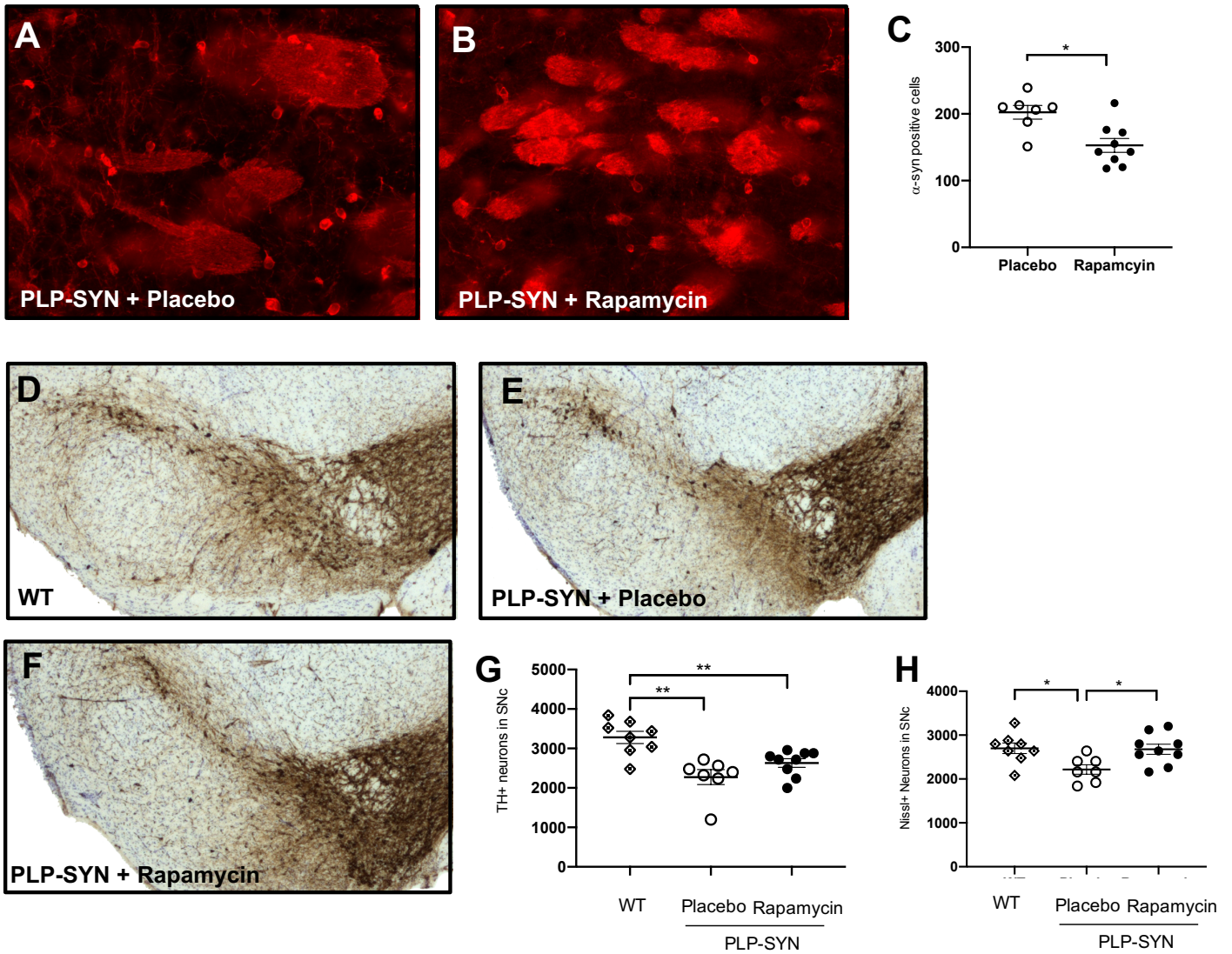


Figure 4: *Effects of chronic treatment with rapamycin on α -synuclein burden and dopaminergic neurodegeneration.*

Representative images of α -syn immunofluorescence in the striatum of PLP-SYN mice treated with normal food (A) and rapamycin-enriched food (B). Cell counts of α -synuclein immunopositive oligodendrocytes revealed that rapamycin reduces striatal α -syn burden (C). Representative images of tyrosine hydroxylase (TH) immunoreactivity in WT mice (D), PLP-SYN in mice treated with normal food (E) and rapamycin-enriched food (F). Stereological counts showing a loss Nissl-stained and TH+Nissl stained neurons in PLP-SYN mice (F, G) indicate that the observed loss of TH-positive neurons is due to a loss of the TH-phenotype.

*, $p < 0.05$; **, $p < 0.01$

Rapamycin inhibits the downstream targets of mTOR

To assess target engagement by rapamycin, we compared striatal levels of total and phosphorylated p70S6K. While a one-way ANOVA analysis revealed no differences between groups in the amount of p70S6K phosphorylated at residue Thr389 (Figure 5), an overall analysis of the phosphorylated-total protein ratio revealed a significant difference between groups [F(3, 23)= 4, p<0.05]. The Tukey's comparison test showed a significant decrease of the ratio in the rapamycin-treated PLP-SYN mice compared to the placebo treated group (p< 0.05) (Figure 5).

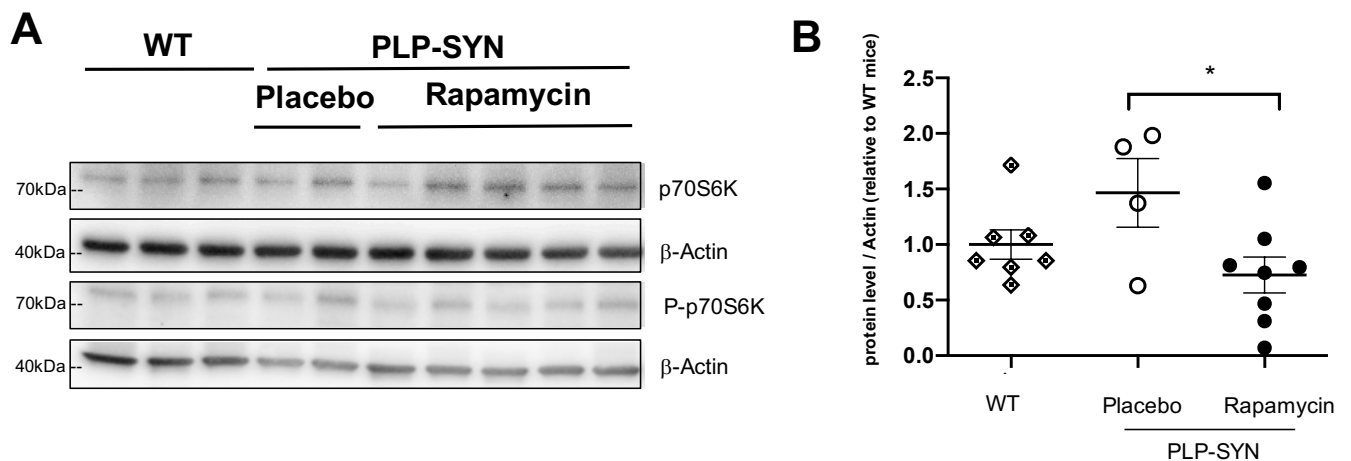


Figure 5: Chronic treatment with rapamycin induces an inhibition downstream of mTOR

Western blot analysis of total and phosphorylated forms of p70S6K, a downstream effector of mTOR (A), demonstrated an inhibition of the kinase activity of mTOR, as shown by a reduced ratio of phosphorylated over total p70S6k compared to placebo treated animals (B). *, p<0.05.

DISCUSSION

Evidence from *in vitro* and *post-mortem* studies point to an impairment of autophagy in MSA (Arotcarena et al., 2019b; Compagnoni et al., 2018; Schwarz et al., 2012; Tanji et al., 2013), thus, enhancing autophagy has emerged as a putative approach for preventing accumulation of

α -syn in synucleinopathies (Arotcarena et al., 2019a; Decressac et al., 2013; Moors et al., 2017; Scrivo et al., 2018b; Torra et al., 2018).

We here have shown an increased activation of the mTOR pathway in the putamen of MSA patients compared to controls. Also, we have found an impaired autophagy, as evidenced by a decrease in beclin-1 levels, unchanged levels of p62 and increased expression of both LC3-I and LC3-II. Further, we showed that autophagy is also impaired in the PLP-SYN mouse model of MSA. While inhibition of mTOR with rapamycin proved to reduce the burden of α -synuclein aggregates in the striatum, rapamycin exerted only a partial neuroprotective effect on dopaminergic neurons.

Dysregulation of the mTOR pathway and autophagy impairment in the putamen of MSA patients

We demonstrated a reduction of mTOR in the putamen, which is in agreement with reduced mRNA levels of mTORC1 in the striatum of MSA patients as previously reported (Valera et al., 2017b). We also found an increase in its phosphorylation ratio, which confers a higher kinase activity. mTOR activates the p70S6K protein through phosphorylation (Hara et al., 1998), and our results confirmed an enhanced activation downstream of mTOR, as illustrated by the elevated ratio of phosphorylated-total p70S6K in MSA patients.

Similar to previous reports, we observed a decrease in the autophagy protein beclin-1 in the putamen of MSA cases (Figure 3) (Valera et al., 2017b). Beclin-1 was found to colocalize with α -syn in GCIs in MSA brains, suggesting an involvement in MSA pathology (Valera et al., 2017b). Expression of beclin-1 has also been shown to be reduced in Alzheimer's disease (AD) (Bieri et al., 2018; Lucin et al., 2013; Pickford et al., 2008). Hence, as we here showed, there is a reduction in autophagy activation and autophagosome elongation, in line with a reduction of beclin-1 (Bové et al., 2011). Regarding p62, we found no difference between controls and

MSA-P (Figure 3). Others have reported reduced levels of p62 in the striatum of MSA patients (Valera et al., 2017b), while increased levels were observed in an *in vitro* model of oligodendroglial pathology (Schwarz et al., 2012). Finally, LC3 is a membrane-bound protein, and its lipidated form, LC3-II, is the signature component of autophagic membranes. We observed increased levels of LC3 in both its forms, LC3-I and LC3-II, in the putamen of MSA-P patients compared to controls (Figure 1). In the context of reduced autophagosome elongation, this could be a result of their defective clearance in MSA (Arotcarena et al., 2019b). Previous literature reported the presence of LC3 inside of GCI, pointing to its involvement in MSA (Schwarz et al., 2012); furthermore, another study found LC3-II accumulation in the cytoplasm of induced-pluripotent stem cells derived from MSA patient skin fibroblasts associated with a defect in lysosomal clearance (Compagnoni et al., 2018).

Taken together, most of the data suggest consistently reported autophagic alterations in MSA, in particular a diminished amount of the autophagy initiating protein beclin-1, secondary to its downregulation (Valera et al., 2017b) or its cleavage by pro-inflammatory proteolytic enzymes (Bieri et al., 2018), and an autophagosome clearance impairment (Compagnoni et al., 2018).

Rapamycin reduces α -syn burden in the striatum of PLP-SYN mice and provides partial neuroprotection against dopaminergic cells death

Rapamycin-treated mice showed a reduced amount of proteinase K-resistant α -syn aggregates in the striatum. Furthermore, we observed that, even though rapamycin-treated mice presented similar counts of TH-positive neurons in the SNpc as placebo-treated animals, the Nissl-positive neuron counts were similar to WT mice. These Nissl-positive neurons represent dopaminergic neurons which have suffered a sufficient stress to impair TH expression, but not enough to induce cell death.

Autophagy dysregulation in PLP-SYN mice

The effects observed in nigral cell survival and α -syn inclusion burden were not paralleled by an improvement in autophagy readouts. We observed a marked decrease of autophagy markers beclin-1, p62 and LC3 (LC3-I and LC3-II) in the striatum of PLP-SYN mice. Interestingly, beclin-1, p62 and LC3 colocalize with α -syn in striatal oligodendroglial inclusions in myelin basic protein α -syn transgenic mice (MBP-SYN), another mouse model of MSA (Valera et al., 2017b). Levels of LC3-I and beclin-1 were also reduced in MBP-SYN mice, while p62 was increased. We found that while chronic treatment with rapamycin inhibited the mTOR downstream cascade, it did not induce similar changes in levels of these markers. One explanation for this discrepancy may be due to alterations of a parallel mTOR-independent autophagy controlling pathways, as (i) it has been the case for the leucine rich repeat kinase 2 (LRRK2) via the beclin-1 pathway, bearing in mind that *LRRK2* mutations are linked to PD and some polymorphisms in MSA (Heckman et al., 2014; Manzoni et al., 2016), and (ii) it could be a consequence of beclin-1 deactivation/enzymatic cleavage, as reported in AD (Lucin et al., 2013; Pickford et al., 2008).

Besides macroautophagy, mTOR also regulates other protein degradation systems (Zhao and Goldberg, 2016; Zhao et al., 2015). Of note, monomeric α -syn is known to be degraded by the ubiquitin-proteasome system (UPS), where proteins are tagged by the attachment of ubiquitin molecules, to be targeted to the proteasome (Nandi et al., 2006). Moreover, it has been demonstrated that pharmacological inhibition of the UPS enhances α -syn induced degeneration in a mouse model of MSA (Stefanova et al., 2012b). Consequently, rapamycin could reduce PK-resistant α -syn aggregates by promoting the action of the aforementioned degradation pathways, such as the clearance of monomeric α -syn by the UPS, thereby preventing its aggregation during the three months of treatment.

Beyond its effects on protein turn-over, rapamycin has been shown to have effects on cell survival, as it has been reported it to reduce neurodegeneration in neurotoxic models of PD (Dehay et al., 2010; Malagelada et al., 2010). Blockade of mTORC1-mediated translation of pro-cell death protein RTP801 has been proposed as a mechanism responsible for preventing dopaminergic neural death in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD (Malagelada et al., 2010). Moreover, mTOR-independent mechanisms have also been reported, such as induction of mitophagy, decreased apoptosis, stabilization of mitochondrial membranes and reduction of oxidative stress (Jiang et al., 2013; Ravikumar et al., 2006; Zimmerman et al., 2018).

CONCLUSION

We report the first study on the effects of rapamycin in the PLP-SYN mouse model of MSA. Rapamycin improved striatal α -syn burden and, to an extent, nigral neural survival, while this was not paralleled by an improvement of autophagy markers. Our data further suggest the existence of a beclin-1-dependent impairment of autophagy in MSA patients and the PLP-SYN model. Additionally, other mTOR-independent autophagy-enhancing strategies need to be further studied in MSA models, likewise combinations of mTOR-dependent and independent approaches could be of interest (Moors et al., 2017; Scrivo et al., 2018a).

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Table 1

Id	Diagnosis	Age (y)	Sex	Postmortem delay (h)
1	Control	84	M	15.5
2	Control	79	M	N/A
3	Control	69	M	6
4	Control	83	F	21
5	Control	80	M	4
6	Control	78	M	23
7	MSA-p	54	M	5.25
8	MSA-p	64	F	6.5
9	MSA-p	60	F	N/A
10	MSA-p	57	F	7
11	MSA-p	75	M	72
12	MSA-p	78	F	48
13	MSA-p	77	M	39

CONCLUSIONS AND PERSPECTIVES

IV- Conclusions and perspectives

As a clinical neurologist, this PhD work has allowed me to immerse myself into the field of basic science and, I expect, will help me bridge the existing gap between basic researchers investigating therapeutic interventions and clinicians who will put this knowledge into practice to try and improve our patients' quality of life. I believe this shall have a positive impact on my clinical practice hereon.

This work is part of a translational approach targeting the rare synucleinopathy MSA. We aimed to assess the neuroprotective effects of different α -synuclein-reducing strategies in transgenic mice of MSA, based on inhibition of post translational modifications of α -synuclein, enhancement of abnormal protein degradation and prevention of α -synuclein aggregation. In parallel, we evaluated the neuropathological features of a rare phenotype of MSA, which, interestingly, added to the existing knowledge that α -synuclein aggregation in neurons may be more relevant to the neurodegenerative process in MSA than previously believed.

1. Deep brain stimulation has no effect on MSA neuropathology

Current diagnostic criteria for MSA that distinguish between MSA-P and MSA-C subtypes have a sensitivity of 90% in late stages of the disease, but in earlier stages it is, at best, 41% (Osaki et al., 2009). If the classical MSA-P phenotype poses such a diagnostic challenge, it is unsurprising that patients affected by a phenotype that more closely resembles the classical progression of PD, i.e. asymmetric, slowly progressive and dopa-responsive parkinsonism with motor fluctuations and long duration (Jellinger, 2012; Masui et al., 2012; Petrovic et al., 2012), could be likely diagnosed as PD.

In the first part of this work we completed a post-mortem neuropathological assessment of a cohort of patients having this rare MSA phenotype, which had been misdiagnosed as PD and

undergone DBS surgery. Typically, DBS alleviates motor symptoms of PD and improves quality of life (Groiss et al., 2009), conversely, all patients in our study suffered a clinical worsening with the appearance of clinical features of MSA, and died, on average, four years after the intervention (Meissner et al., 2016)

We compared our MSA-DBS cohort with a control MSA-P group and, intriguingly, we found no pathological evidence of the surgery having induced morphological, inflammatory, or gliotic changes in the STN and other basal ganglia nuclei that would seem related to the rapid clinical worsening. Since the autopsy was only performed years after the DBS surgery, the ongoing neurodegenerative process could have concealed acute and direct influences on disease progression.

Contrary to PD, where neural accumulation of α -synuclein is mandatory (Spillantini et al., 1997), neuronal α -synuclein accumulation has classically been considered coincidental in MSA, while its deposition in oligodendrocytes is its defining feature (Trojanowski et al., 2007). However, recent studies suggest that accumulation of α -synuclein in neurons is more widespread in MSA than previously believed (Cykowski et al., 2015; Halliday, 2015; Homma et al., 2016; Jellinger, 2007; Rohan et al., 2015; Sekiya et al., 2019). Furthermore, current research indicates the origin of α -synuclein in GCIs is likely of dual origin, a combination of neuron-secreted and oligodendrocyte-expressed α -synuclein (Mavroeydi et al., 2019). Against this backdrop, we here demonstrate that MSA-DBS patients had a larger proportion of neuronal inclusions in the putamen compared to the control group, pointing to the possibility of a continuum between PD and MSA, rather than being them two distinct and independent entities as it is currently believed.

Our results, together with previous reports of MSA patients' failed response to DBS (Chou et al., 2004; Lezcano et al., 2004; Patrick et al., 2006; Tarsy et al., 2003; Thavanesan et al., 2014; Ullman et al., 2012; Zhu et al., 2014) highlight the need for an accurate diagnosis in the

clinical practice in order to provide appropriate therapeutic advice. This requires a high level of clinical suspicion and to look for clinical and paraclinical red flags during DBS eligibility of PD patients (Meissner et al., 2016).

2. Validating therapeutic strategies for MSA

Despite the uncertainties in the aetiology and pathogenesis of MSA, α -synuclein is currently the main target for the development of possible neuroprotective treatments (Meissner et al., 2019). In this PhD work, we have completed two studies aimed at reducing the accumulation of α -synuclein. In spite of the lack of effect against neurodegeneration shown by these compounds, we have garnered new knowledge about the pathogenesis of MSA that will improve our understanding of the underlying pathology.

A growing body of research indicates a link between misfolded α -synuclein and the alteration of the protein degradation machinery; dysfunction of these systems can facilitate α -synuclein aggregation and, conversely, α -synuclein aggregates may disrupt their function (Scriveo et al., 2018a). In particular, monomeric and small soluble oligomeric forms of α -synuclein are degraded through the ubiquitin-proteasome system or chaperone-mediated autophagy, whereas larger oligomeric or aggregated forms are cleared by macroautophagy (Ebrahimi-Fakhari et al., 2011; Lee et al., 2004). A recent review describes the evidence of their impairment in MSA (Arotcarena et al., 2019b). Accordingly, boosting these protein clearance mechanisms has been proposed as a tool to fight this family of neurodegenerative diseases (Arotcarena et al., 2019a; Decressac et al., 2013; Moors et al., 2017; Scriveo et al., 2018b; Torra et al., 2018).

As we set out to assess the validity of the autophagy enhancer rapamycin as a neuroprotective agent in MSA, we found active mTOR levels to be increased in the putamen of MSA patients. As previously mentioned, mTOR suppresses autophagy, so these findings

made the case for rapamycin – an mTOR inhibitor – to be a disease-modifying candidate all the more compelling. In addition, we encountered signs of defected macroautophagy in the putamen of MSA patients. Intriguingly, although rapamycin was able to reduce the accumulation of α -synuclein aggregates in the striatum and had modest neuroprotective effects in the SN of PLP-SYN mice, it did not revert the reduction of macroautophagy markers that we found in untreated PLP-SYN mice. It seems likely that the beclin-1-dependent impairment of macroautophagy, found in both patients and animals, acted as a bottle neck that limited the effects of the upstream macroautophagy-enhancing signal generated by inhibition of mTOR. In this regard, enzymatic deactivation of beclin-1 by caspase-3 has been linked to neurodegeneration (Bieri et al., 2018; Pickford et al., 2008). In addition, the vulnerability shown by dopaminergic neurons to the neurodegenerative process appears to be related to their expression of caspase-3 (Hartmann et al., 2000).

Despite the failure of rapamycin to protect dopaminergic neurons from degeneration, enhancing autophagic clearance of aberrant, aggregated α -synuclein is still a promising strategy for treating MSA. Addressing the beclin-1-dependent defect in macroautophagy could prove of value, as it has been reported in models of AD, PD, spinocerebellar ataxia or brain damage (Bieri et al., 2018; Lonskaya et al., 2013; Nascimento-Ferreira et al., 2011; Pickford et al., 2008; Spencer et al., 2009; Yang et al., 2017). Moreover, it has recently been shown that inducing oligodendroglial overexpression of the autophagy regulator transcription factor EB (TFEB) improves lysosomal biogenesis and autophagic clearance of α -synuclein and prevents neurodegeneration in the PLP-SYN mouse model (Arotcarena et al., 2019a), opening the way to assessing compounds capable of increasing TFEB expression (Kilpatrick et al., 2015; Song et al., 2014; Tan et al., 2019). That being said, before autophagy enhancing therapies can move further into human trials, it is necessary to fully capture the extent and causes related to autophagic dysfunction in MSA.

The preclinical evidence pointing to c-Abl as an putative target for treating α -synucleinopathies appears to be robust (Brahmachari et al., 2016; Brahmachari et al., 2017; Karuppagounder et al., 2014; Mahul-Mellier et al., 2014). Indeed, we found an increased ratio of activated c-Abl in the striatum of PLP-SYN mice, as it has been reported for PD mice (Brahmachari et al., 2016). Likewise, we also found pY39 α -synuclein levels to be increased in the striatum of PLP-SYN mice and to be present in GCI, which parallels findings in PD mice and LB inclusions (Brahmachari et al., 2016); it also appears to have a physiological role, as it can be detected in brains of non-transgenic mice. Despite these similarities and proper target engagement, inhibition of c-Abl by nilotinib had no effect on α -synuclein accumulation and nigral neurodegeneration. We believe this to be due to differences in the roles that c-Abl and pY39 α -synuclein play in oligodendroglial and neural synucleinopathies. It would be interesting for future studies to compare c-Abl kinase activity in different brain cells, and elucidate physiological and abnormal pathways that lead to phosphorylation of Y39 α -synuclein, and its role in health and disease. This underscores the necessity to better understand the intrinsic molecular differences between synucleinopathies in the pursuit of effective disease-modifying treatments.

Beyond phosphorylation, another relevant PTM that α -synuclein undergoes is truncation (see summary at table 4). Proteolytic cleavage of α -synuclein may occur at its carboxyl-terminus (C-terminus) region, and C-terminal truncated α -synuclein can be found in aggregates; further, truncated α -synuclein acts as seed and increases aggregation propensity (Hoyer et al., 2004; Li et al., 2005; Murray et al., 2003; Sorrentino et al., 2018; Tofaris et al., 2003; Ulusoy et al., 2010; Wang et al., 2016; Zhang et al., 2019). Accordingly, belnacasan (previously VX-765), an orally active inhibitor of the pro-inflammatory enzyme caspase-1, which cleaves α -synuclein, has been shown to attenuate α -synuclein accumulation and neuronal loss in vitro and in the PLP-SYN mouse model of MSA (Bassil et al., 2016; Wang et

al., 2016). Four studies have shown that direct inhibition of α -synuclein aggregation by compounds that physically interact with it is a promising approach. The orally available, small molecules CLR01 and PBT434 were revealed to decrease α -synuclein burden and alleviate nigral neural death (Finkelstein et al., 2019; Herrera-Vaquero et al., 2019); and anle138b, another small molecule that directly interacts with amyloidogenic proteins and modulates oligomer formation, reportedly mitigates α -synuclein aggregation and disease progression in animal models of PD and MSA (Antonschmidt et al., 2019; Heras-Garvin et al., 2018; Wagner et al., 2013). A last molecule with similar mechanism of action, SynuClean-D, has been reported beneficial in a mouse model of PD (Pujols et al., 2018); it remains to be seen if it does the same in MSA animals. Our final and ongoing project combines two drugs that reduce α -synuclein aggregation in the PLP-SYN mouse model of MSA. Even though belnacasan and anle138b restored nigral dopaminergic neurons to healthy levels, and similarly reduced aggregates, pathological α -synuclein was still present. We aimed to assess whether combining drugs that target protein oligomerization and aggregation via different mechanisms would prove synergistic, and if a more profound reduction of α -synuclein accumulation could be attained. Briefly, 8 week-old PLP-SYN mice and age-matched non-transgenic littermates were treated with placebo, belnacasan and anle138b alone and in combination for 10 weeks. Study endpoints were the efficacy to decrease α -synuclein burden and alleviate dopaminergic neural loss. We will appraise the drugs' effect on the amount of different species of α -synuclein, namely soluble oligomers and stable aggregates, also their impact on the inflammasome markers caspase-1 and interleukin-1beta (IL-1 β), and the degree of microglial activation. Our preliminary data confirm that both drugs are capable of preserving the number of TH⁺ neurons in the SN to levels equal to those found in non-transgenic mice. We have also found that levels of both forms of caspase-1, pro-caspase and active caspase-1, do not differ in the cortex or striatum of WT and PLP-SYN mice, irrespective of treatment; also, we found no difference of said enzyme in the putamen and

frontal cortex of MSA patients and controls. Once we have a more complete understanding of these drugs and their possible synergistic effects, it seems likely that one or more of them will enter clinical development in the future

Also aimed specifically at α -synuclein, studies evaluating immunization against it, both passive and active, have demonstrated a reduction of α -synuclein burden and neuroprotection in animal models of MSA, supposedly by blocking its cell-to-cell propagation and facilitating its clearance (Mandler et al., 2015; Valera et al., 2017a). This prompted the conduction of a phase I clinical trial of selective active immunization which recently reported a favourable safety and tolerability profile in early MSA patients (Meissner et al., 2018). Through gene therapy, induction of enzymatic proteolysis of α -synuclein has also proven to be effective in MSA mice (Spencer et al., 2015).

Strategies that engage α -synuclein-independent disease mechanisms have shown neuroprotective effects too. Hereof, reversing neuro-oligodendroglial insulin resistance with a glucagon-like peptide 1 agonist prevented dopaminergic neuron loss in MSA mice (Bassil et al., 2017a). Related to the fact that oligodendrocytes produce the myelin sheaths surrounding axons, enhancing myelination with benztropine has been reported to be beneficial (Ettle et al., 2016). Monophosphoryl lipid A, an agonist of the toll receptor 4 (TLR4), induces microglial autophagy, these cells would, in turn, increase their uptake and proteolysis of α -synuclein. It has been shown to be capable of reducing α -synuclein burden and dopaminergic cell loss in PLP-SYN mice (Venezia et al., 2017). Finally, stabilising chromatin by inhibiting histone deacetylases, a toxic mechanism through which α -synuclein interacts with nuclear DNA, has recently been reported to decrease dopamine neural death in MSA mice (Sturm et al., 2016).

DRUGS VALIDATED IN MSA MICE				
Compound	Target	Mechanism	Outcomes	References
AFF1 (corresponds to PD01 in clinical trials)	α -synuclein	Active immunization	Protection of striatal and cortical neurons Induction of anti- α -synuclein antibodies \Downarrow α -synuclein burden \Uparrow microglial activation \Uparrow myelination \Uparrow motor function	(Mandler et al., 2015)
Anle 138b	α -synuclein	Aggregation inhibitor	Protection of nigral neurons \Downarrow α -synuclein burden \Downarrow microglial activation \Uparrow motor function	(Heras-Garvin et al., 2018)
CD5-D5 and lenalidomide	α -synuclein and immunomodulation	Passive immunization, T cell co-stimulation, \Downarrow TNF α	\Downarrow astroglial and microglial activation \Downarrow α -synuclein burden \Downarrow hyperactivity phenotype	(Valera et al., 2017a)
CLR01	α -synuclein	Aggregation inhibitor	Protection of nigral neurons \Downarrow α -synuclein burden \Downarrow anxiety-like behaviour	(Herrera-Vaquero et al., 2019)
Neurosin	α -synuclein	Cleavage	Protection of striatal neurons \Downarrow α -synuclein burden \Uparrow myelination \Downarrow hyperactivity phenotype	(Spencer et al., 2015)
TFEB	α -synuclein	Autophagy enhancer	Protection of nigral neurons \Downarrow α -synuclein burden \Uparrow neurotrophic support \Uparrow autophagy	(Arotcarena et al., 2019a)
Belnacasan (VX-765)	α -synuclein	Inhibition of C-terminal truncation by caspase-1	Protection of nigral neurons \Downarrow truncated α -synuclein \Downarrow α -synuclein burden \Uparrow motor function	(Spencer et al., 2015)
Benzotropine	Myelin	Muscarinic antagonist	Protection of cortical neurons \Uparrow myelination	(Ettle et al., 2016)
Exenatide	Insulin resistance	Glucagon-like peptide-1 analogue	Protection of nigral neurons \Downarrow α -synuclein burden \Downarrow insulin resistance	(Bassil et al., 2017a)
Monophosphoryl lipid A	Toll-like receptor 4 (TLR4)	TLR4 agonist Inducer of phagocytosis	Protection of nigral and striatal neurons \Downarrow α -synuclein burden \Uparrow motor function	(Venezia et al., 2017)
Sodium phenylbutyrate	Histone deacetylase	Histone deacetylase inhibition	Protection of nigral neurons \Downarrow α -synuclein burden \Uparrow motor function	(Sturm et al., 2016)
POSSIBLE THERAPEUTIC APPROACHES TO BE ASSESSED				
Target/compound		Rationale		
G protein-coupled receptor kinase 2 (GRK2)		Main integrator of insulin signalling pathway. Phosphorylates IRS-1, decreasing insulin responsiveness, promoting insulin resistance. Increased levels in animal models of insulin resistance and humans with metabolic syndrome.		
Tubulin polymerization promoting protein (p25 α)		Oligodendroglial protein, present in GCI and involved in early pathological events in MSA. Enhances α -synuclein polymerization.		
Fingolimod		Induces expression of BDNF and enhances myelination. Reverted neurodegeneration and α -synuclein accumulation in PD mice.		

Activated microglia	Drives pathological neuroinflammation, secretes proinflammatory cytokines. Neuroinflammation promotes α -synuclein aggregation and redox imbalances.
Tumour necrosis factor α (TNF α)	Key cytokine in inflammation-induced cell damage. TNF α inhibitors already available for use in humans
Oxidative stress	Enhances neuroinflammation and promotes aggregation of α -synuclein
Iron	Iron imbalance increases oxidative stress. Iron also enhances α -synuclein aggregation

Table 4. Summary of preclinical pipeline in MSA and promising therapeutic approaches to be validated. Brain-derived neurotrophic factor, BDNF; insulin receptor substrate 1, IRS-1.

Apart from molecules already proven to be neuroprotective, there are a number of other possible target candidates for preclinical assessment in MSA. The protein p25 α is involved in early pathogenic events preceding α -synuclein aggregation (Song et al., 2007), and recent in vitro research illustrates how it can interact with α -synuclein to accelerate its seeding and the formation of pathological aggregates in oligodendrocytes (Mavroei et al., 2019). In consequence, targeting p25 α could provide a mechanism to prevent α -synuclein aggregation at the earliest steps of the pathogenesis of MSA. Dysfunction of the insulin signalling cascade in different brain cells has been described in neurodegenerative disorders, including MSA (Bassil et al., 2017a; Bassil et al., 2014). The insulin signalling is notably transduced by an integrator protein named G protein-coupled receptor kinase 2 (GRK2), while mouse models of insulin resistance and humans with metabolic syndromes display increased levels of GRK2 (Vila-Bedmar et al., 2015). Targeted knockdown of GRK2 is currently being evaluated at our lab in MSA mice (<https://www.multiplesystematrophy.org/msa-research/preclinical/>). Fingolimod is an approved oral treatment for relapsing/remitting multiple sclerosis (MS) (Sanford, 2014). Its protective mechanism is due to an increase in the expression of brain-derived neurotrophic factor (BDNF) and improving myelination (Gurevich et al., 2018; Vidal-Martínez et al., 2016). This drug has been shown to alleviate α -synuclein pathology and protect dopaminergic neurons

in PD mice (Vidal-Martinez et al., 2019; Vidal-Martínez et al., 2016), and it appears to be effective in an in vitro model of MSA (Segura-Ulate et al., 2017; Vargas-Medrano et al., 2019).

We have already mentioned the interplay between neuroinflammation and α -synuclein (see page 18 of Introduction), where α -synuclein induces an inflammatory response from microglia and astroglia and, in turn, the inflammatory environment further promotes α -synuclein aggregation (Lee et al., 2010; Vieira et al., 2015). In MSA, the distribution of microglial activation correlates with the known pattern of neurodegeneration and with the GCI burden in affected areas (Ishizawa et al., 2004). Through a PET imaging study assessing microglia, an increased activity was detected in classically affected regions in MSA (Gerhard et al., 2003). A clinical trial assessing minocycline, an antibiotic with anti-inflammatory effects (Clemens et al., 2018), failed to show disease modification in MSA-P patients, while two of the three minocycline-treated patients that underwent PET imaging showed decreased microglial activation (Dodel et al., 2010). In spite of this trial failure, reducing microglial activation remains a very appealing strategy to tackle the neurodegenerative process, and other compounds could prove more effective in restoring microglia to its normal activity. Moreover, abnormally activated glial cells secrete, among other pro-inflammatory cytokines, tumour necrosis factor alpha (TNF α) and IL-1 β (Solleiro-Villavicencio and Rivas-Arancibia, 2018; Vieira et al., 2015). Elevated levels of these two cytokines have been described in cerebrospinal fluid, serum and post-mortem tissue of MSA patients (Kaufman et al., 2013; Salvesen et al., 2015; Yamasaki et al., 2017). Further, a recent study suggests a possible link between TNF α and IL-1 β polymorphisms and MSA (Zhou et al., 2018). In this context, inhibition of TNF α has been suggested as a feasible approach in MSA, given that TNF α inhibitors are readily available in the clinic (Ndayisaba et al., 2019). Interestingly, pro-IL-1 β , is proteolytically activated by caspase-1 (Denes et al., 2012), which is inhibited, as we have mentioned, by belnacasan (Bassil et al., 2016). Also, neuroinflammation is driven by a loss of redox balance

(Solleiro-Villavicencio and Rivas-Arancibia, 2018; Vieira et al., 2015), and iron imbalance has been described as an enhancer of oxidative stress, which promotes α -synuclein aggregation, but iron itself can also accelerate α -synuclein polymerization (Kaindlstorfer et al., 2018). Further, post-mortem and MRI studies have pointed to increased amounts of iron in regions classically damaged by the degenerative process in MSA (Dexter et al., 1991; Kaindlstorfer et al., 2018). Consequently, it would be interesting to explore strategies aimed at restoring redox and iron imbalances in MSA.

Overall, we here have shown that even though MSA presents clinical, neuropathological and molecular features that overlap in some aspects with PD, there are facets that highlight the need to identify and understand their disparities, which range from α -synuclein processing, cellular accumulation, clearance, selective vulnerability or neuropathology, to the clinical presentation. The synucleinopathy puzzle has a millions pieces, to find them and put them together, this hard work has to be carried out in the clinic and the laboratory.

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