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Multiple system atrophy : a translational approach Characterization of the insulin/IGF-1 signaling pathway

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THÈSE PRÉSENTÉE

POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE : Sciences de la Vie et de la Santé

SPÉCIALITÉ : Neurosciences

Par Fares BASSIL

Multiple system atrophy : a translational approach

Characterization of the insulin/IGF-1 signaling pathway

Sous la direction de : Professeur Wassilios MEISSNER

Soutenue le 2 Septembre 2015

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“The element of chance in basic research is overrated. Chance is a lady who smiles only upon those few who know how to make her smile.”

Hans Selye

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Résumé

Titre : ***L'atrophie multisystématisée : Une approche translationnelle***

Ce travail porte sur des approches translationnelles dans les synucléinopathies notamment l'atrophie multisystématisée (AMS). Au-delà de leur rôle dans la régulation du glucose, l'insulin et l'insulin like growth factor-1 (IGF-1) ont des propriétés neurotrophiques. Des études ont montrées que la signalisation de l'insuline/IGF-1 est altérée dans la maladie d'Alzheimer et des données suggèrent l'altération de l'insuline/IGF-1 dans la maladie de Parkinson (MP) et l'AMS. Nous avons mis en évidence une résistance à l'insuline dans les neurones des patients MP et AMS ainsi que dans les oligodendrocytes chez les patients AMS.

Mon travail a également consisté à cibler la troncation de l'α-synuclein (α-syn) comme cible thérapeutique. Nous avons démontré dans un modèle murin d'AMS que la diminution de l'α-syn tronquée permettait de réduire l'agrégation d'α-syn et la dégénérescence des neurones dopaminergiques.

Enfin, nous avons étudié l'implication dans l'AMS des métalloprotéinases matricielles (MMP), des enzymes impliquées dans remodelage de la matrice, la démyélinisation, la troncation de l'α-syn et la perméabilité de la barrière hémato-encéphalique. Ce travail nous a permis de montrer une augmentation de l'expression et de l'activité de MMPs chez les patients AMS. Nous avons également montré que les cellules gliales sont la source de cette augmentation et que la MMP-2 est retrouvée dans les agrégats des patients AMS.

Nous montrons ici de caractéristiques distinctes de l'AMS comme des altérations qui se produisent dans les oligodendrocytes. Nous présentons aussi VX-765 comme un candidat prometteur pour ralentir la progression de la pathologie dans un contexte de synucléinopathie.

Mots clés : Synucléine, résistance à l'insuline, métalloprotéinases matricielles, atrophie multisystématisée, Parkinson, insuline, insulin like growth factor-1, glucagon like peptide-1, inclusions cytoplasmiques gliales, cerveau humain, rongeur, approche translationelle, troncation c-terminal.

Abstract

Title: ***Multiple system atrophy: A translational approach***

This work focused on translational approaches in synucleinopathies and more specifically in multiple system atrophy (MSA). Beyond their role in glucose homeostasis, insulin/IGF-1 are neurotrophic factors in the brain. Studies have shown altered insulin/IGF-1 signalling in Alzheimer's disease and data suggest impaired insulin signaling/IGF-1 in Parkinson's disease (PD) and MSA. The aim of my work was to characterize insulin/IGF-1 signalling in MSA and PD brain tissue. Both groups showed neuronal insulin resistance. Oligodendrocytes in MSA patients were also insulin resistant.

In line with the translational approach, we also targeted α-synuclein (α-syn) truncation pharmacologically in MSA transgenic mice, which led to reduced α-syn aggregation and the protection of dopaminergic neurons.

We also assessed the activity and distribution of matrix metalloproteinases (MMPs) in the brain of MSA patients compared to healthy controls. MMPs are involved in the remodelling of the extracellular matrix, demyelination, α-syn truncation and blood brain barrier permeability. We showed altered expression and activity of MMPs in two distinct structures in MSA brains. We were also able to show that glial cells were the source of increased MMPs and show a unique expression of MMPs in α-syn aggregates of MSA patients compared to PD, evidence that might hint at a mechanism that is differently altered between PD and MSA.

We here show distinct pathological features of MSA such as key alterations occurring in oligodendrocytes, further supporting MSA as a primary oligodendrogliopathy. We also present VX-765 as a candidate drug for disease modification in synucleinopathies.

Keywords: Synuclein, insulin resistance, matrix metalloproteinase, multiple system atrophy, Parkinson's disease, insulin, insulin like growth factor-1, glucagon like peptide-1, glial cytoplasmic inclusions, postmortem human brain study, rodent, translational approach, c-terminal truncation.

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Abbreviations

α-syn	α-synuclein
AAV	Adeno-associated virus
Aβ	Amyloid beta
AD	Alzheimer's Disease
ADAS-cog	Assessment scale - cognitive sub-scale and the Clinical Dementia Rating scale
Akt	Protein kinase B
ALS	Amyotrophic lateral sclerosis
BAD	Bcl-2 associated death promoter
BBB	Blood brain barrier
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma extra large
Bim	Bcl-2 interacting mediator of death
cAMP	Cyclic adenosine monophosphate
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CREB	cAMP response element-binding protein
CSF	Cerebrospinal fluid
DBS	Deep brain stimulation
DJ-1	PARK 7
DLB	Dementia with lewy bodies
DPP-4	Dipeptidyl peptidase 4
ERK	Extracellular signal regulated kinase
FasL	Apoptosis-stimulating fragment ligand
FoxO	Forkhead box O
GCI	Glial cytoplasmic inclusions
GLP-1	Glucagon like peptide-1
GLP-1R	Glucagon like peptide-1 receptor
Grb2	Growth factor receptor-bound protein 2
GSK-3β	Glycogen synthase kinase 3β
IDE	Insulin degrading enzyme
IGF-1	Insulin like growth factor-1
IGF-1R	Insulin like growth factor-1 receptor
IGFBP	Insulin like growth factor binding protein
IR	Insulin receptor

IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinases
LB	Lewy bodies
LV	Lentivirus
MAP-K	Mitogen associated protein kinase
MBP	Myelin basic protein
MMP	Matrix metalloproteinase
mTOR	Mammalian target of rapamycin
MT1-MMP	Membrane type 1-MMP
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple system atrophy
MSA-C	Multiple system atrophy cerebellar phenotype
MSA-P	Multiple system atrophy parkinsonian phenotype
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OPCA	Olivopontocerebellar atrophy
PD	Parkinson's Disease
PI3-K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKR	Protein kinase R
PLP	Myelin proteolipid promoter
PP2A	Protein phophastase 2A
ROS	Reactive oxygen species
Shc	Src homology-2/α-collagen-related protein
SN	Substantia nigra
SND	Striatonigral degeneration
SNc	Substantia nigra pars compacta
Sos	Son of sevenless
TH	Tyrosine hydroxylase
TNF-α	Tumor necrosis factor
T2D	Type 2 diabetes
UPDRS	Unified PD Rating Scale
6-OHDA	6-hydroxydopamine

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Introduction

I - Synucleinopathies: Parkinson's disease and multiple system atrophy

Synucleinopathies encompass multiple system atrophy (MSA), Parkinson's disease (PD) and dementia with Lewy bodies (DLB). In these disorders, the cytopathological hallmark is the aggregation and abnormal accumulation of α-synuclein (α-syn) in different cell types. In MSA, α-syn mainly accumulates in glial cytoplasmic inclusions (GCIs) in oligodendrocytes while it is found in neurons forming Lewy bodies (LBs) in PD and DLB. Synucleinopathies are characterized by a progressive decline in, motor, autonomic and cognitive functions due to neurodegeneration and alteration of several mechanisms implicated in cellular homeostasis and brain physiology.

1- Epidemiology

MSA was previously considered as 3 separate disorders, namely Shy-Drager syndrome, olivopontocerebellar atrophy (OPCA) and striatonigral degeneration (SND). In 1969, Graham and Oppenheimer introduced the term MSA to describe patients showing similar clinical and pathologic findings in SND, OPCA and Shy-Drager syndrome (Graham and Oppenheimer, 1969). Several years later, Papp and colleagues further unified MSA by the discovery of GCIs, now recognized as the hallmark of the disease (Papp *et al.*, 1989). MSA is an orphan disorder with a prevalence of 1.9 to 4.9/100,000 and a yearly incident rate of 3/100,000 in a population older than 50 years old (Chrysostome *et al.*, 2004; Geser *et al.*, 2005; Gilman *et al.*, 2005; Schrag *et al.*, 1999; Tison *et al.*, 2000). MSA mean disease onset is usually in the sixth decade of life but ranges from 30 to 76 years of age and median survival is 6 to 10 years after symptom onset with few exceptions lasting more than 15 years (Ben-Shlomo *et al.*, 1997; Petrovic *et al.*, 2012; Schrag *et al.*, 2008; Wenning *et al.*, 2004). Two subtypes of MSA have been proposed to distinguish the predominant motor parkinsonian phenotype (MSA-P) from the cerebellar phenotype (MSA-C). MSA-P reflects SND and is mostly found in the Western hemisphere. It is known to outnumber with a ratio of 2:1 MSA-C, which is predominant in Asia.

In 1817, James Parkinson described symptoms in six patients of a disease he called 'paralysis agitans' (Parkinson, 1817). Jean-Marie Charcot called this condition in the 1860s Parkinson's disease (PD). PD has an estimated prevalence of 200/100,000 in the general population and reaches up to 1000/100,000 in those aged over 60, making it the second most frequent neurodegenerative disease after Alzheimer's disease (AD). The average age at

symptom onset is 58-60 years (de Lau and Breteler, 2006; de Rijk *et al.*, 1997; Samii *et al.*, 2004). Prevalence is equally distributed between men and women in MSA while a slight trend to male predominance exists in PD (Benito-Leon *et al.*, 2004; de Lau and Breteler, 2006; Fall *et al.*, 1996; Gilman *et al.*, 2005; Kim *et al.*, 2011a; Kollensperger *et al.*, 2010).

2- Etiology

MSA and PD are mainly sporadic neurodegenerative disorders. There is clear evidence for environmental and monogenetic factors in PD while results of genetic studies are inconclusive in MSA. Epidemiologic studies in PD have found that exposure to viral infections and environmental toxicants such as pesticides, solvents or metals might play a role in disease exposure (Goldman, 2014). Due to scarcity of research in MSA and underpowered studies, no substantial evidence implicates environmental factors in this disorder (de Lau and Breteler, 2006; Hanna *et al.*, 1999; Nee *et al.*, 1991; Vidal *et al.*, 2008).

Several mutations have been identified in young-onset and familial forms of PD in the past years (Corti *et al.*, 2011; Martin *et al.*, 2011a; Verstraeten *et al.*, 2015). Gene loci have been associated with autosomal dominant forms of PD due to duplications, triplications and mutations in the *SNCA* gene (Park1, Park4) and Leucine-rich repeat kinase 2 (LRRK2) (Farrer *et al.*, 2004; Polymeropoulos *et al.*, 1997; Singleton *et al.*, 2003; Zimprich *et al.*, 2004). Studies have also described autosomal recessive forms of PD due to mutations in Pink1, DJ-1 and Parkin genes (Bonifati *et al.*, 2003; Kitada *et al.*, 1998).

Studies have failed to show clear evidence of a genetic cause in MSA, as research efforts were unsuccessful in detecting mutations or multiplications in the *SNCA* gene (Lincoln *et al.*, 2007; Ozawa *et al.*, 1999). A study investigating mutations leading to a loss of function of coenzyme Q10-synthesizing enzyme was reported in Japanese familial and sporadic cases, but the mutation was not found in patients from Europe, North America, Korea and China (Chen *et al.*, 2015; Jeon *et al.*, 2014; Multiple-System Atrophy Research, 2013; Quinzii *et al.*, 2014; Schottlaender *et al.*, 2014; Sharma *et al.*, 2014). Similarly, a discordant loss of a copy number of (src homology 2 domain containing)-transforming protein 2 was also found in Japanese but not in American patients (Ferguson *et al.*, 2014; Sasaki *et al.*, 2011).

3- Neuropathology and pathophysiology

The confirmation of the clinical diagnosis requires postmortem histological evaluation. One of the main histological features of PD is the depigmentation of the substantia nigra (SN) pars compacta (SNc), reflecting the degeneration of neuromelanin containing dopaminergic neurons (i.e. tyrosine hydroxylase (TH)) and is associated with LBs (Spillantini *et al.*, 1998b;

Spillantini *et al.*, 1997). In MSA, the depigmentation of the SNc is accompanied by GCIs in oligodendrocytes, overall brain gliosis, neuronal death, tissue vacuolation, and myelin loss (Papp *et al.*, 1989; Spillantini *et al.*, 1998a; Wenning *et al.*, 2008).

a) α-syn, post-translational modification and neurodegeneration

Argylophilic, triangular, sickle or moon-shaped cytoplasmic inclusions in oligodendrocytes known as GCIs are the hallmark of MSA (Papp *et al.*, 1989; Spillantini *et al.*, 1998a; Wenning *et al.*, 2008). In PD, neurodegeneration is accompanied by α-syn containing cytoplasmic inclusions called LBs in perikarya and Lewy Neurites in axons and dendrites (Spillantini *et al.*, 1998b; Spillantini *et al.*, 1997) (Figure 1). In a comparative study, Tong *et al.* (2010) showed abnormal region specific α-syn load in MSA compared to PD patients. MSA patients had higher amounts of membrane fraction α-syn in the nigra and putamen and lower amounts of cytosolic fraction α-syn in the putamen compared to PD patients. Unlike PD, GCIs are mainly composed of loosely packed and randomly aggregated filaments with oligomers forming the core of the inclusions (Campbell *et al.*, 2001; Gai *et al.*, 2003).

α-syn is a 14 kDA protein that can exist *in vitro* as an unfolded monomer (Dehay *et al.*, 2015; Weinreb *et al.*, 1996). Recent research has shown that α-syn can undergo several post-translational modifications such as phosphorylation, tyrosine nitration and truncation, any of which could promote α-syn oligomerization resulting in prefibrillar intermediates leading to high molecular weight protofibrils resembling those that are found in LBs and GCIs. This suggests a highly heterogeneous aggregation process that turns its monomers into multiple oligomeric forms, then protofibrils, fibrils and aggregates (Dehay *et al.*, 2015; Rochet *et al.*, 2012; Wood *et al.*, 1999) (Figure 1).

Although the precise toxic species of α-syn have not been firmly established, several studies point to α-syn oligomerization and aggregation as mediators of neurotoxicity in synucleinopathies. C-terminal truncation has been identified as an enhancer/promoter of α-syn oligomerization and fibrillization (Hoyer *et al.*, 2004; Li *et al.*, 2005b; Liu *et al.*, 2005). Proteases such as cathepsin D, calpain, plasmins, matrix metalloproteinases, neurosin and caspases have been shown to cleave α-syn in its C-terminal portion (Dufty *et al.*, 2007; Kim *et al.*, 2012; Mishizen-Eberz *et al.*, 2003; Sung *et al.*, 2005).

Moreover, despite the difference in α-syn localization, solubility and conformation, several proteins such as PINK1, ubiquitin, FBXO7, 14-3-3, P62, microtubule associated protein light chain-3, SUMO-1 and P25α are found in both GCIs and LBs reflecting potential

overlapping aggregation mechanisms (Kawamoto *et al.*, 2002; Kim *et al.*, 2011b; Kovacs *et al.*, 2004; Murakami *et al.*, 2007; Tanji *et al.*, 2013; Wong *et al.*, 2013; Zhao *et al.*, 2013).

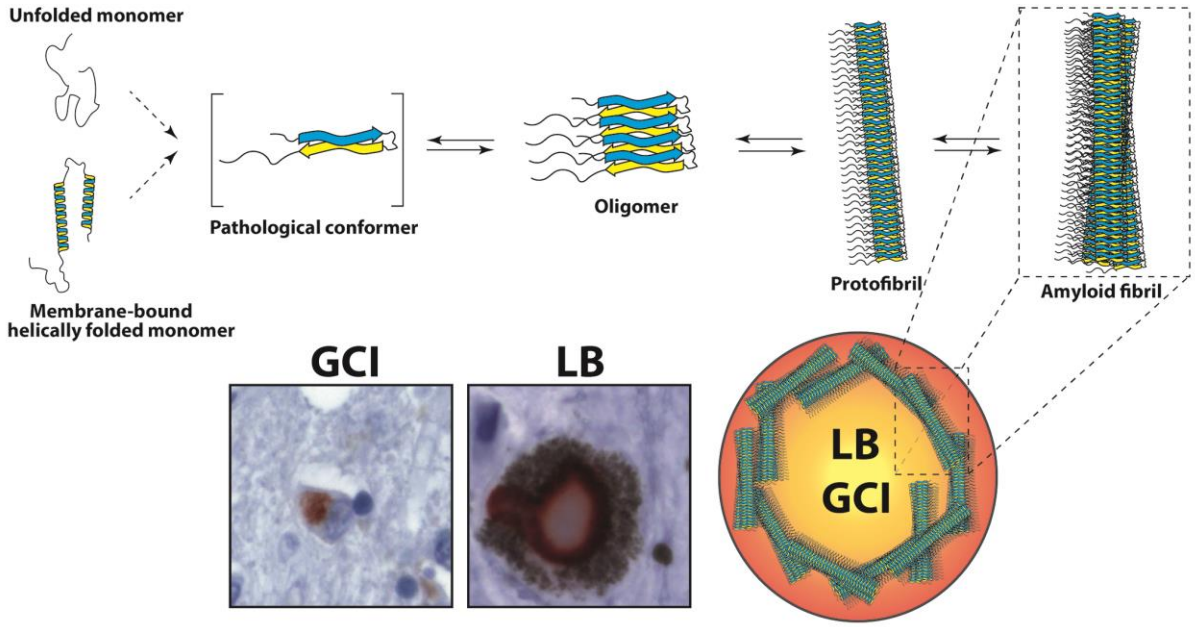

Figure 1 Diagram showing the α-synuclein aggregation pathway leading to the formation of Lewy bodies and glial cytoplasmic inclusions (adapted from Dehay *et al.* (2015)).

b) Pathogenesis

Lately, the "prion-like" hypothesis of α-syn was suggested to be a contributor to the pathogenesis of PD. Studies have shown the capacity of misfolded α-syn to seed the pathological conversion of native α-syn in the recipient cell into toxic forms (Dunning *et al.*, 2012; Luk *et al.*, 2009; Volpicelli-Daley *et al.*, 2011). Neurons are hypothesized to secrete α-syn that is uptaken by the neighbouring cells as the propagation of α-syn through the neuronal networks and axons has been previously reported (Freundt *et al.*, 2012; Lamberts *et al.*, 2015; Luk *et al.*, 2012a; Luk *et al.*, 2012b; Masuda-Suzukake *et al.*, 2014; Recasens *et al.*, 2014). Similar to neurons, astrocytes have shown the capacity to uptake α-syn via endocytosis (Lee *et al.*, 2010).

The reason behind the presence, accumulation and aggregation of α-syn in GCIs of MSA patients is yet to be fully understood. α-syn is produced by neurons and found in synaptic terminals. Studies in MSA have failed to confirm the presence of local production of α-syn in oligodendrocytes using *in-situ* hybridization, while α-syn is transiently expressed in developing oligodendrocytes (Culvenor *et al.*, 2002; Miller *et al.*, 2005; Ozawa *et al.*, 2001; Richter-Landsberg *et al.*, 2000). The uptake of neuronal α-syn by oligodendrocytes in MSA

might explain the presence of α-syn in GCIs as Reyes *et al.* (2014) showed that oligodendrocytes readily take up monomeric and oligomeric forms of α-syn in a dynamin-dependant process. Recently, Asi *et al.* (2014) challenged previous findings by detecting α-syn mRNA in mature oligodendrocytes with a trend to increased α-syn mRNA expression compared to MSA neurons using quantitative reverse transcription polymerase chain reaction. Both techniques were sensitive enough to detect α-syn mRNA in neurons, hence *in-situ* hybridization should have provided enough sensitivity to detect α-syn in GCIs. Regardless of its extracellular or putative oligodendroglial origin, α-syn aggregation in oligodendrocytes has been linked to p25α, an oligodendroglia-specific protein that relocalizes to the nucleus in early stages of MSA. P25α is implicated in the organization of the microtubule system during myelination in the developing brain and in myelin repair conditions (Kovacs *et al.*, 2004; Lehotzky *et al.*, 2010; Skjoerringe *et al.*, 2006; Song *et al.*, 2007). P25α interaction with α-syn is hypothesized to promote phosphorylation and aggregation of α-syn into GCIs (Ota *et al.*, 2014; Song *et al.*, 2007).

c) Neuropathology and histopathology, an emphasis on MSA

In MSA, GCIs are widespread throughout the brain and GCI density is the highest in structures severely affected by the neurodegenerative process such as the putamen, SN and brainstem (Duda *et al.*, 2000; Papp *et al.*, 1989; Papp and Lantos, 1994; Wakabayashi and Takahashi, 2006). The relationship between GCI burden, cell loss or disease severity remains controversial in MSA (Armstrong *et al.*, 2006; Ishizawa *et al.*, 2008; Ozawa *et al.*, 2004; Ozawa *et al.*, 2002; Tong *et al.*, 2010). Some studies have correlated all three factors indicating that the accumulation of GCIs may be an important factor in neuronal death in MSA (Ozawa *et al.*, 2004; Ozawa *et al.*, 2002; Tong *et al.*, 2010). However, regions of the brain, such as the motor cortex known to be relatively spared by the disease process have a remarkably high amount of GCI (Papp and Lantos, 1994).

Neurodegeneration in synucleinopathies, especially in MSA, is also associated with myelin pallor, blood brain barrier (BBB) dysfunction, oxidative stress, mitochondrial dysfunction and neuroinflammation (Beal, 2003; Jellinger, 2014; Kikuchi *et al.*, 2002; Lee *et al.*, 2013; Schapira, 2008; Shibata *et al.*, 2010). Accordingly, structure vulnerability is reflected by the classification of MSA into two subtypes with predominant neurodegeneration in the striatonigral or olivopontocereballar system (Jellinger, 2014). The atrophy of the putamen is considered a key characteristic feature that helps distinguishing MSA-P patients from PD patients, while the loss of dopaminergic neurons in MSA is comparable at early stages to the alterations found in PD patients (Sato *et al.*, 2007; Tison *et al.*, 1995). MSA-P

patients exhibit neuronal loss and atrophy in the caudate nucleus, globus pallidus and loss of striatal afferents to both structures (Brooks *et al.*, 1992; Kume *et al.*, 1993). Examination of MSA-C brains shows a significant atrophy of the cerebellum, pontine base and cerebellar peduncles (Watanabe *et al.*, 2004). MSA-C brains also exhibit pallor of the locus coeruleus in the pons and the inferior olivary nucleus ribbon (Ozawa, 2007). Autonomic failure in these patients is due to neuro-hormonal dysfunction that can precede motor symptoms in MSA (Magalhaes *et al.*, 1995; Sakakibara *et al.*, 2000; Watanabe *et al.*, 2002).

MSA is characterized by an increased number of neuroinflammaotry triggers such as cytokines, reactive oxygen species (ROS) and nitric oxide (Abdo *et al.*, 2004; Gerhard *et al.*, 2003; Ishizawa *et al.*, 2004; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Salvesen *et al.*, 2015; Shibata *et al.*, 2010). Accordingly, gliosis (i.e. increased amounts of activated microglia and reactive astrocytes) is an important contributor to the disease process in MSA (Ozawa *et al.*, 2004; Salvesen *et al.*, 2015; Song *et al.*, 2009). Astrocyte activation correlates with α-syn aggregate proximity and with severity of neurodegeneration (Radford *et al.*, 2015). Moreover, microglia are found in white matter tract regions and are hypothesized to be implicated in myelin phagocytosis (Ishizawa *et al.*, 2008; Ishizawa *et al.*, 2004). The BBB is compromised in MSA (Bartels *et al.*, 2008; Miller *et al.*, 2007; Song *et al.*, 2011) and studies have shown that BBB weakness also correlates with disease severity and progression (Lee *et al.*, 2013; Song *et al.*, 2011)

White matter loss has been reported in several structures in MSA (Papp *et al.*, 1989). No portion of the nervous system is spared in MSA as decreased expression and staining of myelin was observed in the white matter tracts along the cerebellar and nigrostriatal regions (Don *et al.*, 2014; Matsuo *et al.*, 1998; Ozawa, 2007; Papp *et al.*, 1989; Song *et al.*, 2007). Studies have also shown decreased myelin and patches of degraded myelin in the brain of MSA patients (Matsuo *et al.*, 1998; Song *et al.*, 2007). Moreover, myelin atrophy was also observed in structures relatively spared by the disease process (Matsusue *et al.*, 2009).

The mechanisms implicated in the above mentioned alterations are still poorly understood in MSA and evidence from various pathological processes including Alzheimer's disease (AD) (Asahina *et al.*, 2001; Lorenzl *et al.*, 2003; Peress *et al.*, 1995), amyotrophic lateral sclerosis (Fang *et al.*, 2009; Kiaei *et al.*, 2007; Lim *et al.*, 1996; Lorenzl *et al.*, 2006; Yushchenko *et al.*, 2000) and PD (Kim *et al.*, 2007; Lorenzl *et al.*, 2002) suggest a potential involvement of matrix metalloproteinases (MMPs). MMPs are a group of zinc-dependent endopeptidases known for their capacity to degrade several components of the extracellular matrix and basement membranes (Yong *et al.*, 2001).

As previously stated (section 3.a), several MMPs can cleave α-syn in its C-terminal domain (Sung *et al.*, 2005). Proteolytic truncation of α-syn has been shown to act as a promoter/enhancer of α-syn toxicity and aggregation (Levin *et al.*, 2009; Li *et al.*, 2005b). MMPs have also been found to be activated by cytokines, ROS, nitric oxide and other neuroinflammatory triggers; factors that are commonly increased in MSA. (Abdo *et al.*, 2004; Gerhard *et al.*, 2003; Ishizawa *et al.*, 2004; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Shibata *et al.*, 2010). Moreover, some MMPs are primarily secreted by inflammatory cells such as microglia and astrocytes that are activated in MSA (Ishizawa *et al.*, 2004; Salvesen *et al.*, 2015).

Considering the interplay between neuroinflammation and MMPs, their ability to cleave α-syn, and their demonstrated role in neurodegenerative disorders including PD, it is thus tempting to speculate that MMPs might be involved in the pathogenesis of MSA.

4- Symptoms

a) Motor

Owing to symptom similarity, MSA patients could initially be misdiagnosed as PD; nonetheless, symptoms rapidly progress in MSA. The most frequent motor symptoms in MSA and PD are:

1. Rigidity is caused by increased tension and continuous muscle contraction. Rigidity virtually affects all muscles with disease progression with a predominance of the flexor muscles (Delwaide *et al.*, 1986).
2. Bradykinesia, i.e. slowness of voluntary movements, often considered one of the most disabling symptoms in PD and MSA, is observed early in the disease (Halliday, 2007; Samii *et al.*, 2004).
3. Postural instability refers to the poor balance and unsteadiness leading to an increase in the number of falls. Postural instability is often associated with an abnormal gait (Bloem *et al.*, 2001).
4. Resting tremor is the most known and visible symptom of PD as 70-80% of patients suffer from it. It is known as the involuntary, rhythmic and oscillatory movements of body parts (Stanley-Jones, 1956). On the other hand, only 10% of MSA patients show typical parkinsonian resting tremor while 50% display a jerky postural hand tremor (Kaindlstorfer *et al.*, 2013).

b) Non-motor

A large number of non-motor symptoms and/or complications are present in PD and to a higher extent in MSA patients due to the widespread degeneration targeting structures implicated in autonomic functions such as the brainstem and the hypothalamus (Benarroch, 2003, 2007; Benarroch *et al.*, 2006; Benarroch *et al.*, 2007; Dugger *et al.*, 2012; Ozawa, 2007; Schmeichel *et al.*, 2008). Erectile dysfunction occurs early in male MSA patients compared to a late onset in PD patients accompanied in both diseases by urinary dysfunction which is more severe in MSA patients due to incontinence and urinary retention (Beck *et al.*, 1994; Bronner and Vodusek, 2011; Jankovic, 2008; Jost, 2013; Kirchhof *et al.*, 2003; Papatsoris *et al.*, 2008; Robinson *et al.*, 2013; Sakakibara *et al.*, 2000). Moreover, MSA and PD patients also suffer from orthostatic hypotension and are frequently reporting gastrointestinal problems such as gastroparesis and constipation (Gilman *et al.*, 2008; Pfeiffer, 2003). Respiratory failure is considered a primordial aspect in MSA patients since it reflects the extent of neurodegeneration in the pontomedullary respiratory system (Benarroch, 2003, 2007). It may already manifest in early stages of MSA and is the cause of sudden death in some patients (Glass *et al.*, 2006; Kollensperger *et al.*, 2008; Tada *et al.*, 2009). PD and MSA patients show cognitive impairment, most frequently executive dysfunction. In the course of the disease, a significant proportion of PD patients develop dementia, which also concerns 10-15% of MSA patients (Caballol *et al.*, 2007; Stankovic *et al.*, 2014). Patients suffer frequently from depression, mood swings, anxiety, panic attacks and suicidal ideation (Fanciulli and Wenning, 2015; Jankovic, 2008). Finally, sleep-related problems including insomnia, sleep fragmentation, excessive daytime sleepiness and nocturnal agitation during rapid eye movement sleep are also frequent in MSA and PD patients (Boeve *et al.*, 2007; De Cock *et al.*, 2011; Ghorayeb *et al.*, 2002; Knie *et al.*, 2011; Moreno-Lopez *et al.*, 2011; Palma *et al.*, 2015).

5- Animal models

Various animal models have been developed to study the etiopathogenesis of MSA and PD and to test putative neuroprotective treatment strategies before embarking in clinical trials.

a) Toxin based models

Initial attempts to reproduce neurodegenerative disorders in rodents depended on toxin injection aimed at destroying structures implicated in PD and MSA pathology (Bezard *et al.*, 2013; Fernagut and Tison, 2012; Zigmond and Stricker, 1989). Toxins such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP),

rotenone, quinolic acid, 3-nitroproprionic acid and paraquat have been used separately or combined (double toxin-double lesion models) to destroy the nigro-striatal pathway and produce parkinsonian like motor alterations (Fernagut *et al.*, 2004; Stefanova *et al.*, 2003). Toxic lesion models recapitulate neurodegeneration but fail to reproduce the hallmark of these disorders which are intracellular protein aggregates.

b) Genetic models

Since the identification of α-syn aggregates in LBs in PD (Spillantini *et al.*, 1998b; Spillantini *et al.*, 1997) and GCIs in MSA (Spillantini *et al.*, 1998a), genetic models have been developed to gain insight into the molecular mechanisms of PD and MSA. Targeted overexpression of human α-syn (hα-syn) in neurons was achieved under neuronal promoters (Thy1, PDGF, TH, Prion) leading to different transgenic mouse models with widespread expression of the transgene in the brain. Mutated and truncated forms of α-syn were also overexpressed in mice to recapitulate post-transcriptional and post-translational modifications found in the human brain. All models reproduce some aspects of PD, most importantly α-syn expression in neurons but do not show marked neurodegeneration (Bezard *et al.*, 2013; Giasson *et al.*, 2002; Kahle, 2008; Masliah *et al.*, 2000; Matsuoka *et al.*, 2001; Richfield *et al.*, 2002; Rieker *et al.*, 2011; Rockenstein *et al.*, 2002; Tofaris *et al.*, 2006; Wakamatsu *et al.*, 2008).

In MSA, three different promoters (proteolipid promoter (PLP), 2',3' – cyclic nucleotide 3'-phosphodiesterase (CNP) and myelin basic protein (MBP)) were used to target overexpression of hα-syn in oligodendrocytes. All three lines recapitulate MSA pathogenesis via the accumulation of insoluble α-syn, motor and non-motor impairment, neurodegeneration and myelin palor (Fernagut and Tison, 2012; Kahle *et al.*, 2002; Shults *et al.*, 2005; Stefanova and Wenning, 2015; Yazawa *et al.*, 2005). More importantly, these models provided evidence that α-syn overexpression in oligodendrocytes and the subsequent formation of GCIs is sufficient to produce a secondary neurodegeneration in MSA even though cell loss is moderate compared to human disease (Kahle *et al.*, 2002; Shults *et al.*, 2005; Stefanova *et al.*, 2005a; Ubhi *et al.*, 2010; Yazawa *et al.*, 2005).

The PLP-SYN transgenic MSA mouse model was used for this PhD work. PLP-SYN mice recapitulate several aspects of MSA pathology such as triangular/half-moon shaped insoluble α-syn aggregates that are phosphorylated on Ser129 residue (Kahle *et al.*, 2002), loss of dopaminergic neurons in the SNc (Stefanova *et al.*, 2005a) and microglial activation similar to the human disease (Stefanova *et al.*, 2007). These pathological alterations are accompanied by a progressive motor phenotype and autonomic dysfunction as illustrated by

cardiovascular, renal and respiratory dysfunction (Boudes *et al.*, 2013; Fernagut *et al.*, 2014b; Flabeau *et al.*, 2014; Kuzdas *et al.*, 2013; Stemberger *et al.*, 2010).

c) Dual hit models

Alternatives to gene based models are genetic models that are exposed to toxins in order to generate a robust phenotype that is characterized by significant neuronal loss in the nigrostriatal system accompanied by the accumulation of insoluble α-syn in oligodendrocytes or neurons (Norris *et al.*, 2007; Song *et al.*, 2004; Stefanova *et al.*, 2005a; Ubhi *et al.*, 2009).

d) Viral based models

Another approach to generate PD models is based on the expression of α-syn using adeno-associated virus (AAV) and lentiviral (LV) vectors (Decressac *et al.*, 2013; Engeln *et al.*, 2013; Lo Bianco *et al.*, 2008; Xilouri *et al.*, 2013). This approach shows several advantages on previous models such as the expression of the transgene in a targeted cell population or structure, the use of the contralateral hemisphere as control for both histopathological analysis and behavioral analysis and finally the ability to use wild-type animals as hosts increasing the species range of animal models from mouse to rat and macaque.

AAV was used to model PD in this work. Unlike transgenic models, injection of AAV-2/9 encoding the human A53T mutated α-syn in the SN recapitulate several features of the human disease such as behavioral impairments accompanied by nigral dopaminergic cell loss, loss of striatal TH fibers, α-syn inclusions in neurons and neuroinflammation (Engeln *et al.*, 2013; Van der Perren *et al.*, 2015).

6- Treatment

a) Symptomatic treatment

Since its discovery in the 1960s, Levodopa is the standard dopamine replacement therapy for the treatment of PD. Levodopa is a dopamine precursor capable of passing the blood brain barrier. It is metabolized in the striatum by dopaminergic and serotoninergic fibers into dopamine. Even though Levodopa has proven to be successful as a symptomatic treatment for PD, 70-80% of MSA patients are Levodopa unresponsive with the remaining patients becoming unresponsive within short time (Carlsson *et al.*, 1958; Cotzias *et al.*, 1969; Seppi *et al.*, 2006). Beyond Levodopa, dopamine replacement therapy includes monoamine oxidase inhibitors, catechol-O-methyltransferase inhibitors and dopamine agonists. Dopamine

agonists are usually not used in MSA because of the risk to increase orthostatic hypotension (Flabeau *et al.*, 2010; Rascol *et al.*, 2011).

When the above-mentioned treatments become less effective, the medical management of PD becomes more complex with patients often experiencing prolonged wearing-off periods and levodopa-induced dyskinesia. In these patients, deep brain stimulation (DBS) of the subthalamic nucleus or the globus pallidus may be considered (Deuschl *et al.*, 2006; Gillingham, 2000). DBS is not recommended in MSA patients since it is ineffective in most reported cases (Lambrecq *et al.*, 2008; Santens *et al.*, 2006; Talmant *et al.*, 2006; Tarsy *et al.*, 2003).

b) Disease modification

Neuroprotective and disease modifying treatments are urgent unmet needs in PD and more importantly in MSA (Goetz *et al.*, 2005; Meissner *et al.*, 2011). In the early 1990s, transplantation of embryonic cells was undertaken in the striatum of PD patients (Lindvall *et al.*, 1990). Double-blind trials failed to show any significant beneficial effect (Freed *et al.*, 2001; Olanow *et al.*, 2003). Several neuroprotective strategies were successful in preclinical models of PD and MSA, yet these drugs failed to produce and translate beneficial effects to PD and MSA patients in clinical trials. Nevertheless, some candidate drugs such as the anti-diabetic Exendin-4, PRX002 a monoclonal antibody targeting α-syn and AFFITOPE PD01A a vaccine directed against α-syn have proven to be both safe and tolerable in phase I clinical trials and future studies are needed to validate them for therapeutic efficacy and safety in larger cohorts (Dehay *et al.*, 2015; Fernagut *et al.*, 2014a; Meissner *et al.*, 2011).

II- Insulin/IGF-1

Beyond their role in glucose homeostasis in the body and brain, insulin and insulin-like growth factor-1 (IGF-1) have pleiotropic actions in the brain. These actions range from trophic and protective actions on neurons such as housekeeping and anti-apoptotic actions to the modulation of brain excitability, BBB permeability, in addition to oligodendrocyte maturation/functioning, microglial and astrocyte function. Insulin/IGF-1 receptors are widely expressed throughout the brain. Impaired insulin/IGF-1 signalling and brain insulin resistance are well described features of AD (Bomfim *et al.*, 2012; Moloney *et al.*, 2010; Steen *et al.*, 2005; Talbot, 2014; Talbot *et al.*, 2012). Insulin resistance is the inability of cells to use or bind insulin/IGF-1 efficiently, which in turn leads to decreased signalling and modulation of downstream targets (Boura-Halfon and Zick, 2009; Moloney *et al.*, 2010; Zick, 2001, 2004).

Recent studies have reported altered peripheral insulin/IGF-1 levels in MSA and PD patients, as well as abnormal gene expression of insulin, IGF-1 and their receptors in the brains of PD and DLB patients (Godau *et al.*, 2010; Godau *et al.*, 2011; Mashayekhi *et al.*, 2010; Numao *et al.*, 2013; Pellecchia *et al.*, 2010; Picillo *et al.*, 2013a; Tong *et al.*, 2009). Clinical trials targeting insulin/IGF-1 signalling and insulin resistance have recently caught interest in neurodegenerative diseases, especially in AD and PD (Aviles-Olmos *et al.*, 2013a; Aviles-Olmos *et al.*, 2014; Aviles-Olmos *et al.*, 2013b). In this regard, glucagon like peptide-1 (GLP-1) analogues, such as Exendin-4, activate the same signalling mechanisms as insulin/IGF-1 through GLP-1 receptors (Baggio and Drucker, 2007; Baggio *et al.*, 2004).

We recently published a review describing the current state of the art on neurodegeneration and insulin/IGF-1 signalling which will serve here as the second part of the introduction to this PhD work.

Insulin/IGF-1 and neurodegenerative disorders (Review)

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Insulin, IGF-1 and GLP-1 signaling in neurodegenerative disorders: Targets for disease modification?

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ABSTRACT

Insulin and Insulin Growth Factor-1 (IGF-1) play a major role in body homeostasis and glucose regulation. They also have paracrine/autocrine functions in the brain. The Insulin/IGF-1 signaling pathway contributes to the control of neuronal excitability, nerve cell metabolism and cell survival. Glucagon like peptide-1 (GLP-1), known as an insulinotropic hormone has similar functions and growth like properties as insulin/IGF-1. Growing evidence suggests that dysfunction of these pathways contribute to the progressive loss of neurons in Alzheimer's disease (AD) and Parkinson's disease (PD), the two most frequent neurodegenerative disorders. These findings have led to numerous studies in preclinical models of neurodegenerative disorders targeting insulin/IGF-1 and GLP-1 signaling with currently available anti-diabetics. These studies have shown that administration of insulin, IGF-1 and GLP-1 agonists reverses signaling abnormalities and has positive effects on surrogate markers of neurodegeneration and behavioral outcomes. Several proof-of-concept studies are underway that attempt to translate the encouraging preclinical results to patients suffering from AD and PD. In the first part of this review, we discuss physiological functions of insulin/IGF-1 and GLP-1 signaling pathways including downstream targets and receptors distribution within the brain. In the second part, we undertake a comprehensive overview of preclinical studies targeting insulin/IGF-1 or GLP-1 signaling for treating AD and PD. We then detail the design of clinical trials that have used anti-diabetics for treating AD and PD patients. We close with future considerations that treat relevant issues for successful translation of these encouraging preclinical results into treatments for patients with AD and PD.
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Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; Akt, protein kinase B; α-syn, α-synuclein; BAD, Bcl-2 antagonist of death; BBB, blood brain barrier; Bcl-2, B-cell lymphoma 2; Bcl-XL, B-cell lymphoma extra large; Bim, Bcl-2 interacting mediator of death; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; CSF, cerebrospinal fluid; DLB, dementia with lewy bodies; DPP-4, dipeptidyl peptidase 4; ERK, extracellular signal regulated kinase; FasL, apoptosis-stimulating fragment ligand; FoxO, forkhead box O; GCI, glial cytoplasmic inclusions; GLP-1, glucagon like peptide-1; GLP-1R, glucagon like peptide-1 receptor; Grb2, growth factor receptor-bound protein 2; GSK-3β, glycogen synthase kinase 3β; 6-OHDA, 6-hydroxydopamine; IDE, insulin degrading enzyme; IGF-1, insulin like growth factor-1; IGF-1R, insulin like growth factor-1 receptor; IGFBP, insulin like growth factor binding protein; IR, insulin receptor; IRS, insulin receptor substrate; MAP-K, mitogen associated protein kinase; mTOR, mammalian target of rapamycin; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MSA, multiple system atrophy; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PD, Parkinson's disease; PI3-K, phosphoinositide 3-kinase; PKA, protein kinase A; ROS, reactive oxygen species; Shc, Src homology-2/α-collagen-related protein; SN, substantia nigra; Sos, son of sevenless; TH, tyrosine hydroxylase; T2D, type 2 diabetes; UPDRS, Unified PD Rating Scale; ADAS-cog, AD Assessment Scale - Cognitive subscale.

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1. Introduction

Disease modification and neuroprotection are unmet treatment needs in neurodegenerative disorders. A number of epidemiological studies suggest a link between insulin resistance, type 2 diabetes (T2D) and neurodegenerative disorders. Accordingly, diabetes is a risk factor for Alzheimer's Disease (AD) and a significant number of AD patients exhibit diabetes (Leibson et al., 1997; Ott et al., 1999; Schrijvers et al., 2010; Xu et al., 2004). Furthermore, alterations in brain insulin/insulin like growth factor-1 (IGF-1) signaling that are characteristic of T2D have also been documented in animal models (Bjorkqvist et al., 2005; Bomfim et al., 2012; Busiguina et al., 2000; Colin et al., 2005; Ubhi et al., 2010) and patients with AD, Parkinson's Disease (PD) and other neurodegenerative disorders (Table 1) (Aviles-Olmos et al., 2013b; Busiguina et al., 2000; Cereda et al., 2013; D'Amelio et al., 2009; Farrer, 1985; Gatchel et al., 2008; Hu et al., 2007; Lunetta et al., 2012; Mashayekhi et al., 2010; Moloney et al., 2010; Palacios et al., 2011; Pellecchia et al., 2010; Picillo et al., 2013; Podolsky et al., 1972; Saleh et al., 2010; Sandyk, 1993; Santiago and Potashkin, 2013; Steen et al., 2005; Sun et al., 2012; Tong et al., 2009; Torres-Aleman et al., 1998; Vardy et al., 2007).

The insulin/IGF-1 signaling pathway plays a major role in body homeostasis, glucose regulation and has paracrine/autocrine functions in the brain, having in mind that the primary source of both peptides is mostly peripheral and not central (Abbas et al., 2008; Daftary and Gore, 2005; Kappeler et al., 2008; Plum et al., 2005; Porte et al., 2005; Russo et al., 2005; Saltiel and Kahn, 2001; Torres-Aleman, 1999, 2010). It exerts neurotrophic effects in the central nervous system (Knusel et al., 1990; Torres-Aleman, 1999, 2010; Trejo et al., 2001; Ye et al., 2002a; Ye et al., 2002b), acts as a modulator of neuronal excitability and nerve cell metabolism, as well as a prosurvival factor by promoting antiapoptotic action (Barber et al., 2001; Barthwal et al., 2003; Chin et al., 2005; Dudek et al., 1997; Dupraz et al., 2013; Hetman et al., 2000; Leinninger et al., 2004; Miller et al., 2003; Perrini et al., 2010; Pugazhenthi et al., 2000; Russo et al., 2005).

Noteworthy, several drugs used for treating T2D have shown neuroprotective effects in animal models of neurodegenerative disorders and are now being assessed in early phase 2 clinical trials (Aviles-Olmos et al., 2013b; Blonde and Russell-Jones, 2009). These drugs include insulin and IGF-1 on one hand (Table 2) and glucagon like peptide-1 (GLP-1) analogs on the other hand (Tables 3 and 4). The latter have also shown promising neuroprotective effects in preclinical models. These observations are encouraging and suggest that a better understanding of the relationships between diabetes, insulin resistance and neurodegenerative disorders may open new avenues for the development of urgently needed disease-modifying or neuroprotective treatments.

The aim of this review is to provide a comprehensive description of the abnormalities of insulin/IGF-1 and GLP-1 systems in neurodegenerative disorders (Table 1) and to describe recent therapeutic developments for neurodegenerative diseases based on modulation of insulin/IGF-1 and GLP-1 signaling. Other peptides that are involved in glucose homeostasis such as IGF-2 are not treated here because of the scarcity of experimental data with regard to neurodegenerative disorders and the currently unknown potential for future treatment development.

1.1. Insulin/IGF-1 signaling

Insulin and IGF-1 are closely related in terms of biological activity and primary sequence (Werner et al., 2008)(Fig. 1). Insulin is primarily secreted from the pancreas when blood glucose levels are perceived to be high, whereas IGF-1 is mainly secreted by the liver, yet both are also locally synthesized in the brain (Jafferali et al., 2000; Schechter et al., 1996; Schechter et al., 1990; Schechter et al., 1992; Torres-Aleman, 2010).

Insulin classically regulates glucose transport and metabolism. Peripheral actions of insulin/IGF-1 signaling are mediated by a specialized group of glucosensing neurons at the level of the hypothalamus. These neurons respond to peripheral signals that control energy balance and feeding behavior (Baggio and Drucker, 2007; Marino et al., 2011; Niswender and Schwartz, 2003; Porte et al., 2005; Saltiel and Kahn, 2001; Scherer et al., 2011). Alterations in this homeostatic balance through impairment of brain or peripheral insulin release or signaling may cause changes in body weight, hyperinsulinaemia and insulin resistance leading in the long run to harmful effects on the human body including the brain (Abbas et al., 2008; Saltiel and Kahn, 2001; Scherer et al., 2011).

Insulin receptors (IR) are widely expressed throughout tissues of the periphery known to mediate glucose transport into cells and cellular metabolism. In the brain, insulin contributes to synaptic maintenance, neuronal outgrowth and survival, learning and memory, as well as weight and sexual maintenance and regulation (Banks et al., 2012; Craft and Watson, 2004; De Felice et al., 2009; Dudek et al., 1997; Niswender and Schwartz, 2003; Ott et al., 2012; Plum et al., 2005; Porte et al., 2005). IGF-1 exerts its functions mainly through binding to the IGF-1 receptor (IGF-1R) and is reported to be essential for normal growth and development, neuron survival, myelin sheath synthesis, astrocyte function, vessel growth, neuronal excitability and oligodendrogenesis (Chesik et al., 2008; Freude et al., 2008; Kappeler et al., 2008; Lagarde et al., 2007; Liu et al., 2009; Russo et al., 2005; Torres-Aleman, 2010; Ye et al., 2002b).

Insulin and IGF-1 are formed from an ancestral origin for they have common homology (Navarro et al., 1999; Werner et al., 2008). Both receptors show structural homologies, including a transmembrane domain, a kinase domain, and an extracellular binding domain (Fig. 1). Upon activation by ligand binding, the intrinsic tyrosine kinase activity of the IGF-1R or IR phosphorylates the intramembrane domains that serve as docking site for insulin receptor substrate (IRS) and Src homology-2/α-collagen-related

Table 1
Alterations in peripheral and central insulin/IGF-1 signaling pathway in patients with Alzheimer's or Parkinson's disease (and related disorders). Aβ: Amyloid β; AD: Alzheimer's disease; CSF: Cerebrospinal fluid; DLB: Dementia with Lewy bodies; GFAP: Glial fibrillary acidic protein; IGF-1: Insulin like growth factor-1; IGF-1R: Insulin like growth factor-1 receptor; IR: Insulin receptor; IRS-1: insulin receptor substrate 1; IRS-2: insulin receptor substrate 2; IGFBP: Insulin like growth factor-1 binding protein; GSK-3β: Glycogen synthase 3 beta; MSA: Multiple system atrophy; PD: Parkinson's disease; PSP: Progressive Supranuclear Palsy.

Reference	Disease	Sample	Effects
Frölich et al. (1998)	AD	Brain tissue	Decreased insulin tissue levels and IR binding in normal aging in frontal, temporal, parietal and occipital cortex. No difference in insulin tissue levels between AD and age-matched controls, while occipital IR binding was higher in AD. No significant difference in IGF-1 binding between AD and young adults as well as age-matched controls.
Steen et al. (2005)	AD	Brain tissue	In AD compared to controls: - Decreased mRNA expression of IR and IGF-1R in hippocampus and hypothalamus. No changes in IGF-2R expression. - Decreased mRNA expression of insulin and IGF-2 in hippocampus and hypothalamus, and of IGF-1 in frontal cortex. - Less insulin, IGF-I, IR, and IGF-IR-positive neurons in hippocampus due to loss of neurons and reduced neuronal expression. - Decreased levels of hippocampal IR and IGF-1R protein, tyrosyl-phosphorylated IR and IGF-1R protein, as well as substrates IRS-1 (frontal cortex, hippocampus and hypothalamus) and IRS-2 protein (hippocampus). - Reduced levels of p85-associated IRS-1 in hippocampus and hypothalamus as marker of impaired IRS-1 signaling. - Total Akt and GSK-3β levels unchanged but pAkt and pGSK-3β concentrations reduced indicating reduced levels of Akt kinase activity and increased levels of GSK-3β activity.
Vardy et al. (2007)	AD	Serum	Increase in total and free circulating blood IGF-1 levels in AD compared to controls. No difference in IGFBP-3 levels between AD and controls.
Salehi et al. (2008)	AD	Serum/CSF	Increase in IGF-1 and IGFBP CSF levels compared to controls.
Gil-Bea et al. (2010)	AD	CSF	Decrease in insulin CSF levels in patients with mild AD and women with mild cognitive impairment. Positive association between insulin and Aβ1-42 CSF levels.
Moloney et al. (2010)	AD	Brain tissue	IGF-1R levels increased and IGFBP-2 levels decreased in temporal cortex of AD brains. Although IGF-1R levels are increased, fewer neurons express IGF-1 levels. In controls, IGF-1R expression is higher in neurons than in GFAP-positive astrocytes. In AD, the increase in IGF-1R levels is mainly due to increased expression in astrocytes, neurofibrillary tangle-immunoreactive dystrophic neuritis and Aβ plaques, while the number of IGF-1R expressing neurons is reduced. No difference in IR levels between AD and controls, but predominant internal and nuclear staining in AD with reduced cytoplasmic and dendritic expression. No redistribution between neurons and glia. Reduced IRS-1 and IRS-2 levels in AD with concomitant increase of inactive phosphorylated IRS1 (serine 312 and 616). Decrease in regulatory subunits p85α and p110α of PI3-kinase.
Bomfim et al. (2012)	AD	Brain tissue	Increase in IRS-1pSer636/639 and decrease in IRS-2 levels compared to controls.
Talbot et al. (2012)	AD	Brain tissue	Reduced responses to insulin signaling in the IR→IRS-1→PI3K signaling pathway and IGF-1 signaling in the IGF-1R→IRS-2→PI3K signaling pathway. Increase in phosphorylated IRS-1 (serine 616 and 636/639).
Tong et al. (2009)	PD/DLB	Brain tissue	In PD and DLB: - Decreased mRNA expression of insulin and IR in frontal white matter and amygdala, IGF-1R and IGF-2R in frontal white matter. - Increased mRNA expression of IGF-1R and IGF-2R in amygdala. In DLB: - Additional increase in insulin mRNA expression in basal ganglia and decrease in IGF-1R and IGF-2R mRNA expression in frontal cortex. - Decreased IGF-1R and IGF-2R binding in frontal cortex.
Mashayekhi et al. (2010)	PD	Serum/CSF	Increased IGF-1 and IGFBP 1-6 levels in CSF and serum compared to controls.
Godau et al. (2010)	PD	Serum	Increase in IGF-1 levels in patients relative to controls.
Pellecchia et al. (2010)	MSA	Serum	Increase in IGF-1 and insulin levels in patients relative to controls. No difference in IGF-2, IGFBP-1 and IGFBP-3 levels between patients and controls.
Godau et al. (2011)	PD	Serum	Increase in IGF-1 levels in patients relative to controls.
Picillo et al. (2013)	PD	Serum	Increase in IGF-1 levels in patients relative to controls.
Numao et al. (2013)	PD/MSA/PSP	Serum	Increase in IGF-1 levels in MSA, PD and PSP relative to controls. IGF-1 levels were highest in MSA compared to PD, PSP and controls.

protein (Shc). In most cases, phosphorylation of IRS on serine residues results in the uncoupling of IRS from the activated insulin or IGF-1 receptor and a subsequent decrease in insulin/IGF-1 signaling and degradation of the IRS. The fact that IRS contains dozens of potential phosphorylation sites indicates that IGF-1 or IR signaling can be regulated by ligand-independent processes implicated in normal functioning of the cells and also in pathological alterations of the cascade (Boucher et al., 2010; Draznin, 2006; Duarte et al., 2012; Moloney et al., 2010; Talbot et al., 2012).

Phosphorylation of IRS leads to the activation of phosphoinositide 3-kinase (PI3-K) known as one of the important phosphorylated substrate activators. PI3-K activates Akt (also known as protein kinase B), whose phosphorylation modifies the activity of several downstream effectors, leading to enhanced protein synthesis and antiapoptotic effects through direct or indirect inactivation of glycogen synthase kinase 3β (GSK-3β), caspase-9, mixed lineage kinase and B-cell lymphoma 2 (Bcl-2) antagonist of death (BAD) by phosphorylation and activation of Bcl-2 in addition to phosphorylation and activation of B-cell lymphoma extra-large (Bcl-XL) through the activated cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Barber et al., 2001; Barthwal et al., 2003; Brunet et al., 1999; Chin et al., 2005; Datta et al., 1997; Dudek et al., 1997; Erol, 2008; Hetman et al., 2000; Kulik et al., 1997; Plum et al., 2005; Pugazhenthi et al., 2000). PI3-K serves as an activator of many other cascades. It is essential for axonal growth, regeneration and protein synthesis enhancement through activation of mammalian target of rapamycin

Table 2
Preclinical studies assessing the effects of insulin/IGF-1 in models of neurodegenerative disorders. PD: Parkinson's disease; AD: Alzheimer's disease; IGF-1: Insulin like growth factor-1; sc.t.: IGF-1 producing stem cells and transplantation; i.c.: Intracarotidal; s.c.: Subcutaneous; i.c.v.: Intracerebroventricular; i.p.: Intraperitoneal; GPE: Tripeptide glycine-proline-glutamate; MPP+: 1-methyl-4-phenylpyridinium; APP: Amyloid Precursor Protein; 6-OHDA: 6-Hydroxydopamine; TH: Tyrosine hydroxylase; Aβ: Amyloid Beta; Bcl-2:B-cell lymphoma 2; PI3-K: Phosphoinositide 3-kinase.

Reference	Substance	Species route	Model	Duration/dose	Main result
Carro et al. (2002)	IGF-1	Mouse/rat i.c.	AD (Tg 2576 mice overexpressing mutant APP695; Rat: Aβ induced toxicity)	Mouse: 50 μg/kg/d for 1 month Rat: 10 μg, 2 days before Aβ injection	Treatment decreases Aβ burden and gliosis in aging rats and AD mice. IGF-1 stimulates Aβ clearance by increasing the transport of Aβ binding agents such as albumin and transthyretin into the brain.
Carro et al. (2006)	IGF-1	Mouse s.c./i.p.	AD (APP/PS2 mutant)	s.c.: 3 months at 50 μg/kg/day i.p.: 50 μg/kg	Reversal of cognitive deficits and memory impairments. Decrease in astrogliosis and amyloid burden, normalization of synapse viability markers.
Ebert et al. (2008)	IGF-1	Rat sc.t.	PD (6-OHDA)	One week after 6-OHDA lesioning, transplantation of IGF-1 transgenic neurospheres (250000 cells/μl; 500000 cells per hemisphere)	Reduction in amphetamine-induced rotations. Increased survival of grafted human neural progenitor cells and nigral TH positive neurons but not of striatal TH positive fibers.
Gasparini et al. (2001)	Insulin	In-vitro	AD (APP695 mutant)	0.3-1 μM for 4-16 h	Reduction of intracellular accumulation of Aβ by accelerating its trafficking to the plasma membrane.
Guan et al. (2000)	IGF-1(GPE)	Rat i.c.v.	PD (6-OHDA)	GPE treatment (3 μg/15 μl) over 2 h after 6-OHDA lesioning at a rate of 2 μl/min	Prevention of loss of TH positive neurons in the substantia nigra and TH positive fibers in the striatum.
Kao (2009)	IGF-1	In-vitro	PD (WT, A30P and A53 T mutant)	100 ng/ml	Rescue from α-synuclein toxicity and suppression of α-synuclein aggregation.
Krishnamurthi et al. (2004)	IGF-1(GPE)	Rat i.p.	PD (6-OHDA)	GPE treatment (0.3, 3, 30 mg/kg) 3 h after 6-OHDA lesioning at a rate of 2 μl/min	Improvement of parkinsonian motor deficits, no effect on loss of TH positive neurons and fibers.
Niikura et al. (2001)	IGF-1/Insulin	In-vitro	AD (WT-APP and V642I-APP mutant)	10 nM	IGF-1 and insulin protected cells from APP induced apoptosis. IGF-1 suppressed the cleavage of procaspase-3.
Offen et al. (2001)	IGF-1	In-vitro	PD (dopamine)	IGF-1 (0.5 μg/ml) along with or after dopamine addition	Decrease in apoptosis accompanied by an increase in Bcl-2 levels.
Quesada et al. (2008)	IGF-1	Rat i.c.v.	PD (6-OHDA)	100 μg/ml for 7 days right after 6-OHDA lesioning	Significant increase in TH positive neurons and improvement in motor performance, protective effect dependent on PI3-K/Akt signaling.
Sun et al. (2010)	IGF-1	In-vitro	PD (MPP^+)	IGF-1 (0, 0.33, 1, 3.3, 10, 33, 100 nM) along with MPP^+	Increase in cell viability and decrease in cell apoptosis.
Zawada et al. (1996)	IGF-1	In-vitro	Proliferation assay	20–1200 ng/ml	Reduction in TH positive neurons undergoing apoptosis. Proliferation of astrocytes but not of dopamine neurons.

(mTOR) (Banks et al., 2012; Brunet et al., 1999; Delcommenne et al., 1998; Dupraz et al., 2013; Kennedy et al., 1999). The activation of Akt by insulin and IGF-1 promotes the phosphorylation and inhibition of Forkhead box O (FoxO) which is retained in the cytoplasm (Dong et al., 2008; Duarte et al., 2012; Matsuzaki et al., 2003; Polter et al., 2009). FoxO is part of the transcriptional activator family known to be implicated in the regulation of genes and to have effects ranging from pro-survival to apoptotic effects. Activation of FoxO in the brain has been linked to apoptosis through the induction of apoptosis-stimulating fragment ligand (FasL) promoter and Bcl-2 interacting mediator of death (Bim) (Barthelemy et al., 2004; Dijkers et al., 2000; Lam et al., 2006).

Another important cascade activated by insulin/IGF-1 is the Raf-1/MEK-MAP-K (mitogen associated protein kinase)/ERK (extracellular signal regulated kinase) pathway and their downstream targets. MAPK inhibits apoptosis and promotes neuronal survival through the inhibition of caspase-9 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) but also through its antagonistic effects on oxidative stress and neuroinflammation (Dagon et al., 2005; Giri et al., 2004). The upstream activators of this pathway known as Shc and IRS actually compete for binding growth factor receptor-bound protein 2 (Grb2), the recruiting agent of Ras also known as the activator of this pathway (Fig. 1). The amount of Shc/Grb2 binding correlates with ERK activation (Duarte et al., 2012; Quesada et al., 2008; Yamauchi and Pessin, 1994). Once activated, ERK is translocated to the nucleus where it phosphorylates a wide range of transcription factors involved in cell growth and mitogenesis (Feldman et al., 1997). Shc protein is found in several isoforms, which can be phosphorylated upon stimulation by growth factors. The P46/P52Shc isoform modulates growth transduction and survival signals while P66Shc plays an important role in mediating oxidative stress-dependent cell damage and apoptosis (Migliaccio et al., 1999; Su et al., 2012). P46/P52Shc activates the ERK pathway via Grb2-Son of sevenless (Sos)-Ras while P66Shc exerts an inhibitory effect on P46/P52 activated ERK marked by decreased P66Shc levels when ERK activation is high. Clearly these phosphorylation series control how ERK regulates neural activity and cell death (Fasano et al., 2010; Okada et al., 1997; Spescha et al., 2013; Su et al., 2012).

It is currently hardly possible to define the respective weight/contribution of each cascade implicated in insulin/IGF-1 signaling due to the multiple interactions of these pathways. Computational science will help in the future to better understand the respective weight/contribution of the different cascades but models are still primitive. Activating PI3-K and MAPK separately is not evident since they interact and may also compensate for each other. Recent studies have shown a dichotomic action by insulin and/or IGF-1 binding which initiate a segregated response from the earliest signaling elements to the ones that come far downstream (see also chapter 2.2 and 2.4).

Table 3
Preclinical studies assessing effects of GLP-1 in models of neurodegenerative disorders. PD: Parkinson's disease; AD: Alzheimer's disease; GLP-1: Glucagon-like peptide 1; s.c.: Subcutaneous; i.c.v.: Intracerebroventricular; i.p.: Intraperitoneal; p.o.: Per os administration; TH: Tyrosine hydroxylase; Aβ: Amyloid Beta; APP: Amyloid Precursor Protein, LTP: Long term potentiation; LTD: Long term depression; VMAT2: Vesicular monoamine transporter 2; 6-OHDA: 6-hyroxydopamine; IRS: Insulin receptor substrate; IL1β: Interleukin-1 beta; JNK: c-Jun N-terminal kinase; TNFα: Tumor necrosis factor α; GSK3β: Glycogen synthase 3 beta; t.i.d.: Three times a day; IDE: Insulin degrading enzyme.

Reference	Substance	Species route	Model	Dose/duration	Result
Qin et al. (2008)	GLP-1	In-vitro	AD (SH-SY5Y cells exposed to Aβ)	GLP-1 (0.02, 0.1, 0.5, 2.5 ng/ml) added with Aβ	Reduction in Aβ induced apoptosis.
Perry et al. (2003)	GLP-1 Exendin-4	In-vitro mice (i.c.v.)	AD (Aβ induced toxicity)	In-vitro: cells pretreated for 2 h with GLP-1 (3 ng/ml (1 M), 16 ng/ml (5 M), 33 ng/ml (10 M) and 66 ng/ml (20 M)) or Exendin-4 (0.2 μg/ml (50 nM), 0.4 μg/ml (100 nM), 0.8 μg/ml (200nM) and 2 μg/ml (500 nM)); Mice: 3.3 ng of GLP-1 or 0.2 ng of Exendin-4	Treatment reduced Aβ in mouse brain and βAPP in cell cultures. Protected against Aβ and iron induced toxicity in mouse model.
Ma et al. (2012)	GLP-1(9-36)^{amide}	In-vitro mice (s.c.)	AD (Aβ toxicity in hippocampal slices, APP/PS1 mutant)	In-vitro: 100 pM; Mice: 500 ng/g/day for 2 weeks	Slice: reversal of LTP and LTD impairment. Mice: reversal of memory deficits, decrease in reactive oxygen species and improvement in Akt-GSK3β signaling.
Bertilsson et al. (2008)	Exendin-4	Rat (i.p.)	PD (6-OHDA)	0.1 μg/kg for 21 days	Reduction of amphetamine-induced rotations, increase in TH positive and VMAT2 positive neurons in the substantia nigra.
Bomfim et al. (2012)	Exendin-4	In-vitro mouse (i.p.)	AD (Aβ oligomer toxicity in hippocampal neurons; APP/PS1 mutant mice; Aβ oligomer toxicity in non-human primates)	In-vitro: 300 nM 30 min before Aβ injection; Mice: 25 nmol/kg for 21 days	Cell culture: prevention of the Aβ oligomer induced increase in IRS-1pSer636 and decrease in IRS-1pTyr465 brain levels, attenuation of defects in axonal transport of dense core vesicles. Mice: reduction in brain levels of IRS-1pSer636, IRS-1pSer312 and pJNK, improvement in cognition.
Harkavyi et al. (2008)	Exendin-4	Rat (i.p.)	PD (6-OHDA or LPS)	Injected with 0.1 or 0.5 μg/kg t.i.d. for 7 days. Start of injection 7 days after lesioning with 6-OHDA or LPS.	Reduction of amphetamine-induced rotations, increase in striatal tissue dopamine concentrations and the number of nigral TH positive neurons.
Kim et al. (2009)	Exendin-4	Mouse (i.p.)	PD (MPTP)	10 μg/kg 30 min before each of four MPTP injections that were separated by a 2 h interval	Reduction in loss of TH positive striatal fibers and nigral neurons, attenuation of microglial activation and MPTP-induced expression of matrix metalloproteinase-3, TNFα and IL1β.
Li et al. (2009)	Exendin-4	In-vitro mouse (i.c.v.)	PD (6-OHDA in-vitro, MPTP mouse model)	In-vitro: 10 nM to 1 μM Mice: 20nM, 0.25 μl/hr for 7 days. Injection began 2 h before MPTP treatment	Increase in the number of TH positive striatal fibers and nigral neurons as well as striatal dopamine levels, improvement in motor performance (rotarod, pole test).
Li et al. (2010)	Exendin-4	In-vitro mouse (s.c.)	AD (Aβ toxicity in rat primary neurons and SH-SY5Y cells; mutant mice (3xTg-AD))	In-vitro: 0, 50, 100, 200, and 500 nM for 2 h and later injected with Aβ; Mice: 3.5 pM/kg/min for 16 weeks	Cell culture: reduction of vulnerability to Aβ oxidative stress-induced cell death. Mice: decrease in Aβ and Aβ protein precursor levels, no effect on total tau levels.
Gault and Holscher (2008)	(Val^{8})GLP-1	Rat (i.c.v.)	AD (Aβ 25-35 fragment injection)	3 nmol/μl either at the same time, 15 or 30 min before Aβ injection	Reversal of LTP abnormalities when applying (Val^{8})GLP-1 30 min before Aβ.
Gengler et al. (2012)	(Val^{8})GLP-1	Mouse (i.p.)	AD (APP/PS1 mutant)	2.5 or 25 nmol/kg for 21 days	Rescue of hippocampal LTP, decrease in cortical dense core plaque load.
Wang et al. (2013)	(Val^{8})GLP-1	In-vitro	AD (Aβ induced toxicity)	10 nM	Reversal of Aβ induced reduction in excitatory and inhibitory postsynaptic currents, prevention of Aβ induced increase in intracellular calcium.
McClean et al. (2011)	Liraglutide	Mouse (i.p.)	AD (APP/PS1 mutant)	25 nM/kg for 8 weeks prior to conduction of tests	Improvement of cognition, increase in LTP and paired pulse facilitation, reduction in amyloid plaque and dense core plaque load, reduction in microglial activation.
McClean and Holscher (2013)	Liraglutide	Mouse (i.p.)	AD (APP/PS1 mutant)	25 nM/kg for 8 weeks prior to conduction of tests	Enhanced spatial memory, increase in LTP along with an increase in the number of synapses in the hippocampus and cortex, reduction in amyloid plaque load, reduction in total brain APP and Aβ, reduction in microglial activation, increase in neuronal progenitor cells, increase in IDE levels.

Table 3 (*Continued*)

Reference	Substance	Species route	Model	Dose/duration	Result
Parthsarathy and Holscher (2013)	Liraglutide	Mouse (i.p.)	AD (APPswe, PSEN1dE9 mutant)	25 nM/kg for 7 or 37 days	Acute treatment: increase in newly generated cells in dentate gyrus in AD mice but not in wildtype mice. Increase in cell proliferation and neuroblast differentiation in AD and wildtype mice. No difference in the number of mature neurons. Chronic treatment: increase in newly generated cells, neuroblast differentiation and neurogenesis in AD and wildtype mice.
D'Amico et al. (2010)	Sitagliptin	Mouse (p.o.)	AD (APPswe, PSEN1dE9 mutant)	5,10 or 20 mg/kg for 12 weeks	Increase in brain levels of GLP-1, decrease in hippocampal Aβ and APP levels and formation of amyloid plaques, reduction in IL1β and nitrotyrosine, improvement in cognition.

Table 4
Main pharmacokinetic characteristics of GLP-1 analogs and DPP-4 inhibitors.

Drug	Trade name	Function	Half-life	BBB penetration	Excretion	Tissue distribution	References
Albiglutide	Syncria®	GLP-1 Analog	6–8 days	None to very limited	No data	GLP-1 binding sites: kidney, lung, pancreas, stomach, blood, spleen, liver and brain	Baggio et al., 2004; Bush et al., 2009; Rosenstock et al., 2009
Exendin-4	Byetta® Bydureon®	GLP-1 analog	2–3 h	High	Renal		Copley et al., 2006; EMEA, 2009a, 2010; Kastin and Akerstrom, 2003; Wild et al., 2010
Liraglutide	Victoza®	GLP-1 analog	4–15 h	Moderate to high	None		Elbrond et al., 2002; Hunter and Holscher, 2012; Malm-Erjefalt et al., 2010; McClean et al., 2010
Lixisenatide	Lyxumia®	GLP-1 analog	2–4 h	Moderate to high	Renal		EMEA, 2013; Hunter and Holscher, 2012
Alogliptin	Nesina®	DPP4 inhibitor	12–21 h	No data	Renal	Kidney, lung, liver, intestine, adrenal gland, testis, pancreas, spleen; low amounts in brain; Surface of endothelial cells lining blood vessels and found in a soluble form, freely circulating in the blood	Baetta and Corsini, 2011; Baggio and Drucker, 2007; Christopher et al., 2008; Scheen, 2010
Linagliptin	Tranjenta®	DPP4 inhibitor	36 h	Low	Fecal		Baggio and Drucker, 2007; Blech et al., 2010; Deacon, 2011; EMEA, 2011; Fuchs et al., 2009; Scheen, 2010
Saxagliptin	Onglyza®	DPP4 inhibitor	2–4 h	Low	Renal		Baggio and Drucker, 2007; Deacon, 2011; EMEA, 2009b; Fura et al., 2009; Scheen, 2010
Sitagliptin	Januvia®	DPP4 inhibitor	8–14 h	Low	Renal		Baggio and Drucker, 2007; Chu et al., 2007; Deacon, 2011; Herman et al., 2005; Vincent et al., 2007
Vildagliptin	Galvus®	DPP4 inhibitor	2–3 h	Low	Renal		Baggio and Drucker, 2007; Deacon, 2011; EMEA, 2007; He et al., 2009; Scheen, 2010

1.2. GLP-1 signaling

GLP-1 is an endogenous insulinotropic hormone that has an important role in the balance between insulin and glucose levels. Primarily secreted by intestinal endocrine L-cells, GLP-1 functions include stimulation of glucose-dependent insulin secretion and insulin biosynthesis, as well as inhibition of glucagon secretion, gastric emptying and food intake (Baggio and Drucker, 2007; Doyle and Egan, 2001; Nauck et al., 1993; Rachman et al., 1996; Sarkar et al., 2003; Toft-Nielsen et al., 1999; Willms et al., 1996; Zander et al., 2002). Interestingly, GLP-1 is expressed in neurons and acts as a neurotransmitter (Sarkar et al., 2003). It has trophic effects on cell proliferation, neurogenesis and apoptosis (Brubaker and Drucker, 2004). GLP-1 reduces cell death in islets β-cells, fibroblasts and neurons (Farilla et al., 2002; Li et al., 2005; Li et al., 2003; Perry et al., 2002a; Perry et al., 2002b).

GLP-1 mediates its actions through the GLP-1R, a 7-transmembrane-spanning G protein-coupled receptor (GPCR). It activates the α subunit of the GPCR leading to adenyl cyclase activation and increased production of cAMP. As a result, cAMP activates protein kinase A (PKA) a central component which phosphorylates and activates several downstream effectors that act on protein synthesis and antiapoptotic effectors (see further chapter 1.1) (Fig. 1) (Baggio and Drucker, 2007; Drucker et al., 1987). Major pathways through which GLP-1 exerts its functions are PI3-K and MAPK pathways (Baggio and Drucker, 2007; Li et al., 2005; Perry et al., 2002b). The effects of GLP-1 on lowering peripheral glucose levels are limited by dipeptidyl peptidase 4 (DPP-4) which metabolizes GLP-1 within two minutes (Deacon et al., 1995; Drucker, 2003a,b). DPP-4 is a serine protease known to specifically cleave dipeptides from the amino terminus of proteins that contain an alanine or proline residue thereby inhibiting or modifying their

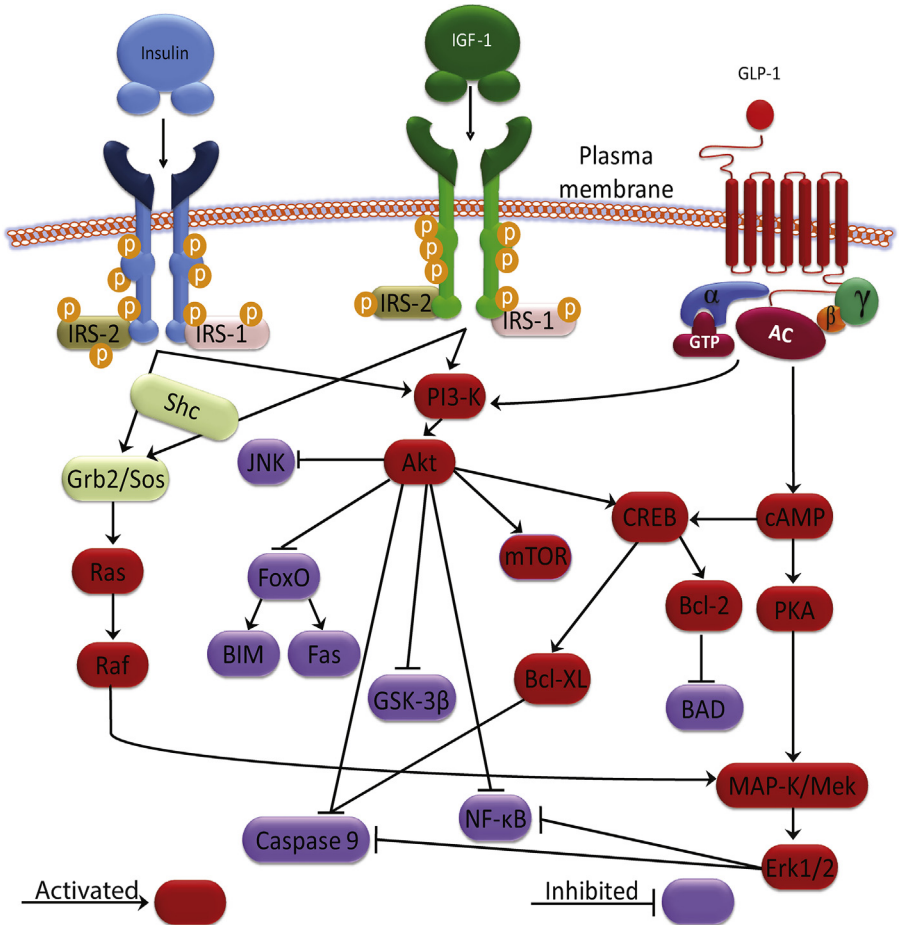

Fig. 1. Insulin, IGF-1 and GLP-1 dependent intracellular signaling transduction pathways showing overlapping downstream targets. AC: Adenyl cyclase; Akt: Protein kinase B; Bcl-2: B-cell lymphoma 2; BAD: (Bcl-2) antagonist of death; Bcl-XL: B-cell lymphoma 2 extra-large; BIM: Bcl-2 interacting mediator of death; cAMP: Cyclic adenosine monophosphate; CREB: cAMP response element-binding protein; ERK: Extracellular signal regulated kinase; Fas: Apoptosis-stimulating fragment; Foxo: Forkhead box O; GLP-1: Glucagon like peptide-1; GRB2: growth factor receptor-bound protein 2; GSK3β: Glycogen synthase 3 beta; GTP: Guanosine triphosphate; IGF-1: Insulin like growth factor-1; IRS-1: Insulin receptor substrate 1; IRS-2: Insulin receptor substrate 2; JNK: c-Jun N-terminal kinase; MAP-K: Mitogen associated protein kinase; MEK: MAPK kinsase; mTOR: Mammalian target of rapamycin; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; PI3-K: Phosphoinositide 3-kinase; PKA: Protein kinase A; Shc: Src homology-2/α-collagen-related protein.

activity and function (Baetta and Corsini, 2011; Demuth et al., 2005; Scheen, 2010). DPP-4 metabolizes GLP-1 into GLP-1 (9-37) and GLP-1 (9-36) NH2, the latter accounting for 75% of circulating GLP-1 in the body. DPP-4 circulates in the blood as a soluble protein and is expressed in brain tissue, liver, pancreas, endothelial cells and other areas (Baggio and Drucker, 2007; Demuth et al., 2005).

Synthetic GLP-1 analogs such as exendin-4, liraglutide, albiglutide and lixisenatide (Table 4) are resistant to DPP-4, pass the blood brain barrier (BBB) similar to GLP-1, except for albiglutide, and bind to the GLP-1R (Baetta and Corsini, 2011; Deacon, 2011; Hunter and Holscher, 2012; Lovshin and Drucker, 2009; Martin et al., 2011; McIntyre et al., 2013; Rosenstock et al., 2009). They exert neurotrophic and neuroprotective actions and enhance cognitive functions (see further chapter 3.2 and Table 3) (During et al., 2003; Perry and Greig, 2002; Perry et al., 2002a; Perry et al., 2002b).

1.3. Expression of IR, IGF-1R and GLP-1R in the brain

IGF-1R is differentially expressed throughout the brain (Table 5). Levels are highest in the olfactory bulb and cortex, while moderate to low amounts are found in the hippocampus, amygdala, thalamus and substantia nigra (Bondy, 1991; Cardona-Gomez et al., 2000; De Keyser et al., 1994; Ferrari et al., 2003; Gammeltoft et al., 1985; Garcia-Segura et al., 1997; Matsuo et al., 1989; Quesada et al., 2007; Rotwein et al., 1988; Sonntag et al., 1999; Werther et al., 1989; Zhao et al., 2004). IGF-1R is also highly expressed in the choroid plexus, suggesting that these receptors may serve as a transport system for circulating IGF and thereby regulate IGF-1 levels in the cerebrospinal fluid (Bondy, 1991; De Keyser et al., 1994). Brain white matter shows the lowest expression of IGF-1R in line with the current assumption that the majority of IGF-1R positive cells are neurons and to a lower extent glial cells, most of the latter being in inflammatory activating states (Cardona-Gomez et al., 2000; Garcia-Segura et al., 1997; Rotwein et al., 1988). Neurons synthesize IGF-1 under physiological conditions, while astrocytes produce it after local injury (Jafferali et al., 2000; Moloney et al., 2010; Salehi et al., 2008; Torres-Aleman, 2010), which may represent an active defense mechanism in degenerating parts of the brain. IGF-1R and IR densities decrease with age both in humans and rodents (Adamo et al., 1989; Daftary and Gore, 2005; Garcia-Segura et al., 1991; Jafferali et al., 2000; Rotwein et al., 1988).

Controversy remains regarding the source of brain insulin. Although circulating insulin crosses the BBB (Banks, 2004), several studies have also shown local synthesis of insulin in cultured neurons, immature nerve cells, as well as positive staining for insulin or its precursors in synapses, axons and dendrites of neurons (Clarke et al., 1986; Schechter et al., 1996; Schechter et al.,

Table 5
Receptor distribution in the brain. IR: Insulin receptor; IGF-1R: Insulin like growth factor-1 receptor; GLP-1R: Glucagon like peptide-1 receptor; PET: Positron emission tomography.

References	Receptors	Model	Methods	Result/distribution
Bondy (1991)	IGF-1R	Rat	*In-situ* hybridization	Olfactory bulb (mitral, tufted and granule cells), cerebral cortex (pyramidal neurons and interneurons), granule layer of Purkinje cells, choroid plexus, granule layer of dentate gyrus and pyramidal cells of CA1 of hippocampus, hypothalamus.
Cardona-Gomez et al. (2000)	IGF-1R	Rat	Immunofluorescence	Colocalization with estrogen receptor. Expression in glia.
De Keyser et al. (1994)	IGF-1R	Human	Binding	Found in pituitary gland, choroid plexus, olfactory bulb, pineal gland, cerebral cortex, hippocampus, amygdala, subtantia nigra, thalamus.
Ferrari et al. (2003)	IGF-1R	Rat	Immunohistochemistry Immunofluorescence	Characterization of IGF-1R on olfactory receptor neurons and expression through growth and maturation.
Gammeltoft et al. (1985)	IGF-1R	Rat Human	Binding	Olfactory bulb, cerebellum, hippocampus, amygdala, cortex, striatum, hypothalamus.
Garcia-Segura et al. (1997)	IGF-1R	Rat	Electron microscopy	Cerebellar cortex (soma and dendrites of Purkinje cells),mediobasal hypothalamus (cell soma and dendrites), astrocytes, oligodendrocytes and endothelial cells.
Matsuo et al. (1989)	IGF-1R	Rat	Binding	High densities in the olfactory nerve layer, olfactory glomerular layer, anterior pituitary gland, choroid plexus, CA3 and CA4 of the hippocampus, basolateral amygdaloid nucleus, and endopiriform nucleus. Moderate levels in the cerebral cortex (layer II and VI), nucleus stria terminalis, nucleus accumbens, lateral septum, median preoptic nucleus, supraoptic nucleus, paraventricular hypothalamic nucleus, and ventroposterior thalamic nucleus.
Quesada et al. (2007)	IGF-1R	Rat	Immunocytochemistry immunofluorescence *in-situ* hybridization	Presence on glial cells and neurons of substantia nigra.
Rotwein et al. (1988)	IGF-1R	Rat	Binding	Olfactory bulb, cerebellum, cortex, striatum, hippocampus, hypothalamus.
Sonntag et al. (1999)	IGF-1R	Rat	Autoradiography *in-situ* hybridization	Showed that IGF-1R in cortex, hippocampus and hypothalamus decreases with aging.
Werther et al. (1989)	IR and IGF-1R	Rat	Binding	IGF-1R: olfactory bulb, choroid plexus, cerebellum, thalamus, hippocampus, amygdala, cortex, basal ganglia and hypothalamus. Insulin: high expressions in same locations. Hypothalamus and CA1 of hippocampus with higher expression than the thalamus and the other regions of the hippocampus.
Baskin et al. (1986)	IR	Rat	Binding	High amounts in the choroid plexus, olfactory bulb and cerebral cortex.
Dorn et al. (1981)	IR	Mouse	Immunohistochemistry	High amounts in the cerebellum and hypothalamus. Moderate amounts in brain stem and hippocampus. Low amounts in the thalamus and cortex (localization not specified).
Dorn et al. (1982)	IR	Human	Immunohistochemistry	Distribution throughout the brain mainly in the hypothalamus, hippocampus, medulla oblongata, cerebral cortex and amygdala.
Havrankova et al. (1978)	IR	Rat	Binding	High amounts in the olfactory bulb and the cerebral cortex. Moderate amounts in the cerebellum, hypothalamus and brain stem.
Iozzo et al. (2002)	IR	Rat	PET	High amounts in the olfactory bulb, cerebellum and hypothalamus. Moderate amounts in the hippocampus, cerebral cortex, medulla oblongata and thalamus.
Unger et al. (1989)	IR	Rat	Immunohistochemistry	Hippocampus, hypothalamus, habenula, olfactory bulb, subthalamic nucleus, cerebral cortex, amygdala, basal ganglia.
Zhao et al. (2004)	IR	Rat	*In-situ* hybridization	Highest in Cerebellum, choroid plexus and lateral ventricles.
Alvarez et al. (2005)	GLP-1R	Human	*In-situ* hybridization	Hypothalamus (arcuate and ventromedial nuclei), caudate putamen, globus pallidus, hippocampus, thalamus and cerebral cortex.
Chowen et al. (1999)	GLP-1R	Rat	*In-situ* hybridization binding	Cerebral cortex, hippocampus, thalamus, hypothalamus, choroid plexus and pituitary gland; present in glia after injury.
Goke et al. (1995)	GLP-1R	Rat	Binding	High amounts in hypothalamus, medulla and pons; Moderate amounts in septum, thalamus, basal ganglia, amygdala and mesencephalon.
Hamilton and Holscher (2009)	GLP-1R	Mouse	Immunohistochemistry	Neocortex: expressed in medium to large pyramidal cells and in dendrites. Glia, stellate cells and interneurons are negative for GLP-1R. Hippocampus: pyramidal neurons and dendrites were stained. Basal ganglia: few amounts. Cerebellum: staining in Purkinje cells.
Jin et al. (1988)	GLP-1R	Rat	Immunoreactivity	High amounts in hypothalamus. Moderate amounts in thalamus and mesencephalon. Low amounts in pons, basal ganglia, olfactory bulb, medulla, cortex, septum and amygdala.
Kanse et al. (1988)	GLP-1R	Rat	Binding	High amounts in the hypothalamus, medulla and midbrain. Moderate amounts in pons, cerebellum and pituitary gland. Low amounts in the olfactory bulb and cerebral cortex.
Merchenthaler et al. (1999)	GLP-1R	Rat	*In-situ* hybridization	High amounts in hypothalamus and medulla. Moderate amounts in septum and thalamus. Low amounts in pons, basal ganglia and olfactory bulb.

1990; Schechter et al., 1992; Schechter et al., 1998; Steen et al., 2005; Woods et al., 2003). Having a similar distribution pattern and a shared signaling system with IGF-1 (Baskin et al., 1986; Baskin et al., 1993; Baskin et al., 1983; Dorn et al., 1981; Dorn et al., 1982; Havrankova et al., 1978; Iozzo et al., 2002; Unger et al., 1991; van Houten et al., 1980; Zhao et al., 2004), IR is widely distributed in the brain with highest concentrations in the olfactory bulb, cerebral cortex, hypothalamus, hippocampus and cerebellum (Banks, 2004; Dorn et al., 1981; Dorn et al., 1982; Havrankova et al., 1978; Iozzo et al., 2002; Plata-Salaman, 1991; Unger et al., 1989; Unger et al., 1991; Werther et al., 1989; Zhao et al., 2004). IR levels are higher in neurons than in glial cells, while local synthesis of insulin only occurs in neurons but not in glia (Duarte et al., 2012; Unger et al., 1991).

GLP1-R is also expressed in the brain (Table 5) (Alvarez et al., 2005; Chowen et al., 1999; Goke et al., 1995; Hamilton and Holscher, 2009; Jin et al., 1988; Kanse et al., 1988; Merchenthaler et al., 1999; Perry and Greig, 2003). Sustained GLP1-R expression is reported for the cerebral cortex, especially the occipital and frontal lobes, the hypothalamus and the thalamus while lower levels are found in the caudate putamen, the globus pallidus and the hippocampus (Alvarez et al., 2005). More recent investigations showed that GLP1-R is highly expressed in the pyramidal layer of the hippocampus, the granule layer of the dentate gyrus, and Purkinje cells of the cerebellum but not in glia of normal animals (Hamilton and Holscher, 2009). Noteworthy, GLP-1R and its ligand are expressed by glial cells in pathological conditions (Chowen et al., 1999; Kappe et al., 2012).

2. Insulin/IGF-1, GLP-1 and neurodegenerative disorders

The alteration of the insulin/IGF-1 signaling pathway in AD, PD and other neurodegenerative disorders (Table 1) raises several questions: Does insulin/IGF-1 signaling alteration represent a risk factor for these disorders? Are these alterations a primary contributing cause to the underlying neurodegenerative process or a secondary phenomenon (Aviles-Olmos et al., 2013b; Chen et al., 2012; Finkelstein et al., 2011; Frölich et al., 1998; Gatchel et al., 2008; Lunetta et al., 2012; Morselli et al., 2006; Numao et al., 2013; O'Neill et al., 2012; Ono et al., 2000; Pellecchia et al., 2010; Saleh et al., 2010; Steen et al., 2005; Torres-Aleman et al., 1998)?

2.1. Findings from epidemiological studies

T2D increases the risk for different neurodegenerative diseases. Accordingly, T2D patients have a 65% increased risk of developing AD later in their life (Arvanitakis et al., 2004; Haan, 2006; Leibson et al., 1997; Ott et al., 1999; Schrijvers et al., 2010; Xu et al., 2004), while results remain conflicting for PD (Arvanitakis et al., 2004; Becker et al., 2008; D'Amelio et al., 2009; Driver et al., 2008; Haan, 2006; Hu et al., 2007; Leibson et al., 1997; Noyce et al., 2012; Ott et al., 1999; Palacios et al., 2011; Powers et al., 2006; Pressley et al., 2003; Sandyk, 1993; Schrijvers et al., 2010; Simon et al., 2007; Xu et al., 2011; Xu et al., 2004) (Table 1). Nevertheless, growing evidence shows that PD patients exhibit altered peripheral and cerebral insulin/IGF-1 signaling (Godau et al., 2010; Godau et al., 2011; Mashayekhi et al., 2010; Santiago and Potashkin, 2013; Tong et al., 2009) (Table 1).

2.2. Alzheimer's disease

AD is a common neurodegenerative disease affecting around 27 million people worldwide (Brookmeyer et al., 2007). It is characterized by progressive deterioration of cognitive functions, dominated by a decline in memory. The two pathological hallmarks of AD are extracellular plaques containing amyloid β (Aβ) aggregates and intracellular neurofibrillary tangles that are formed of hyperphosphorylated tau protein (Claeysen et al., 2012; Ittner and Götz, 2011). Mutations in genes encoding amyloid precursor protein (APP) and its cleaving enzymes presenilin-1 or 2 have been described in familiar AD. APP is processed via the amyloidogenic and the non-amyloidogenic pathways. In the latter, APP is cleaved within the Aβ sequence while in the amyloidogenic pathway the action of β and γ secretase cleave APP to 40 and 42 amino acid long peptides. These peptides form toxic Aβ species including dimers, oligomers and fibrils that cause synapse and spine loss, impair long term depression and potentiation, and induce NMDA receptor-mediated excitotoxicity (Ittner and Götz, 2011; Shankar et al., 2008). Hyperphosphorylation of tau protein interferes with microtubule stabilization and axonal transport. Aβ and tau contribute synergistically to the progressive neurodegenerative process (Ittner and Götz, 2011).

Several factors interact in the development of AD, such as the growing evidence of T2D as an independent risk factor (Arvanitakis et al., 2004; Leibson et al., 1997; Ott et al., 1999; Schrijvers et al., 2010). Impaired brain glucose consumption and energy production in AD have been linked with altered insulin/IGF-1 signaling (Chen and Zhong, 2013; Fernandez and Torres-Aleman, 2012). Moreover, T2D and transgenic AD mice show similar cognitive deficits, vascular dysfunction, mitochondrial impairment, and increased Aβ burden in cortex and hippocampus (Carvalho et al., 2012; Carvalho et al., 2013). Intracerebroventricular administration of streptozotocin induces an insulin resistant brain state in mice and non-human primates, which is associated in mice with memory impairment, mitochondrial dysfunction, as well as a significant increase in hippocampal Aβ and hyperphosphorylated tau protein levels reminiscent of sporadic AD in humans (Salkovic-Petrisic et al., 2013; Correia et al., 2013; Lee et al., 2014). Finally, AD transgenic mice that receive a high fat diet show T2D-like peripheral insulin resistance together with decreased IR signaling in the brain, higher hippocampal amyloid burden and more severe cognitive deficits compared to normoglycemic AD mice (Ho et al., 2004). This suggests that conditions leading to T2D-like peripheral insulin resistance may increase the hippocampal amyloid burden via activation of GSK-3β as a consequence of attenuated IR signaling in the brain. These observations were confirmed in another transgenic AD model where high fat diet increased the amount of cortical detergent-insoluble Aβ as well as soluble and insoluble tau protein, decreased levels of the dendritic spine protein drebrin, and induced a trend for increased reactive astrocytosis (Julien et al., 2010). Similar abnormalities were also described in senescence-accelerated mice receiving a high fat diet (Mehla et al., 2014).

In contrast to healthy controls, IR expression in cortical neurons of AD patients increases in internal and nuclear compartments while cytoplasmic and dendritic staining is reduced. Simultaneously, less neurons express IGF-1R while expression in astrocytes occurs in a higher proportion than in healthy controls. In neurons of AD patients, IGF-1R co-localizes with neurofibrillary tangles (Moloney et al., 2010). Furthermore, the expression of insulin, IGF-1, IGF-2 and their respective receptors and downstream substrates are reduced in brains of AD patients (Table 1) (Erol, 2008; Frölich et al., 1998; Gil-Bea et al., 2010). This has led some authors to propose the term "type 3 diabetes" to describe these abnormalities (Steen et al., 2005). One striking observation in brains of AD patients is insulin/IGF-1 resistance as illustrated by decreased insulin→IRS-1→PI3-K and IGF-1R→IRS-2→PI3-K signaling (Bomfim et al., 2012; Moloney et al., 2010). The underlying mechanism seems to be inactivation of IRS-1 via phosphorylation at serine 312, serine 616 and serine 636/639 (Bomfim et al., 2012; Moloney et al., 2010). As discussed by Talbot et al. (2012), there is no evidence of hyperglycemia in AD brains, insulin resistance at

the level of IRS occurs in the absence of T1D or T2D and does not affect glucose uptake in neurons contrary to what is observed in muscle, fat and liver in the setting of peripheral insulin resistance. Therefore, they suggest using the term "insulin-resistant brain state" (Correia et al., 2011) instead of T3D.

Insulin activates PI3-K via IR and IRS-1, while IGF-1 activates the same downstream target via IGF-1R and IRS-2 (Nadjar et al., 2009; Talbot et al., 2012). Inactivation of IRS-1 or IGF-1 gene expression has positive effects on survival and motor performance in mice, while inactivation of IRS-2 gene expression leads to accumulation of neurofibrillary tangles containing phosphorylated tau and reduced survival in most studies (Holzenberger et al., 2003; Schubert et al., 2003; Selman et al., 2008; Taguchi et al., 2007). The cross of transgenic AD mice overepressing APP with IRS-2 KO mice shows increased tau phosphorylation but reduced Aβ burden (Freude et al., 2009; Killick et al., 2009). Interestingly, APP overexpressing IRS-2 KO mice have less severe cognitive deficits and reduced mortality compared to APP mice suggesting that inactivation of IRS-2 gene expression is beneficial in this transgenic model of AD. Crossing APP overexpressing animals with IGF-1R KO mice is also beneficial in terms of survival, while no such effect was observed when crossing with IR-KO mice (Freude et al., 2009).

Insulin and IGF-1 activate Aβ trafficking and clearance via PI3-K/MAPK dependent pathways by increasing the presence of Aβ transporters in the cerebrospinal fluid (Carro et al., 2006; Carro et al., 2002; Claeysen et al., 2012; Costa et al., 2008a; Costa et al., 2008b; Gasparini et al., 2001; Stein and Johnson, 2002). They also protect neurons against Aβ induced toxicity and enhance memory in AD patients (Reger et al., 2006; Reger et al., 2008a; Reger et al., 2008b) and preclinical models (Table 2) (Carro et al., 2006; Carro et al., 2002; De Felice et al., 2009; Freude et al., 2009; Niikura et al., 2001). While insulin degrading enzyme (IDE) catabolizes insulin, Aβ is also a substrate of IDE but with lower affinity. Thus, an increase in insulin is expected to inhibit IDE-mediated degradation of Aβ (Farris et al., 2003; Kurochkin, 2001; Li and Holscher, 2007; Selkoe, 2001). Aβ can however be degraded by several other proteases (Saido and Leissring, 2012), i.e. insulin blocking of IDE is only considered as a minimal concern. Taken together, insulin has a dual effect with high levels providing enhanced metabolic and trophic support to the brain, while prolonged hyperinsulinemia may block IDE functioning in protecting against Aβ accumulation and in the long run desensitize insulin receptors and alter the postsynaptic signaling cascade.

Insulin and IGF-1 inhibit phosphorylation of tau through the inhibition of GSK-3β and enhance the binding of tau to microtubules, having in mind that hyperphosphorylated forms are considered to be the toxic tau species (Hong and Lee, 1997; Schubert et al., 2004; Tokutake et al., 2012). In neuroblastoma cells, insulin treatment induces a transient increase in tau phosphorylation followed by a decrease that correlates with a sequential activation and deactivation of GSK-3β (Lesort and Johnson, 2000; Lesort et al., 1999). In agreement, impaired insulin signaling in IRS-2 KO mice causes accumulation of hyperphosphorylated tau that was attributed to inactivation of tau protein phosphatase 2a, a tau dephosphorylating enzyme (Schubert et al., 2003). However, decreased phosphorylation of GSK-3β, a kinase playing an important role in tau phosphorylation, was also reported in the same model suggesting that GSK-3β may be involved in accumulation of hyperphosphorylated tau in IRS-2 KO mice (Freude et al., 2009). Importantly, no change in tau aggregation was observed in IRS-2 KO mice; these animals also show enhanced cognitive performance and reduced amyloid burden (Cheng et al., 2005; Freude et al., 2009; Killick et al., 2009). Taken together, these results suggest overall positive effects of insulin/IGF-1 signaling on Aβ burden and tau aggregation in AD.

2.3. Synucleinopathies

PD, dementia with Lewy bodies (DLB) and multiple system atrophy (MSA) are neurodegenerative disorders belonging to the synucleinopathy family that is characterized by abnormal accumulation of α-synuclein (α-syn) (Beyer and Ariza, 2007; Spillantini et al., 1998). In PD and DLB, α-syn accumulates in neurons in form of Lewy bodies and dystrophic neurites (Beyer and Ariza, 2007; Spillantini et al., 1998). By contrast, α-syn aggregates are mainly found as glial cytoplasmic inclusions (GCI) in MSA (Papp et al., 1989; Ubhi et al., 2011; Wenning et al., 2008). α-syn is a protein that is yet believed to have a role in neurotransmitter release and synaptic plasticity (Lashuel et al., 2013).

PD is the predominant form of synucleinopathies and the second most common neurodegenerative disorder after AD (Alves et al., 2008). PD is dominated by a progressive loss of dopaminergic neurons in the substantia nigra (SN), but other regions within and outside the brain are also affected by the widespread neurodegenerative process explaining the occurrence of multiple motor and non-motor symptoms in the course of the disease (Braak and Braak, 2000; Parkinson, 2002). PD and diabetes, both age related chronic diseases, share similar deregulated pathways (Chung et al., 2011; Numao et al., 2013; Santiago and Potashkin, 2013; Tong et al., 2009) (Table 1). Growing evidence connects impaired insulin/IGF-1 signaling to the pathophysiology of PD: (i) IGF-1R is expressed in the substantia nigra (Quesada et al., 2007); (ii) IGF-1 protects dopaminergic neurons from toxin-induced damage *in-vitro* (Table 2) (Beck et al., 1993; Offen et al., 2001; Sun et al., 2010; Zawada et al., 1996); (iii) IGF-1 increases the survival of neurons in the brainstem including the SN and/or improves functional deficits (Guan et al., 2000; Krishnamurthi et al., 2004; Quesada et al., 2008; Quesada and Micevych, 2004; Schulingkamp et al., 2000; Trejo et al., 2001); (iv) mutant α-syn promotes Akt aggregation resulting in Akt deactivation while IGF-1 rescues α-syn toxicity by activating the PI3-K/Akt pathway (Chung et al., 2011; Kao, 2009), (v) transgenic T2D mice show increased levels of α-syn monomers and toxic oligomers in midbrain homogenates, and are more vulnerable to administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) with higher loss in tyrosine hydroxylase (TH)-immunoreative dopamine neurons in the substantia nigra pars compacta compared to wildtype mice (Wang et al., 2014), (vi) high fat diet exacerbates MPTP induced neurotoxicity on nigrostriatal dopamine neurons in mice (Bousquet et al., 2012; Choi et al., 2005). Similarly, high fat diet aggravates 6-hydroxydopamine (6-OHDA) induced dopamine depletion in the substantia nigra and the striatum (Morris et al., 2010).

Several cohort studies have reported increased serum IGF-1 levels in PD patients compared to controls (Godau et al., 2010; Godau et al., 2011; Numao et al., 2013; Picillo et al., 2013) (Table 1). It remains to be understood how these findings reflect and contribute to impaired brain metabolism or compensatory mechanisms, having in mind that both cerebrospinal fluid and serum levels of IGF-1 are increased in PD patients (Mashayekhi et al., 2010). Similarly, IGF-1 serum levels are increased in patients with MSA (Numao et al., 2013; Pellecchia et al., 2010) while brain tissue levels are reduced in a transgenic animal model of MSA (Ubhi et al., 2010). Brain insulin/IGF-1 signaling is decreased in DLB patients (Tong et al., 2009).

Possible interactions and the respective contribution of peripheral versus central IGF-1 release to brain dysfunction in synucleinopathies remain to be determined.

2.4. Insulin and IGF-1: angels or demons?

Decreasing insulin/IGF-1 signaling may also have positive and beneficial effects in neurodegenerative disorders. This hypothesis

is supported by the observation that decreased IRS-2 signaling in aging brains promotes healthy metabolism, attenuates meal-induced oxidative stress and increases life span of insulin resistant mice (Taguchi et al., 2007). In AD, beneficial effects of IRS-2 deletion on cognitive deficits, amyloid deposition and survival have been documented in a transgenic mouse model (Freude et al., 2009; Killick et al., 2009). Reduced IGF-1 signaling was further related to longevity and increased resistance to oxidative stress (Holzenberger et al., 2003; Kappeler et al., 2008). Moreover, decreasing IGF-1 signaling delays proteotoxicity in AD mice (Cohen et al., 2009; Freude et al., 2009) and IGF-1R+/- mice produce less reactive oxygen species (ROS) after injection of MPTP, a toxin-based model of PD (Nadjar et al., 2009).

The apparent contradiction in the literature with regard to beneficial effects of increasing or decreasing IGF-1 signaling may be explained in several ways. First, IGF-1 is widely known as a growth factor providing cell growth and trophic support. Reduced levels have negative effects on cell function and viability, while growing evidence suggests that insulin/IGF-1 signaling is deficient in neurodegenerative disorders. Thus, restoring this altered signaling cascade and improving its functioning may be of therapeutic use. Second, IGF-1 speeds up the cell cycle and aging which leads to cell death. IGF-1 antagonists have already been tested for treating cancer and a way of slowing down aging may be by decreasing IGF-1 signaling. Third, IRS-1 and IRS-2 have a dichotomous action on brain function. While IRS-1 regulates memory, IRS-2 signaling is more directly related to longevity. Postmortem studies have reported decreased IRS-2 levels in the brains of AD patients which may either contribute to, or being an attempt to compensate the abnormal aggregation of Aβ and tau protein (Bomfim et al., 2012; Moloney et al., 2010). A very elegant study confirmed the dichotomy in insulin/IGF-1 signaling by showing that impaired insulin signaling was related to altered IRS-1 function while abnormal IRS-2 signaling led to IGF-1 resistance (Talbot et al., 2012). The results of this study further suggest distinct mechanisms of insulin and IGF-1 resistance in AD. Accordingly, insulin resistance may be the consequence of phosphorylation of IRS-1 by Aβ oligomer activation, while IGF-1 resistance may be compensatory since it delays Aβ accumulation. One may speculate that bypassing the insulin and IGF-1 receptor while improving insulin signaling could be a way by which GLP-1 and its analogs may exert beneficial effects in neurodegenerative disorders.

Cell-type and region specific differences in insulin/IGF-1 signaling may also contribute to differing effects. Indeed, glial cells are also implicated in neurodegenerative disorders (Desplats et al., 2009; Lee et al., 2010; Moloney et al., 2010). For instance, GCIs are found in MSA but some inclusions are also found in astrocytes of PD and DLB patients (Braak et al., 2007; Papp et al., 1989) and glial cells are critically involved in Aβ pathology (Nagele et al., 2003; Wegiel et al., 2001; Wegiel et al., 2000). The resulting activation of glia in neurodegenerative disorders is believed to play a role in disease initiation and/or progression (Halliday and Stevens, 2011; Nagele et al., 2004). Hence, increased insulin/IGF-1 signaling in neurons could have a different effect to that of glial cells.

3. Targeting insulin/IGF-1 and GLP-1 in preclinical models of neurodegenerative disorders

3.1. Insulin and IGF-1

Guan et al. (1993) showed two decades ago that central administration of IGF-1 to hypoxic-ischemic adult rats provides trophic support to cells within the cerebral structures. In AD models (Table 2), insulin and IGF-1 rescue from Aβ or APP-induced cell death (Carro et al., 2006; Carro et al., 2002; Gasparini et al., 2001; Niikura et al., 2001), reduce intraneuronal Aβ levels (Carro et al., 2006; Carro et al., 2002; Gasparini et al., 2001), decrease amyloid burden and astrogliosis (Carro et al., 2006; Carro et al., 2002) and enhance cognitive performances (Carro et al., 2006). Beneficial effects of IGF-1 were also observed *in-vitro* on toxicity induced by α-syn (Kao, 2009), dopamine (Offen et al., 2001) and 1-methyl-4-phenylpyridinium ion, an active metabolite of MPTP (Sun et al., 2010), while IGF-1 rescues dopaminergic neurons from programmed cell death (Zawada et al., 1996). Similar results were found in *in-vivo* models of PD where IGF-1 administration prevented the loss of TH-positive neurons in the substantia nigra (Ebert et al., 2008; Guan et al., 2000) and reversed motor behavior abnormalities (Ebert et al., 2008; Krishnamurthi et al., 2004; Quesada et al., 2008).

3.2. GLP-1

Both GLP-1 and GLP-1 analogs (Table 4) have positive effects on cell survival in preclinical models of neurodegenerative disorders (Table 3) (Cabou et al., 2008; Nakade et al., 2006). One main limitation in using GLP-1 is the short half-life due to rapid degradation by DPP-4 (Baetta and Corsini, 2011; Baggio and Drucker, 2007; Deacon, 2011). By contrast, GLP-1 analogs resist DPP-4 and easily pass the BBB which makes them suitable for treating brain disorders (Martin et al., 2011). New GLP-1 analogs allow less frequent dosing and have a better safety profile (Baker et al., 2011; Blonde and Russell-Jones, 2009; Kim et al., 2007; McIntyre et al., 2013).

In preclinical models of AD, GLP-1 decreases Aβ toxicity in-vitro (During et al., 2003; Perry et al., 2003; Qin et al., 2008). GLP-1(9-36)^{amide}, the natural cleavage product of GLP-1 also exhibits beneficial effects in AD models by reversing impairments of long term potentiation and depression in hippocampal slices incubated with Aβ. It further attenuates memory deficits, restores impaired signaling within the Akt/GSK-3β pathway and reverses elevated levels of ROS in APP/PS1 mutant mice (Ma et al., 2012).

Exendin-4, a GLP-1 analog, reduces levels of Aβ and APP *in-vitro* and in streptozocin-treated 3xTg-AD mice without modifying total levels of tau protein (Li et al., 2010). Aβ oligomers cause insulin resistance via c-Jun N-terminal kinase-mediated phosphorylation of IRS-1 at different serine residues in hippocampal neurons *in-vitro* and in normal non-human primates after intracerebroventricular injection (Bomfim et al., 2012). In this study, exendin-4 reversed Aβ oligomer induced insulin resistance *in-vitro*, and improved insulin signaling and cognition in APP/PS1 mutant mice. Preclinical trials in PD models showed that exendin-4 increases the number of TH-immunoreactive neurons and improves motor performance in 6-OHDA-lesioned rats (Bertilsson et al., 2008; Harkavyi et al., 2008). In a mouse model of PD, exendin-4 decreased the MPTP-induced loss of nigral neurons and striatal dopaminergic fibers, decreased proinflamatory markers and improved motor function (Kim et al., 2009; Li et al., 2009). (Val^{8})GLP-1, another stable GLP-1 analog, was found to rescue Aβ-induced synaptic dysfunction in models of AD (Gault and Holscher, 2008; Gengler et al., 2012; Wang et al., 2013).

The novel GLP-1 agonist liraglutide decreases the amount of Aβ pathology and microglial activation, increases IDE levels, improves measures of synaptic plasticity in hippocampal neurons together with cognition and promotes cell proliferation and differentiation into neurons in an AD model (McClean et al., 2010; McClean and Holscher, 2013; McClean et al., 2011; Parthsarathy and Holscher, 2013).

These findings illustrate the utility for further drug development of GLP-1R analogs for treating neurodegenerative disorders. Both exendin-4 and liraglutide are approved treatments for diabetes (Table 4) that are currently being tested in early clinical trials for treating patients with AD and PD (see chapter 4).

Two additional compounds (lixisenatide, albiglutide) are in advanced clinical development or close to market approval for treating diabetes. Their interest for treating neurodegenerative disorders remains to be determined.

3.3. DPP-4 inhibitors

DPP-4 inhibitors are oral antidiabetic drugs used to enhance GLP-1 levels (Baetta and Corsini, 2011; Deacon, 2011; Scheen, 2010). DPP-4-inhibtors have only low penetration of the BBB, i.e. they mainly act by increasing peripheral GLP-1 levels (Table 4). This may be a limitation compared to GLP-1 analogs such as exendin-4 and liraglutide that easily cross the BBB.

One preclinical study has assessed the efficacy of the DPP-4 inhibitor sitagliptin in mutant AD mice (Table 3). The drug decreased Aβ burden and improved performance in the contextual fear conditioning test (D'Amico et al., 2010). These findings were accompanied by increased GLP-1 brain levels as a result of peripheral DPP-4 inhibition with subsequent passage of GLP-1 from the blood to the brain.

4. Clinical trials in neurodegenerative disorders

One clinical trial is underway to assess the effect and efficacy of exendin-4 treatment in AD (NCT01255163). This study is a National Institute on Aging (NIA)-sponsored phase 2 randomized, placebo-controlled trial that has recruited 230 patients with early-stage Alzheimer's disease or mild cognitive impairment (MCI; Mini Mental State Examination score > 20). Patients receive exendin-4 twice daily (b.i.d) and are followed up to 36 months. Primary outcomes evaluate safety, but also efficacy based on the Alzheimer's Disease Assessment scale–cognitive sub-scale (ADAS–cog) and the Clinical Dementia Rating scale. The last visit for final data collection is expected for December 2015.

The results of a small open label, randomized phase 2 clinical trial evaluating the safety and efficacy of exendin-4 in patients with moderate PD were recently published (Aviles-Olmos et al., 2013a). Twenty-one patients were randomized to the treatment group (5 μg b.i.d for 1 month and 10 μg b.i.d for 11 months), while 24 served as controls. The primary outcome of this study to show a difference in Unified PD Rating Scale (UPDRS) motor scores in the defined OFF-medication condition at 12 months reached significance (mean improvement of 2.7 points in treatment group vs. mean worsening of 2.2 points in control group). In the secondary outcomes, patients receiving exendin-4 treatment showed improved cognitive efficiency as assessed by the Mattis Dementia Rating Scale (mean improvement of 2.8 points in treatment group vs. mean worsening of 3.5 points in control group) but no difference in health-related quality of life. In terms of safety, exendin-4 induced weight loss in the treatment group (mean loss of 3.5 kg in treatment group vs. 0.8 kg in control group), an observation that may become a relevant issue when treating patients with neurodegenerative disorders for years. The results of this preliminary open-label trial have set the grounds for a randomized, double blind, placebo-controlled study (EXENATIDE-PD trial, NCT01971242) in 60 PD patients that has started its enrollment in December 2013. This study compares the effects of exendin-4 (2 mg subcutaneously given once a week) with placebo. Similar to the open-labeled pilot study, the primary outcome is to compare the effectiveness of exendin-4 with placebo on UPDRS motor scores in the defined OFF-medication condition at 60 weeks. Secondary outcomes include safety and health-related quality of life. The completion of this study is expected for March 2016.

The effect of liraglutide on cerebral amyloid deposits in the brain is currently being assessed in a small randomized clinical trial in patients with earlyAD (NCT01469351). Seventeen patients received 1.8 mg liraglutide once a day over 26 weeks, while the other half served as control. The primary outcome is the change in the amount of cerebral amyloid deposits as assessed by Pittsburg compound B positron emission tomography scan. The expected final data collection date for the primary outcome measure was April 2013, i.e. the study results should soon be available. A large randomized, placebo-controlled phase 2 trial that assesses the safety and efficacy of liraglutide in 206 patients with early AD was launched in June 2013 (NCT01843075). Patients will receive for 12 months either liraglutide (1.8 mg per day) or placebo. The primary outcome is the change in cerebral glucose metabolic rate from baseline to follow up in the treatment group compared with the placebo group. Secondary outcomes include ADAS, MRI changes, microglial activation, and CSF markers. The expected final data collection date for the primary outcome measure is June 2016.

GLP-1 analogs have shown promising effects in preclinical models which await confirmation in clinical trials. Potential limitations for further clinical development may be the peripheral actions of GLP-1 analogs including weight loss. Intranasal administration may be one way to bypass unwanted peripheral actions. In this line, several clinical trials assessing the effect of intranasal administration in patients with MCI or AD were recently completed or are underway (Craft et al., 2012; Reger et al., 2008b). Craft et al. (2012) evaluated in a randomized, placebo-controlled phase 2 trial the effect of intranasal insulin (10 IU or 20 IU b.i.d for 4 months) in 111 patients with MCI or AD. Patients receiving insulin showed improved delayed memory (only 20 IU group) and preserved caregiver-rated functional ability. In secondary analyses, patients receiving insulin showed less worsening of ADAS-Cog and Alzheimer's Disease Cooperative Study–Activities of Daily Living Scale (ADCS–ADL) scores (only AD but not MCI patients). The same authors have recently completed another randomized, placebo-controlled phase 2 trial in 90 MCI and AD patients assessing the effect of intranasal 40 IU insulin in a design close to their prior study (NCT01595646). The final data collection date for the primary outcome measure was March 2013, i.e. the study results should soon be available. Based on these encouraging findings, a large randomized, double blind placebo-controlled phase 2/3 study is currently being conducted by the Alzheimer's Disease Cooperative Study in 240 patients with MCI or AD (NCT01767909). Patients will receive insulin (20 IU b.i.d) or placebo for 12 months after an open-label period of 6 months where all study participants will be given active drug. Primary outcome measures include ADAS-Cog, ADCS-ADL, as well as imaging and cerebrospinal fluid biomarkers. The expected data collection date for the primary outcome measure is October 2014.

5. Future considerations and conclusion

There is growing evidence of impaired signaling of insulin/IGF-1 and GLP-1 in brains of patients with neurodegenerative disorders suggesting that targeting these cascades may be beneficial. In preclinical models of AD and PD, administration of insulin, IGF-1 and GLP-1 agonists reverses these signaling abnormalities and has positive effects on surrogate markers of neurodegeneration and behavior. If anything, preclinical studies have focused on neuronal survival while data remain sparse about the effects of insulin, IGF-1 and GLP-1 on glial cell function, the latter is increasingly being recognized as relevant for normal brain metabolism and in neurodegenerative disorders.

Hitherto, only toxin-based preclinical models of PD were used (Tables 2 and 3). These models have limited translational value because of their clear limitations for studying putative neuroprotective effects of a tested drug (Meissner et al., 2004). Therefore, future studies should be conducted in models based on the overexpression of α-syn, the pathological hallmark of PD.

The distinct regulation of IRS-1 and IRS-2 by insulin and IGF-1 receptor stimulation (Talbot et al., 2012) warrants further investigation in preclinical models, having in mind the negative regulatory effect of IRS-2 on cognition and the compensatory decrease of IRS-2 in preclinical models of AD and brains of AD patients (Bomfim et al., 2012).

Positive findings of preclinical proof-of-concept studies have already been translated in early phase clinical trials in AD and PD assessing the safety and efficacy of the GLP-1 analogs exendin-4 and liraglutide. Despite the encouraging results of preclinical studies, positive translation in AD and PD patients is far from being guaranteed. Beyond species differences, trial design is a critical issue that has to be considered when preparing future clinical trials. For instance, no objective surrogate markers are approved by the US Food and Drug Administration or the European Medicines Agency as primary outcomes for disease-modifying or neuroprotective trials in AD and PD. Primary end points currently rely on clinical rating scales such as the ADAS-Cog in AD and the UPDRS in PD, together with other outcomes such as the need for symptomatic treatment in drug-naive patients with PD. In light of the symptomatic effects of insulin and GLP-1 on cognition through improving synaptic plasticity, the separation of potential symptomatic effects from disease-modifying or neuroprotective actions may not only be an issue in trials involving AD patients but also in PD where the separation of symptomatic and putative disease-modifying effects of monamine oxidase inhibitors has been challenging in the past (Meissner et al., 2011).

Search strategy

References for the review were found through PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) with the terms: insulin, insulin-like growth factor-1, neurodegeneration, incretins, glucagon like peptide-1, Parkinson's disease, Alzheimer's disease, Multiple System Atrophy, insulin resistance, brain, central nervous system, therapy, diabetes, type 2 diabetes. Clinical trial data was identified through the website of clinical trials (http://clinicaltrials.gov/). Only English papers were reviewed and the final list of reference was generated on the basis of the scope of this review.

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III- Thesis objectives

This work was part of a translational approach in synucleinopathies with an emphasis on MSA. It consists of five main projects aimed at assessing disease mechanisms in the brain of MSA and PD patients in addition to establishing proof of concept studies in view of disease modification in MSA.

1. The main thesis project consisted of characterizing insulin/IGF-1 resistance in the putamen of MSA and PD patients and the underlying alterations in different cellular populations. Insulin/IGF-1 signalling in the brain is implicated in several functions ranging from apoptosis to pro-survival actions. Studies have shown altered insulin/IGF-1 signalling and insulin resistance in Alzheimer's disease while recent evidence has also pointed to a potential involvement of insulin/IGF-1 signalling in the pathophysiology of synucleinopathies. Indeed, several downstream effectors of the insulin/IGF-1 signalling are altered in synucleinopathies. We hypothesize that insulin/IGF-1 signalling is altered in the putamen of MSA and PD patients. This work will allow us to assess insulin resistance in neurons and glial cells in the putamen, a key structure implicated in MSA and PD pathology.

2. In the second project we aimed at studying the effect of α-syn overexpression on insulin resistance and its implication in PD pathogenesis in AAV-A53TSyn injected rats. Until now, no study has shown a relationship between α-syn overexpression and insulin resistance *in vivo*. We hypothesize that α-syn aggregates alter insulin signalling and induce insulin resistance in surviving dopaminergic neurons in PD.

3. Based on the translational approach we are following, we aim in this project at characterizing insulin/IGF-1 signalling and insulin resistance in PLP-SYN transgenic MSA mice. Our objective is providing a proof of concept study to evaluate the therapeutic efficacy of exendin-4 on α-syn burden and α-syn induced neurodegeneration. We hypothesize that insulin/IGF-1 signalling is altered in PLP-SYN mice and modulating this signalling mechanism could reverse insulin resistance and mitigate α-syn induced neurodegeneration. Beyond MSA, this work represents relevance for the group of synucleinopathies as previous studies assessing the therapeutic efficacy and underlying mechansims of action of exendin-4 were done on toxic models of PD that lacked α-syn induced neurodegeneration.

4. A second proof of concept study was conducted using VX-765, an inhibitor of caspase-1, the latter known to cleave α-syn at its C-terminal. Since C-terminal truncation of α-syn is believed to be a promoter or enhancer of aggregation, we here aim at limiting C-terminal truncation of α-syn and its subsequent oligomerization, thus preventing neurodegeneration in PLP-SYN transgenic mice. We hypothesize that VX-765 could mitigate α-syn pathology and mediate neuroprotection in PLP-SYN mice.

5. The last major part of my PhD work consisted of measuring alterations of several MMPs in the brain of MSA patients. MSA pathogenesis is still poorly understood and several studies in neurodegenerative disorders such as PD, AD and ALS point to MMP implication in tissue remodeling, BBB alteration, myelin breakdown, α-syn truncation and neuroinflammation. We here studied the expression and activity of MMPs in the putamen and frontal cortex of MSA patients. We hypothesize that MMP activity and expression is altered in MSA.

Results

IV- Results

Article 1:

Brain insulin resistance in Parkinson's disease and multiple system atrophy

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(soumis)

La maladie de Parkinson (MP) et l'atrophie multisystématisée (AMS) sont des maladies neurodégénératives progressives d'étiologie inconnue. Elles sont caractérisées par la présence d'agrégats intracytoplasmiques de la proteine α-synucléine. Au-delà de leur rôle dans la régulation du glucose, l'insulin et l'insulin like growth factor-1 (IGF-1) ont des propriétés neurotrophiques et leurs récepteurs sont largement exprimés dans le cerveau. Des études ont montré une altération de la signalisation de l'insuline/IGF-1 dans la maladie d'Alzheimer et des données récentes suggèrent une altération de la signalisation de l'insuline / IGF-1 dans MP et AMS, comme illustré par l'augmentation des concentrations périphériques de l'insuline / IGF-1.

Nous émettons l'hypothèse que la signalisation de l'insuline / IGF-1 est altérée dans MP et AMS. Nous avons donc étudié la résistance à l'insuline dans les neurones et les cellules gliales dans le putamen des patients MP (n = 7) et AMS (n = 7) par rapport aux contrôles (n = 7) en mesurant deux marqueurs bien connus pour la résistance à l'insuline. Ces deux marqueurs sont IRS-1pS312 et IRS-1pS616.

Dans notre étude, toutes les cellules étaient positives pour IRS-1pS312 / 616. La quantification de l'immunofluorescence a révélé une diminution des neurones positifs pour IRS-1pS312 / 616 chez les patients AMS comparés à des sujets sains et des patients MP. L' intensité neuronale de IRS-1pS312 était augmentée chez les patients AMS et MP par rapport aux contrôles. Nous avons également observé une tendance pour l'augmentation de l'intensité du marquage IRS-1pS616 dans les neurons des patients AMS par rapport au patients MP et contrôles. De plus, nous avons remarqué une augmentation des astrocytes et des cellules microgliales positives pour IRS-1pS312/S616 dans les patients AMS par rapport aux patients MP et contrôles. L'intensité de IRS-1pS312 dans la microglie des patients AMS était considérablement diminuée par rapport aux patients MP et contrôles. La quantifications des oligodendrocytes positifs pour IRS-1pS312 / S616 était similaire dans les 3 groupes étudiés. De plus, les oligodendrocytes des patients AMS ont montré une augmentation de l'intensité de IRS-1pS312 par rapport aux témoins et aux patients MP.

Nous montrons ici une alteration de la signalisation de l'insuline/IGF-1 dans les neurones des patients MP et AMS, ainsi que dans les oligodendrocytes des patients AMS. L' alteration de l'axe insuline / IGF-1 dans les neurones peut contribuer au dysfonctionements neuronaux chez les patients MP et l'AMS. De plus l'axe insuline / IGF-1 peut contribuer à la mort neuronale dans l'AMS en diminuant l'apport de facteurs trophiques fournis aux neurones par les oligodendrocytes qui sont eux-mêmes altérés. Ces résultats confirment que les analogues de l'insuline /IGF-1 (anti-diabétiques) pourraient faire l'objet d'une approche thérapeutique dans les synucléinopathies.

Brain insulin resistance in Parkinson's disease and multiple system atrophy

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Key words: alpha-synuclein, parkinsonism, multiple system atrophy, insulin resistance, IGF-1, insulin.

Abstract

Parkinson's disease and multiple system atrophy are progressive neurodegenerative disorders of unknown etiology characterized by the presence of intracytoplasmic α-synuclein aggregates. Beyond their role in glucose homeostasis, insulin and insulin like growth factor-1 (IGF-1) have neurotrophic properties and their receptors are widely expressed throughout the brain. Brain insulin/IGF-1 signalling is impaired in Alzheimer's disease and emerging evidence suggest impaired insulin/IGF-1 signaling in Parkinson's disease and multiple system atrophy as illustrated by increased peripheral insulin/IGF-1 concentrations in both disorders. We hypothesized that insulin/IGF-1 signalling is altered in the brain in Parkinson's disease and multiple system atrophy. We investigated insulin resistance in neurons and glial cells in the putamen of Parkinson's disease (n=7) and multiple system atrophy patients (n=7) compared to healthy controls (n=7) by measuring two known insulin resistance markers, insulin receptor susbtrate-1 phosphorylation on serine 312 and 616 (IRS-1pS312, IRS-1pS616). We report that all cells stained positive for IRS-1pS312/616. Immunofluorescence quantification revealed a decrease in IRS-1pS312/616 neuronal counts in multiple system atrophy compared to healthy controls and Parkinson's disease patients. Neuronal IRS-1pS312 staining intensity was increased in multiple system atrophy and Parkinson's disease patients compared to healthy controls. A trend for increased neuronal IRS-1pS616 staining intensity in multiple system atrophy compared to Parkinson's disease patients and healthy controls was also noted. IRS-1pS312 and IRS-1pS616 positive astrocytes were increased in multiple system atrophy compared to Parkinson's disease patients and controls. Microglial IRS-1pS312/616 quantification showed a significant increase in multiple system atrophy patients compared to healthy controls and showed a trend for an increase compared to Parkinson's disease. IRS-1pS312 staining intensity in multiple system atrophy microglia was significantly decreased compared to healthy controls. Oligodendroglial IRS-1pS312/S616 cell counts were similar in all 3 groups but oligodendrocytes from multiple system atrophy patients showed increased IRS-1pS312 staining intensity compared to healthy controls and Parkinson's disease. These results demonstrate insulin resistance in neurons in the putamen in Parkinson's disease and multiple system atrophy, as well as in oligodendrocytes in multiple system atrophy. Altered insulin/IGF-1 signalling in neurons and oligodendrocytes may contribute to neuronal death in multiple system atrophy by decreasing the neurotrophic support provided by insulin and IGF-1 and by altering oligodendrocytes maturation and functioning. These results further support the use of insulin/IGF-1 analogues (i.e. anti-diabetics) as possible candidates for disease modification in synucleinopathies.

Introduction

Parkinson's disease (PD) and multiple system atrophy (MSA) are progressive neurodegenerative disorders characterized by the accumulation and aggregation of α-synuclein (α-syn). In PD, α-syn mainly accumulates in neurons forming Lewy bodies, while it is found as glial cytoplasmic inclusions (GCIs) in oligodendrocytes in MSA patients (Gilman *et al.*, 2008; Goedert *et al.*, 2013; Papp *et al.*, 1989; Spillantini *et al.*, 1998b; Wenning *et al.*, 2008).

Several studies have demonstrated impaired insulin/insulin like growth factor-1 (IGF-1) signalling in neurodegenerative disorders, particularly in Alzheimer's disease (AD) (for review Bassil *et al.* (2014)) and emerging evidence suggests a potential involvement of insulin/IGF-1 signalling in the pathophysiology of synucleinopathies. Indeed IGF-1 levels are increased in the serum and cerebrospinal fluid in PD patients (Godau *et al.*, 2010; Godau *et al.*, 2011; Mashayekhi *et al.*, 2010; Picillo *et al.*, 2013b). Moreover, gene expression of insulin and IGF-1 receptors are significantly decreased in frontal white matter and amygdala in PD patients (Tong *et al.*, 2009). IGF-1 serum levels are also increased in MSA compared to PD patients and controls (Numao *et al.*, 2014; Pellecchia *et al.*, 2010). Furthermore, several lines of evidence point to peripheral insulin resistance and type 2 diabetes (T2D) as possible risk factors for PD (Abbott *et al.*, 2002; Aviles-Olmos *et al.*, 2013b; Hu *et al.*, 2007; Hu *et al.*, 2006; Hu *et al.*, 2000).

The primary source of insulin and IGF-1 is peripheral, but local production also exists in the brain (Jafferali *et al.*, 2000; Schechter *et al.*, 1996; Schechter *et al.*, 1990; Schechter *et al.*, 1992). In the brain, the insulin/IGF-1 signalling pathway is involved in numerous biological processes including myelin sheath synthesis, astrocyte glycogen storage, cholesterol production, oligodendrogenesis and maturation, as well as neuronal survival. The effects of insulin and IGF-1 are mediated through the activation of insulin receptor substrate (IRS-1) and its downstream target Akt, which acts as a central hub that modulates the activity of several effectors (for review Bassil *et al.* (2014)). Among these effectors, caspases, cAMP response element-binding protein (CREB), mammalian target of rapamycin (mTOR), forkhead box O (FoxO), nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB), Bcl-2 interacting mediator of death (Bim), apoptosis-stimulating fragment (Fas) and glycogen synthase 3 beta (GSK-3β) are implicated in oxidative stress, apoptosis, protein synthesis, gene expression, autophagy, and inflammation. Interestingly, these biological processes are altered and crucially involved in the pathophysiology PD and MSA (Bassil *et al.*, 2014; Chen *et al.*, 2009; Chu *et al.*, 2009; Dehay *et al.*, 2010; Genis *et al.*, 2014; Golpich *et al.*, 2015; Kawamoto *et al.*, 2014; Kleinridders *et al.*, 2014; Kragh *et al.*, 2013; Levy *et al.*,

2009; Liu *et al.*, 2009; Muhic *et al.*, 2015; Nakamura *et al.*, 2001; Nakamura *et al.*, 1998; Schwarz *et al.*, 1998; Wilkins *et al.*, 2001).

Insulin resistance is the decreased responsiveness of cells to insulin/IGF-1 signalling or the inability of cells to bind insulin/IGF-1 efficiently, which in turn leads to decreased signalling and modulation of downstream targets (Boura-Halfon and Zick, 2009; Moloney *et al.*, 2010; Zick, 2001). Previous studies have described increased serine phosphorylation of IRS-1 as a surrogate marker of insulin resistance in the brain of patients with AD (Bomfim *et al.*, 2012; Moloney *et al.*, 2010; Talbot *et al.*, 2012; Yarchoan *et al.*, 2014).

Although the underlying mechanisms are not yet understood, a small open-label clinical trial assessing the effects of exendin-4, a glucagon-like peptide 1 (GLP-1) analogue, in 45 PD patients reported a significantly better motor and cognitive outcome in patients receiving exendin-4 compared to placebo, underlining the potential of antidiabetics for treatment development in PD (Aviles-Olmos *et al.*, 2013a; Aviles-Olmos *et al.*, 2014).

To provide insight into the potential contribution of insulin/IGF-1 signalling in the pathophysiology of synucleinopathies, we here investigated brain insulin resistance in synucleinopathies by measuring two well-characterized serine phosphorylations (Ser312 and Ser616) of IRS-1 (Bomfim *et al.*, 2012; Boura-Halfon and Zick, 2009; Gual *et al.*, 2005; Moloney *et al.*, 2010; Talbot *et al.*, 2012) in neurons, astrocytes, oligodendrocytes and microglia of PD and MSA patients.

Materials and Methods

Patient Samples

Human brain samples were obtained from the French national brain repository (Comité Protection des Personnes N° CEBH 2009/03; Ministère Enseignement Supérieur et Recherche: DC-2008-337). The present study was declared and approved by the local ethics committee ("Comité de Protection des Personnes du Sud-Ouest et Outre Mer III"). Patient characteristics are given in **Table 1**.

Immunofluorescent labelling

Sequential immunofluorescence labelling was performed with insulin resistance markers IRS-1pS312 (rabbit polyclonal antibody, 1:200; Invitrogen, USA) or IRS-1pS616 (rabbit polyclonal antibody, 1:200; Invitrogen, USA) coupled to anti-glial fibrillary acidic protein (GFAP, mouse monoclonal antibody, 1:500; Millipore, France) combined with S100β (mouse monoclonal antibody, 1:1000; Abcam, England), the microglial marker anti-HLA-DR (mouse monoclonal antibody, clone TAL.1B5, 1:500; Dako, Denmark), the oligodendrocyte marker

anti-CNPase (mouse monoclonal antibody, clone 11-5B, 1:500; Abcam, England) or the neuronal marker anti-microtubule-associated protein 2 (MAP-2 mouse monoclonal antibody, clone AP20, 1:500; Millipore, France). Following antigen retrieval with citrate buffer pH=6 (DAKO, France) and blocking with 5% normal goat or donkey serum containing 1% BSA in 0.1M phosphate buffered saline (PBS), sections were incubated overnight at room temperature with the primary antibodies. Sections were then washed in PBS and incubated with secondary antibodies goat anti-mouse Alexa Fluor 488 targeting MAP-2, GFAP/S100β, CNPase and HLA-DR. For IRS-1pS312 and IRS-1pS616 labelling, Alexa Fluor 568 goat anti-rabbit was used (All secondary antibodies from Invitrogen, France). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, France).

To lower the intensity of lipofuscin auto-fluorescence, slides were incubated for 10 min in 0.1% Sudan Black B (Sigma-Aldrich, France) in 70% ethanol. After thorough washing in PBS, slides were mounted in permafluor mounting medium (Thermo scientific, USA). Immunofluorescence was visualized by using a Zeiss Axioplan 2 epifluorescent microscope at x40 and x63 magnification.

Quantitative analysis

Analysis of the number of immunopositive cells was done using a computerized image analysis system (Morphostrider, Explora Nova, France) linked to a Zeiss fluorescence microscope Imager M2. For colocalization analysis, 9 images were taken randomly from MSA, PD and healthy patients and image analysis was done using Image J v1.47 (Abramoff *et al.*, 2004) implemented with the colocalization threshold plugin. A threshold was applied to all images in green (MAP-2, HLA-DR, CNPase and GFAP/S100β) and another was used for the red (IRS-1pS312 and IRS-pS616) filter to assess colocalization. Quantitative analysis was carried out on all images and results are expressed as a proportion of immunopositive cells over total cells per mm^2.

After colocalization analysis, IRS-1pS312 or IRS-1pS616 positive cells' intensity was measured and values were organized according to cell type. For cell fluorescence intensity, Image J software was used to measure pixel intensity and area with respect to background intensity and cell surface respectively according to the following formula: Intensity of stained cell – (sample of background/area of sample background) X area of stained cell. To minimize the inherent variability in the immunofluorescence procedure, sections from all cases were processed simultaneously for a given marker.

Statistical analysis

Comparison of cell counts and intensity of staining between MSA patients, PD patients and healthy controls were performed using a one-way ANOVA test followed by Holm-Sidak's post-hoc analysis whenever appropriate. When data did not follow a Gaussian distribution a Kruskal-Wallis test was performed followed by Dunn's post-hoc test. Statistical analysis was performed with Graphpad Prism 6.0 (GraphPad, U.S.A). Data are presented as mean ± SEM. For all statistical tests, the level of significance was set at $p < 0.05$.

Results

Neurons in synucleinopathies are insulin resistant

Using immunofluorescence staining with the neuronal marker MAP-2, we quantified the number of neurons positive for the insulin resistance markers IRS-1pS312 and IRS-1pS616 in the putamen of PD, MSA patients and healthy controls **(Fig. 1A-C, F-H)**. Colocalization analysis revealed that all neurons expressed IRS-1p312 and IRS-1pS616. Accordingly, the quantification of IRS-1pS312 and IRS-1pS616 in MAP-2 immunopositive neurons correlated with the number of neurons in the putamen (r^2=0.99, $p<0.0001$). As the result of severe neuronal loss in MSA, one-way ANOVA analysis showed a significant difference between groups for the number of IRS-1pS312 ($F(2,20)=16.63$; $p<0.0001$, **Fig. 1D)** and IRS-1pS616 immunopositive neurons ($F(2,20)=10.36$; $p<0.001$, **Fig. 1I)**. Post-hoc analysis revealed that the numbers of IRS-1pS312 and IRS-1pS616 positive neurons were decreased in MSA compared to healthy controls (IRS-1pS312: -66%, $p<0.0001$; IRS-1pS616: -55%, $p<0.001$) and PD patients (IRS-1pS312: -55%, $p<0.01$; IRS-1pS616: -46%, $p<0.05$). Post-hoc analysis did not show significant differences between PD patients and healthy controls (IRS-1pS312: -22%, p=0.14; IRS-1pS616: -16%, p=0.4) **(Fig. 1A-D, F-I)**.

We then measured the fluorescence intensity of insulin resistance markers in neurons of PD, MSA and healthy subjects. A Kruskal-Wallis test revealed a significant difference in IRS-1pS312 ($p<0.01$) and IRS-1pS616 staining intensity ($p<0.05$) between groups with significantly increased IRS-1pS312 levels in MSA (+89%, $p<0.001$) and PD patients (+89%, $p<0.05$) compared to healthy controls **(Fig. 1E)**. There were also trends for increased neuronal IRS-1pS616 staining intensity in MSA compared to PD patients (+174%, p=0.07) and healthy controls (+156%; p=0.1) **(Fig. 1J)**. Staining intensity was not different for IRS-1pS312 between MSA and PD patients (-5%, p=0.95) and for IRS-1pS616 between PD patients and healthy controls (-6%, p=0.99) **(Fig. 1E, J)**.

Microglia but not astrocytes display decreased IRS-1pS312 staining in MSA

Colocalization analysis showed that all astrocytes stained positive for IRS-1pS312 and IRS-1pS616 (r^2=1, p<0.0001). One-way ANOVA revealed differences between groups for IRS-1pS312 (F(2,20)=8.8; p<0.01) and IRS-1pS616 (F(2,20)=14.26; p<0.001) GFAP positive cell counts (**Fig. 2A-D, F-I**). Post-hoc analysis showed that the numbers of IRS-1pS312 and IRS-1pS616 positive astrocytes were increased in MSA compared to PD patients (IRS-1pS312: +71%, p<0.01; IRS-1pS616: +70%, p<0.01) and healthy subjects (IRS-1pS312: +79%, p<0.01; IRS-1pS616: +127%, p<0.001). No differences in the number of IRS-1pS312 and IRS-1pS616 positive astrocytes were observed between PD patients and healthy subjects (IRS-1pS312: +4%, p=0.9; IRS-1pS616: +30%, p=0.4) (**Fig. 2A-D, F-I**). Kruskal-Wallis revealed that IRS-1pS312 staining intensity was not different between groups (p=0.7) (**Fig. 2E**), while a one-way ANOVA revealed a trend for a significant difference in staining intensity was observed in IRS-1pS616 positive astrocytes (F(2,20)=2.77; p=0.08) (**Fig. 2J**).

IRS-1pS312 and IRS-1pS616 co-localized with HLADR staining in all microglial cells (r^2=1, p<0.0001). One-way ANOVA revealed a significant difference between groups for IRS-1pS312 (F(2,20)=4.59;.p<0.05) and IRS-1pS616 (F(2,20)=4.07; p<0.05). IRS-1pS312 and IRS-1pS616-positive microglial cells counts were increased in MSA compared to healthy controls (IRS-1pS312: +163%, p<0.05; IRS-1pS616: +70%, p<0.05) and showed a trend for an increase compared to PD (IRS-1pS312: +81%, p=0.09; IRS-1pS616: +53%, p=0.07). No differences were observed between PD patients and healthy controls (IRS-1pS312: +45%, p=0.42; IRS-1pS616: +11%, p=0.69) (**Fig. 3A-D, F-I**).

One-way ANOVA showed that IRS-1pS312 staining intensity in microglia was different between groups (F(2,20)=5.6; p<0.05). Specifically, IRS-1pS312 staining intensity in MSA microglia was significantly decreased compared to healthy controls (-41%, p<0.05) but not compared to PD (-18%, p=0.31). PD patients microglia showed a trend for decreased IRS-1pS312 staining intensity compared to healthy controls (-28%, p=0.07) (**Fig. 3E**). IRS-1pS616 staining intensity was not different between groups (F(2,20)=0.026; p=1.64) (**Fig. 3J**).

Oligodendrocytes are insulin resistant in MSA

Double immunofluorescence showed that all oligodendrocytes stained positive for IRS-1pS312 and IRS-1pS616. One-way ANOVA revealed no significant difference in CNPase positive cell counts and CNPase positive IRS-1pS312 (F(2,20)=0.026; p=0.9) and IRS-1pS616 (F(2,20)=2.74; p=0.1) oligodendrocytes between PD, MSA and healthy controls (**Fig. 4 A-D, F-I**).

Interestingly, the intensity of IRS-1pS312 in CNPase positive oligodendrocytes in MSA was significantly different between groups (F(2,20)=7.493; p<0.01, **Fig. 4E**). Post-hoc

analysis showed a significant increase in IRS-1pS312 staining intensity in MSA patients oligodendrocytes compared to healthy controls (+88%, $p<0.01$) and PD (+101%, $p<0.01$), while no difference was found between oligodendrocytes from PD patients and healthy controls (-7%, $p=0.13$). IRS-1pS616 intensity remained unchanged in all three groups ($F(2,20)=0.1$; $p=0.9$, **Fig. 4J**)

Discussion

Accumulating evidence indicate that altered insulin/IGF-1 signalling resulting in brain insulin resistance is implicated in the pathophysiology of AD (Moloney *et al.*, 2010; Talbot *et al.*, 2012). Here we demonstrate that insulin resistance also occurs in different cell types in the putamen of PD and MSA patients. Specifically, expression of the insulin resistance marker IRS-1pS312 was increased in neurons of PD and MSA patients compared to healthy controls. Moreover, in MSA patients, increased IRS-1pS312 staining intensity was detected in oligodendrocytes.

Phosphorylation of IRS-1 on serine residues (S312/S616) is a dynamic feedback loop that negatively regulates the activity of the insulin/IGF-1 signalling pathway (Boura-Halfon and Zick, 2009; Gual *et al.*, 2005; Harrington *et al.*, 2005; Zick, 2005). IRS-1 phosphorylation prevents its activation and binding to insulin and IGF-1 receptors, in addition to directing it to the proteasome for degradation (Aguirre *et al.*, 2002; Boura-Halfon and Zick, 2009; Gual *et al.*, 2005; White, 2006; Zick, 2001, 2005).

Neurons in synucleinopathies are insulin resistant

We observed severe neuronal loss in the putamen of MSA patients in accordance with the literature (Salvesen *et al.*, 2015; Sato *et al.*, 2007). As a result, MSA patients had lower IRS-1pS312/S616 positive neuronal counts compared to PD and healthy controls, while neurons in PD and surviving neurons in MSA showed increased IRS-1pS312 staining intensity. Moreover, MSA neurons also showed a trend for increased IRS-pS616 staining intensity compared to PD and healthy controls.

Insulin resistance, as assessed by phosphorylation of IRS-1 on serine residues 312, 616 and 636 is increased in the hippocampus in postmortem brain tissue of AD patients and in preclinical models of AD (Bomfim *et al.*, 2012; Moloney *et al.*, 2010; Talbot *et al.*, 2012). We here show altered insulin/IGF-1 signalling and insulin resistance in the putamen of MSA patients and PD patients. Yarchoan *et al.* (2014) recently reported an increased area with IRS-1pS616 staining in the hippocampus and midfrontal gyrus cortex of AD and tauopathies. This study did not show any difference in IRS-1pS616 in the hippocampus and midfrontal gyrus

cortex between synucleinopathies patients and healthy controls. Together with the findings of Yarchoan et al., our results suggest that synucleinopathies and tauopathies are associated with distinct and disease-specific regional patterns of insulin resistance.

Intact insulin/IGF-1 signalling is pivotal to neuronal survival in the brain since it modulates the activity of several prosurvival or proapoptotic effectors such as FoxO, GSK-3β, caspases and Bcl-2 (Bassil *et al.*, 2014). Insulin/IGF-1 signalling is a repressor of FoxO activity in the brain, while increased FoxO activity has been linked to apoptosis through activation of FasL promoter and Bim (Barthelemy *et al.*, 2004; Dijkers *et al.*, 2000; Matsuzaki *et al.*, 2003). Moreover, insulin/IGF-1 signalling is essential for axonal growth, regeneration and protein synthesis through the activation of mTOR and inhibition of GSK-3β (Delcommenne *et al.*, 1998; Dupraz *et al.*, 2013; Leibinger *et al.*, 2012; Yang *et al.*, 2011). Insulin resistance in neurons of MSA and PD patients may contribute to neuronal dysfunction by decreasing the activity of prosurvival activity effectors such as Bcl-2 and mTOR and gene expression in neurons via decreased CREB activity (Chen *et al.*, 2009; Chu *et al.*, 2009; Dehay *et al.*, 2010; Golpich *et al.*, 2015; Kawamoto *et al.*, 2014; Kragh *et al.*, 2013; Levy *et al.*, 2009; Nakamura *et al.*, 2001; Nakamura *et al.*, 1998). In addition, altered insulin/IGF-1 signalling in MSA and PD patients may lead to decreased repression of proapoptotic effectors such FoxO and caspases leading to cell death. Since there is no loss of neurons in the putamen in PD contrary to MSA, these results indicate that insulin resistance in putaminal neurons is not sufficient *per se* for neurodegeneration and suggest that additional factors, possibly including other contributing sources of insulin resistance could account for the differential neuronal vulnerability observed between PD and MSA.

Microglia but not astrocytes in MSA display decreased IRS-1pS312 staining

As previously shown, neuronal loss was accompanied by increased neuroinflammation in the putamen of MSA patients (Gerhard *et al.*, 2003; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Shibata *et al.*, 2010). Increased non-neuronal IRS-1pS312 and IRS-1pS616 cell counts in MSA patients were mainly due to the increased number of astrocytes and microglia.

Insulin/IGF-1 signalling in astrocytes is required for proliferation, glutamate transporter expression, glycogen synthesis and neuroprotection by decreasing oxidative stress (Bassil *et al.*, 2014; Genis *et al.*, 2014; Heni *et al.*, 2011; Muhic *et al.*, 2015). In this regard, glycogen, the main energy source in the brain, is almost exclusively regulated by insulin/IGF-1 signalling in astrocytes (Brown and Ransom, 2007; Muhic *et al.*, 2015). Moreover, activated astrocytes produce less IGF-1 compared to naïve astrocytes which might also contribute to decreased IGF-1 availability in the brain (Muhic *et al.* (2015). However, our data did not reveal significant differences in astrocytic insulin resistance between groups.

Microglia are implicated in the production of neuronal growth factors such as IGF-1 (Beilharz *et al.*, 1998; Butovsky *et al.*, 2006; Suh *et al.*, 2013). The number of microglial cells that stained for IRS-1pS312 and IRS-1pS616 were increased in the putamen of MSA patients compared to healthy controls and PD patients. Microglial cells of MSA patients further showed decreased IRS-1pS312 staining intensity compared to PD and healthy controls. The PI3-K/Akt pathway is implicated in the response of microglia to inflammatory conditions (Saponaro *et al.*, 2012). More specifically, the activation of the PI3-K/Akt pathway mediates the transition of microglia from a pro-inflammatory role to an anti-inflammatory (Tarassishin *et al.*, 2011). Hence, decreased IRS-1pS312 in microglia of MSA patients suggest that microglial cells in MSA might be in an anti-inflammatory state in late stages of the disease.

Oligodendrocytes are insulin resistant in MSA

Oligodendrocyte counts were similar in MSA, PD and healthy controls, while oligodendrocytes in MSA patients were insulin resistant compared to PD and healthy controls. As previously mentioned, oligodendrocytes are of particular importance in MSA as being the cells hosting GCIs due to the accumulation and formation of α-syn aggregates in their cytosol (Papp *et al.*, 1989; Spillantini *et al.*, 1998b; Wakabayashi and Takahashi, 2006). Whether insulin resistance precedes α-syn inclusions or is merely a result of α-syn aggregation in oligodendrocytes remains an unanswered question. *In vitro* studies support the former hypothesis since transient overexpression of α-syn in human neuroblastoma cells alters insulin/IGF-1 signalling and induces insulin resistance via phosphorylation of IRS-1 on serine residues (Boura-Halfon and Zick, 2009; Gao *et al.*, 2015; Harrington *et al.*, 2005; White, 2006; Yang *et al.*, 2013; Zick, 2005). Insulin/IGF-1 signalling plays a prominent role in oligodendrocyte survival, proliferation, differentiation and functioning (Carson *et al.*, 1993; Chesik *et al.*, 2007; De Paula *et al.*, 2014; Goddard *et al.*, 1999; Zeger *et al.*, 2007). Studies haves also shown that insulin/IGF-1 signalling acts as a myelin synthesis and maturation factor in several demyelinating disorders (Liu *et al.*, 1995; Mason *et al.*, 2000; Yao *et al.*, 1995). Several studies have reported myelin loss, fragmentation and alteration in MSA (Ishizawa *et al.*, 2008; Matsuo *et al.*, 1998; Papp *et al.*, 1989; Papp and Lantos, 1994; Song *et al.*, 2007). In this line, mRNA and protein levels of myelin basic protein (MBP), a main constituent of myelin, are decreased in the brain of MSA patients pointing to a possible deficit in MBP synthesis (Salvesen *et al.*, 2015; Song *et al.*, 2007). Interestingly, IGF-1 has been shown to play a pivotal role in myelin synthesis by increasing transcripts for MBP, myelin proteolipid protein and 2’,3’-Cyclic-nucleotide 3’-phosphodiesterase, all known to be critical for myelin formation (Mozell and McMorris, 1991; Yao *et al.*, 1995, 1996). Moreover, MSA patients also exhibit decreased levels of myelin associated lipids that are main constituents of

myelin sheath and are implicated in myelin stability (Don *et al.*, 2014; O'Brien and Sampson, 1965), while IGF-1 has been shown to stimulate *de-novo* fatty acid biosynthesis via PI3-K/Akt activation (Liang *et al.*, 2007). Early oligodendroglial dysfunction may include altered insulin/IGF-1 signalling and insulin resistance contributing to abnormal oligodendrocyte functioning and myelin alteration. As a result, oligodendroglial trophic support to neurons may also be compromised in MSA and contribute to degeneration of neurons that also show insulin resistance. Besides its potential contribution to neurodegeneration through altered myelinisation and trophic support, decreased insulin signalling in oligodendrocytes may also contribute to the impaired maturation of oligodendrocytes progenitors occurring in MSA (May *et al.*, 2014). Indeed, IGF-1 and PI3-Kinase/Akt activation promote the differentiation of oligodendrocyte progenitors and myelinisation (De Paula *et al.*, 2014).

Implications for treatment development

Several studies have shown that administration of insulin and/or IGF-1 can reverse disease severity in preclinical models of neurodegeneration. With regard to PD models, *in vitro* studies have shown beneficial effects of IGF-1 against α-syn, dopamine and 1-methyl-4-phenylpyridinium ion-induced cytotoxicity (Kao, 2009; Offen *et al.*, 2001; Sun *et al.*, 2010). Similarly IGF-1 administration in *in vivo* models of PD prevented the loss of dopaminergic neurons in the substantia nigra and associated motor impairments (Ebert *et al.*, 2008; Guan *et al.*, 2000; Krishnamurthi *et al.*, 2004; Quesada *et al.*, 2008).

Glucagon like peptide-1 (GLP-1) analogues are FDA approved treatments for type 2 diabetes. They activate the same downstream effectors as insulin/IGF-1, rendering them suitable candidate drugs for targeting insulin resistance (Bassil *et al.*, 2014). Exendin-4, a GLP-1 analogue, improved motor performance and rescued dopaminergic neurons from 6-OHDA induced cell death (Bertilsson *et al.*, 2008; Harkavyi *et al.*, 2008). Similarly, Exendin-4 decreased the loss of nigral neurons and striatal dopaminergic fibers, proinflammatory markers and improved motor function in mouse models of PD (Kim *et al.*, 2009b; Li *et al.*, 2009).

Successful studies in preclinical models of PD lead the way to a small, open-label clinical trial assessing the effects of Exendin-4 in 45 PD patients who were followed for 14 months. Motor and cognitive outcomes were significantly improved in patients receiving Exendin-4 (Aviles-Olmos *et al.*, 2013a; Aviles-Olmos *et al.*, 2014). The promising results of this preliminary open-label trial have set the grounds for a randomized, double blind, placebo-controlled study (NCT01971242) in 60 PD patients that has started its enrollment in December 2013.

Conclusions

We here show insulin resistance as evidenced by increased IRS-1 phosphorylation at serine residues 312 and 616 in the putamen of MSA and PD patients. Specifically, neurons in PD and MSA patients, as well as oligodendrocytes in MSA patients were insulin resistant, while in MSA, microglia showed lower IRS-1pS312 staining intensity. Abnormal insulin/IGF-1 signalling in oligodendrocytes may lead to impaired oligodendrocyte maturation and functioning, thus contributing to secondary neurodegeneration in the putamen of MSA patients. Our results further support insulin/IGF-1 analogues (i.e. anti-diabetics) as possible candidates for disease modification in synucleinopathies.

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Figure legends

Patient	Identity tag	Type	Age	Sex	Postmortem Delay	Disease Duration
P1	N13-199	Control	23	M	< 48	-
P2	N13-212	Control	54	F	< 48	-
P3	N13-54	Control	35	M	< 48	-
P4	N07-164	Control	84	F	16	-
P5	N07-576	Control	69	M	< 48	-
P6	99N10	Control	55	M	< 48	-
P7	N08-710	Control	57	M	< 48	-
P8	N08-907	PD	75	M	< 48	11
P9	N03-817	PD	63	M	< 24	11
P10	N10-864	PD	77	F	< 48	NA
P11	N10-1132	PD	69	M	< 48	NA
P12	N11-491	PD	74	M	26	14
P13	N09-121	PD	67	F	7	22
P14	N14-00296	PD	76	M	36	18
P15	N10-569	MSA-C	73	F	24	3
P16	N10-794	MSA-P	57	F	24	2
P17	N11-982	MSA-P	57	F	7	7
P18	N11-441	MSA-C	59	M	24	8
P19	N10-1157	MSA-P	83	F	12	6
P20	N07-1159	MSA-P	71	F	24	3
P21	N02-99	MSA-P	72	M	48	6

Table 1: Demographic and neuropathological characteristics of cases used in this study.

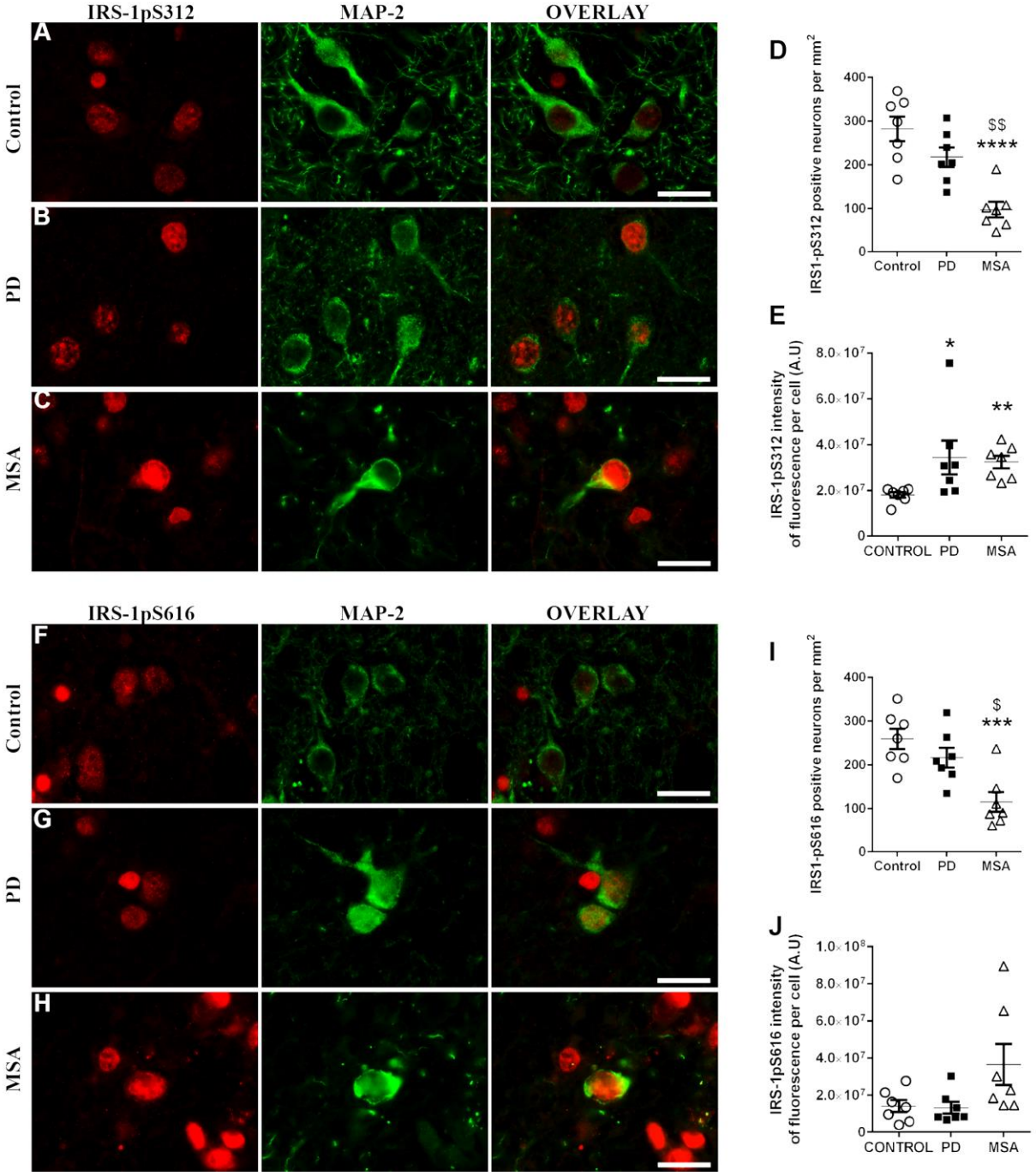

Figure 1: Neurons in synucleinopathies are insulin resistant. (**A-C**) and (**F-H**) are representative images of IRS-1pS312 and IRS-1pS616 staining in neurons of control (**A, F**), PD (**B, G**) and MSA patients (**C, H**). Decreased IRS-1pS312 and IRS-1pS616 positive neurons in MSA patients compared to PD and healthy patients (**D, I**). Quantification of IRS-1pS312 staining intensity in PD and MSA neurons showed increased staining in neurons of PD and MSA patients compared to healthy patients (**E**). A trend to increased IRS-1pS616 staining intensity in MSA neurons compared to healthy and PD patients (**J**). Scale bar = 20µm. Error bars indicate standard error. MSA or PD compared to control: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; MSA compared to PD: $p<0.05, $$p<0.01.

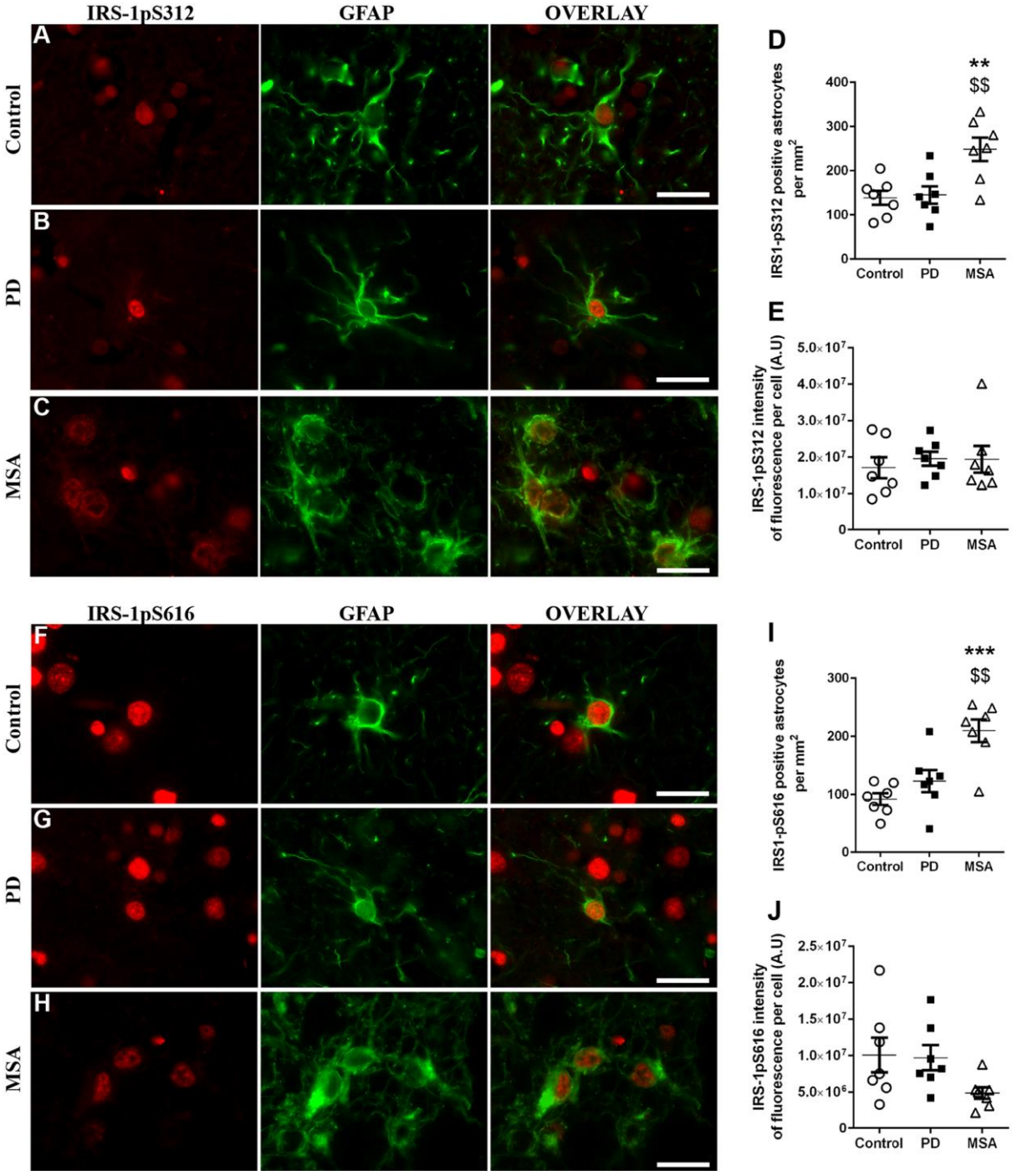

Figure 2: Increased astrocyte counts in the putamen of MSA patients. (**A-C**) and (**F-H**) are representative images of IRS-1pS312 and IRS-1pS616 staining in astrocytes of control (**A, F**), PD (**B, G**) and MSA patients (**C, H**). Increased IRS-1pS312 and IRS-1pS616 positive astrocytes in MSA patients compared to PD and healthy patients (**D, I**). Quantification of IRS-1pS312 staining intensity showed no significant difference between all three groups (**E**). A trend to decreased IRS-1pS616 staining intensity in MSA astrocytes compared to healthy and PD patients (**J**). Scale bar = 20µm. Error bars indicate standard error. MSA compared to control: **p<0.01, ***p<0.001; MSA compared to PD: $$p<0.01.

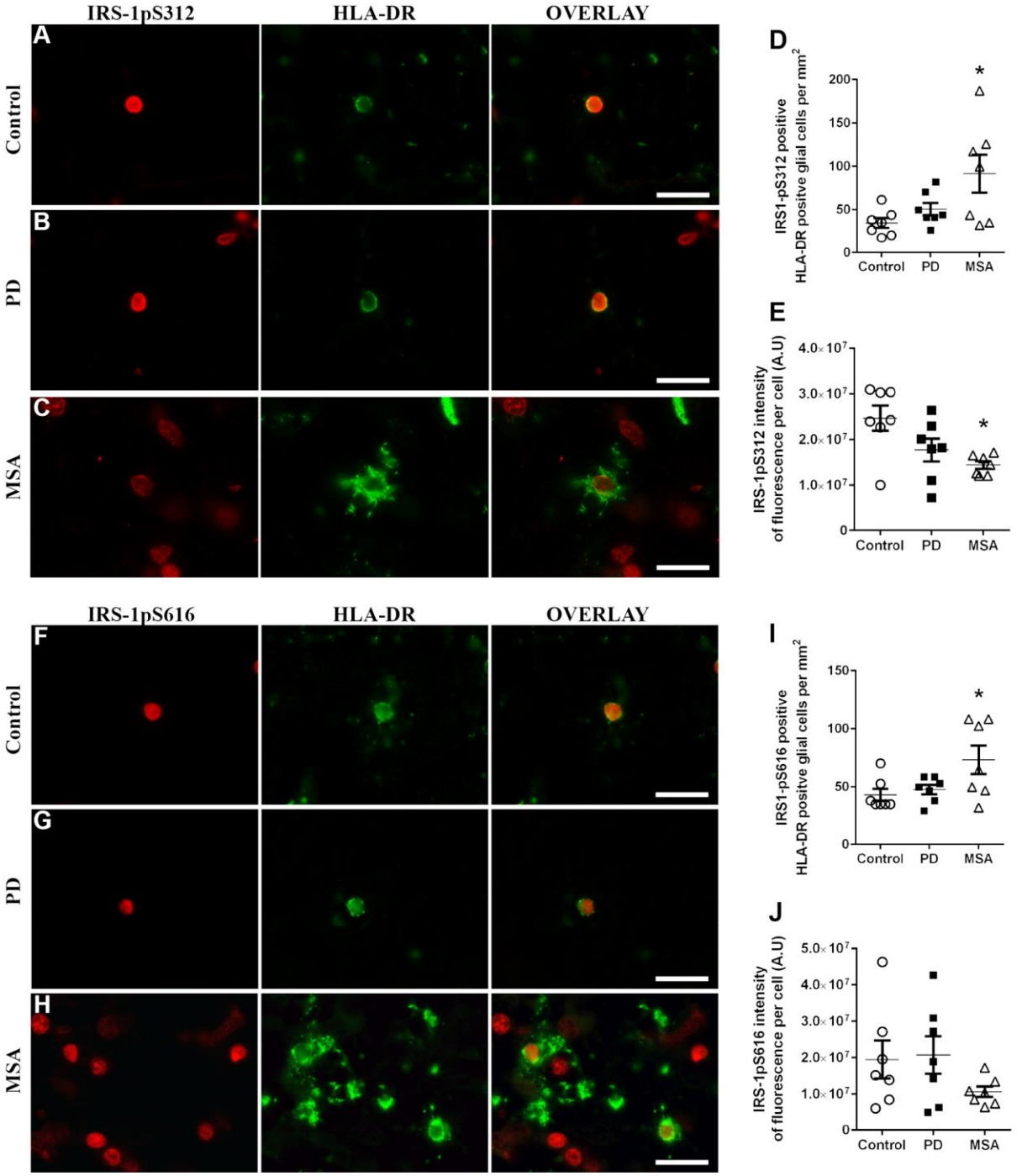

Figure 3: Microglia show decreased IRS-1pS312 staining in MSA. (**A-C**) and (**F-H**) are representative images of IRS-1pS312 and IRS-1pS616 staining in microglia of control (**A, F**), PD (**B, G**) and MSA patients (**C, H**). Increased IRS-1pS312 and IRS-1pS616 positive microglia in MSA patients compared to PD and healthy patients (**D, I**). Quantification of IRS-1pS312 staining intensity in MSA microglia showed decreased staining in microglia of MSA patients compared to PD and healthy patients (**E**). Quantification of IRS-1pS616 staining intensity showed no significant difference between all three groups (**J**). Scale bar = 20µm. Error bars indicate standard error. MSA compared to control: *p<0.05.

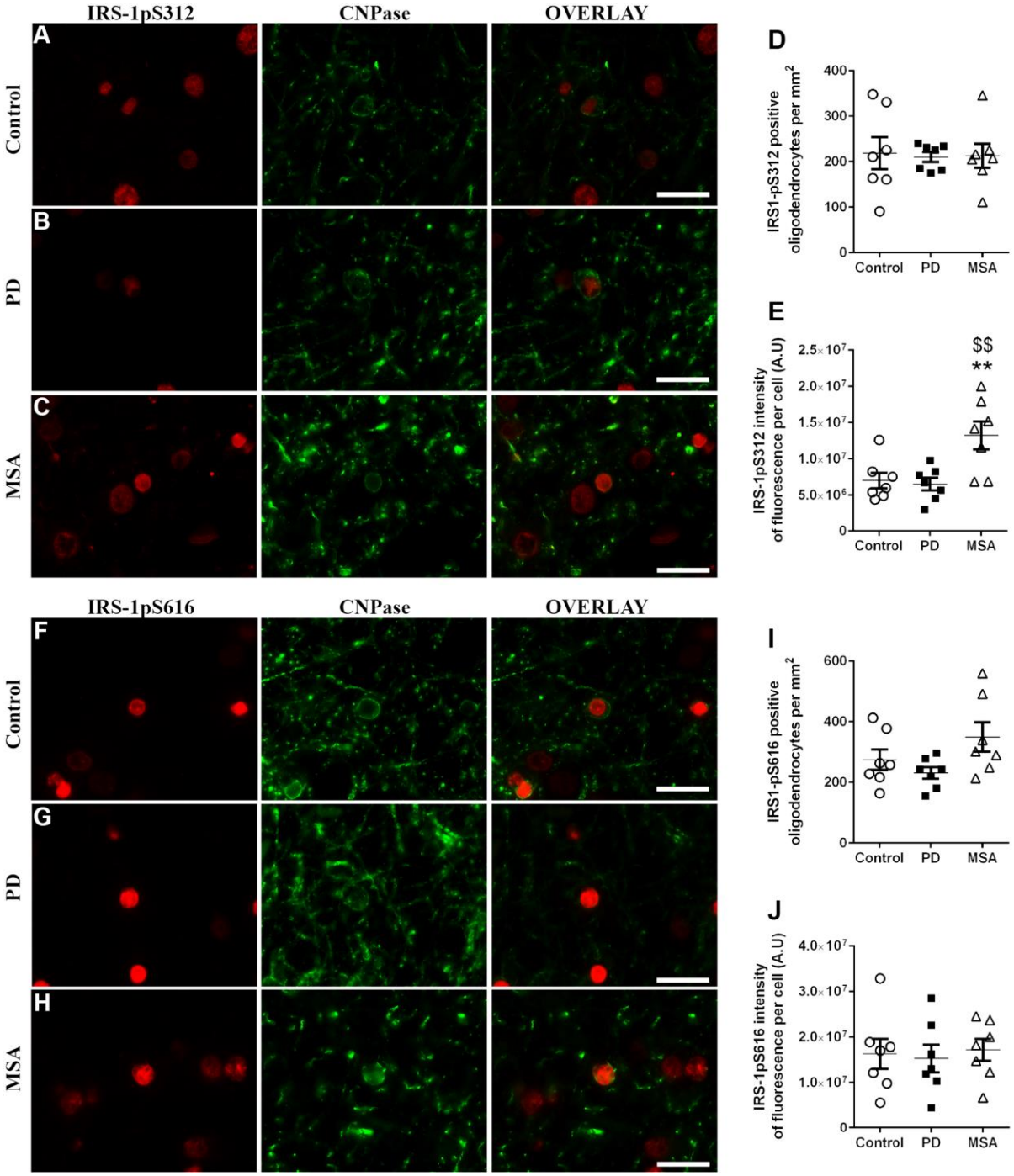

Figure 4: Oligodendrocytes are insulin resistant in MSA. (**A-C**) and (**F-H**) are representative images of IRS-1pS312 and IRS-1pS616 staining in oligodendrocytes of control (**A, F**), PD (**B, G**) and MSA patients (**C, H**). No change in IRS-1pS312 and IRS-1pS616 positive oligodendrocyte quantification between all three groups (**D, I**). Quantification of IRS-1pS312 staining intensity in PD and MSA neurons showed increased staining in oligodendrocytes of MSA patients compared to PD and healthy patients (**E**). Quantification of IRS-1pS616 staining intensity showed no significant difference between all three groups (**J**). Scale bar = 20µm. Error bars indicate standard error. MSA compared to control: **p<0.01; MSA compared to PD: $$p<0.01.

Article 2:

Reducing C-terminal truncation mitigates synucleinopathy and neurodegeneration in a transgenic model of multiple system atrophy

Fares Bassil, Pierre-Olivier Fernagut, Erwan Bezard, Quyen Hoang, Dagmar Ringe, Gregory A. Petsko and Wassilios G Meissner*

(soumis)

Dans cet article, nous avons cherché à évaluer les effets thérapeutiques de la réduction de la troncation de l'α-synucléine (α-syn) sur l'agrégation et l'insolubilité de l'α-syn ainsi que la neurodégénérescence dans un modèle préclinique murin de l'AMS.

Plusieurs études ont pu mettre en évidence le rôle de l'oligomérisation et de l'agrégation de l'α-syn dans la neurotoxicité des synucléinopathies. Plus récemment, la troncation de l'α-syn dans la partie C-terminale de la protéine a été montrée comme favorisant l'agrégation de l'α-syn *in vitro*. La caspase-1 est une protéase capable de cliver l'α-syn en C-terminal. Afin de déterminer le potentiel thérapeutique de la diminution de la troncation de l'α-syn par la caspase-1, nous avons traité des souris PLP-SYN et des souris sauvages sur une période de 11 semaines avec VX-765, un inhibiteur de la caspase-1, ou son placebo.

Le traitement avec VX-765 a diminué les déficits moteurs chez les souris PLP-SYN par rapport à aux animaux traités par le placebo. De plus, le VX-765 a diminué la toxicité induite par l'agrégation de l'α-syn, notamment en réduisant la charge protéique de l'α-syn dans le striatum des souris PLP-SYN traitées. Non seulement le traitement avec VX-765 a réduit la forme tronquée de l'α-syn, mais il a également diminué les formes monomériques et oligomériques. Enfin, VX-765 a montré un effet neuroprotecteur par la préservation des neurones dopaminergiques dans la substance noire des souris PLP-SYN. En conclusion, nos résultats suggèrent que VX-765 est un candidat prometteur pour ralentir la progression de la pathologie dans un contexte de synucléinopathie en limitant notamment l'accumulation α-syn.

Reducing C-terminal truncation mitigates synucleinopathy and neurodegeneration in a transgenic model of multiple system atrophy

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Abstract

Multiple system atrophy (MSA) is a sporadic orphan neurodegenerative disorder. No treatment is currently available to slow down the aggressive neurodegenerative process and patients die within a few years after disease onset. The cytopathological hallmark of MSA is the accumulation of alpha-synuclein (α-syn) aggregates in affected oligodendrocytes. Several studies point to α-syn oligomerization and aggregation as a mediator of neurotoxicity in synucleinopathies including MSA. C-terminal truncation by the inflammatory protease caspase-1 has recently been implicated in the mechanisms that promote aggregation of α-syn *in vitro* and in neuronal cell models of α-syn toxicity. We here present an *in vivo* proof of concept of the ability of the caspase-1 inhibitor prodrug VX-765 to mitigate α-syn pathology and to mediate neuroprotection in PLP-SYN mice, a transgenic mouse model of MSA. PLP-SYN and age-matched wild-type mice were treated for a period of 11 weeks with VX-765 or placebo. VX-765 prevented motor deficits in PLP-SYN mice compared to placebo controls. More importantly, VX-765 was able to limit the progressive toxicity of α-syn aggregation by reducing its load in the striatum of PLP-SYN mice. Not only did VX-765 reduce truncated α-syn but also decreased its monomeric and oligomeric forms. Finally, VX-765 showed neuroprotective effects by preserving tyrosine hydroxylase positive neurons in the substantia nigra of PLP-SYN mice. In conclusion, our results suggest that VX-765, a drug that was well tolerated in a six-week-long phase 2 trial in patients with epilepsy, is a promising candidate to achieve disease modification in synucleinopathies by limiting α-syn accumulation.

Significance Statement

Multiple system atrophy (MSA) is a fatal neurodegenerative disorder associated with the accumulation of alpha-synuclein (α-syn) aggregates in oligodendrocytes. There is currently no treatment to slow down the aggressive neurodegenerative process. C-terminal truncation of α-syn promotes the formation of oligomers and aggregates that, in turn, mediate neurotoxicity in synucleinopathies including MSA. We here present an *in vivo* proof of concept of the ability of the caspase-1 inhibitor VX-765 to mitigate α-syn pathology and provide neuroprotection in a transgenic mouse model of MSA through reduction of α-syn C-terminal truncation. These findings suggest that VX-765, a well-tolerated drug in a six-week-long phase 2 trial in patients with epilepsy, is a promising candidate to achieve disease modification in MSA by limiting α-syn accumulation.

\body

Introduction

Multiple system atrophy (MSA) is a sporadic adult-onset orphan neurodegenerative disorder clinically characterized by a combination of parkinsonism, cerebellar impairment and autonomic dysfunction (Gilman *et al.*, 2008). The cytopathological hallmark of MSA is the accumulation of alpha-synuclein (α-syn) aggregates in oligodendrocytes, forming glial cytoplasmic inclusions (GCIs) (Papp *et al.*, 1989; Spillantini *et al.*, 1998a).

The 14 kDA protein α-syn can exist *in vitro* as an unfolded monomer; although other oligomeric species have been reported (Fauvet *et al.*, 2012). Full-length α-syn undergoes several post-translational modifications such as phosphorylation, tyrosine nitration and truncation, any of which could promote the formation of toxic α-syn aggregates (Lashuel *et al.*, 2013; Muntane *et al.*, 2012; Rochet *et al.*, 2000). Although the precise toxic species of α-syn have not been firmly established, several studies point to α-syn oligomerization and aggregation as a mediator of neurotoxicity in synucleinopathies (Auluck *et al.*, 2010; Cremades *et al.*, 2012; Lashuel *et al.*, 2013; Winner *et al.*, 2011). Hence decreasing aggregation might be an effective approach to disease modification. Among the mechanisms that promote aggregation of α-syn, C-terminal truncation has been identified as an enhancer/promoter of α-syn oligomerization and fibrillization (Hoyer *et al.*, 2004; Li *et al.*, 2005b; Liu *et al.*, 2005; Ulusoy *et al.*, 2010). Accordingly, inhibiting α-syn truncation could alter the disease course in MSA (and other synucleinopathies) (Fernagut *et al.*, 2014a) by decreasing α-syn oligomerization and aggregation. Interestingly, the inflammatory protease caspase-1 cleaves α-syn at Asp121, promoting its aggregation into amyloid fibrils similar to those previously found both *in vitro* and *in vivo* (Wang *et al.*, 2015). In turn, the caspase-1 inhibitor prodrug VX-765 decreases α-syn truncation and aggregation *in vitro* and rescues cells from α-syn-induced toxicity (Wang *et al.*, 2015). Therefore, VX-765 could exert neuroprotective effects on MSA pathogenesis, by reducing α-syn cleavage hence limiting its toxicity and its ability to form aggregates. VX-765 is an orally active, well-tolerated, brain-penetrant prodrug that is hydrolyzed by esterases in vivo to produce a potent and selective caspase-1 inhibitor (Boxer *et al.*, 2010; Wannamaker *et al.*, 2007) that was initially developed for the treatment of inflammatory diseases such as psoriasis and rheumatoid arthritis and later also tested as possible therapeutic for epilepsy (Vertex, 2011). Thus, the drug is readily available for further clinical development in MSA.

Here we show that VX-765 mitigates progressive synucleinopathy and neurodegeneration in a transgenic mouse model of MSA.

Results

VX-765 prevents motor impairments in transgenic MSA proteolipid protein α-syn (PLP-SYN) mice.

Transgenic MSA PLP-SYN mice display progressive motor impairment with aging, as shown with an increased number of errors on the traversing beam task (Fernagut *et al.*, 2014b). Motor performance of wild-type (WT) mice was not affected by VX-765 treatment (100mg/kg/d over 11 weeks; p>0.5), while VX-765-treated PLP-SYN mice showed significant improvement in the traversing beam task compared to placebo-treated PLP-SYN mice (P<0.01) (Fig. 1*A*).

VX-765 decreases α-syn burden in the striatum of PLP-SYN mice.

GCIs are the cytopathological hallmark of MSA (Papp *et al.*, 1989). PLP-SYN mice overexpress α-syn under the PLP promoter, leading to the formation of GCIs (Fernagut *et al.*, 2014b; Kahle *et al.*, 2002). To investigate whether reducing C-terminal truncation affects α-syn load in PLP-SYN mice, we first measured the quantity of α-syn in the striatum (Fig. 1) and the cortex (Fig. 2) of PLP-SYN mice by western blot. VX-765 treatment decreased both oligomeric (-43%, P<0.05) and monomeric α-syn (-37%, P=0.001) (Fig. 1*B, C, E*) in the striatum but not in the cortex (P=0.93 and P=0.23, respectively, Fig. 2*A, B, D*) of PLP-SYN mice. Interestingly, VX-765-treated mice had a 53% decrease in α-syn truncation in the striatum (P=0.01) (Fig. 1*B, C, F*), while no significant effect was found on the formation of the C-terminally truncated protein in the cortex of PLP-SYN mice (P=0.7) (Fig. 2*A, B, E*). The amount of oligomeric α-syn positively correlated with truncated α-syn (r^2=0.53, p<0.05) in the placebo group, while VX-765 treatment abolished this correlation in PLP mice (r^2=0.0007, p=0.84).

We then assessed the density of α-syn inclusions in PLP-SYN mice and whether VX-765 affects α-syn aggregate solubility using immunohistochemistry on adjacent sections, with or without proteinase-K pre-treatment, in the striatum (Fig. 1*G-L*) and in the cortex *(Fig.* 2*F-K*). VX-765-treated PLP-SYN mice showed a significant decrease in the density of α-syn-immunopositive GCIs in the striatum (-40%, P<0.001) (Fig. 1*G-I*) but not in the cortex (P=0.19) (Fig. 2*F-H*) compared to placebo PLP-SYN mice. The amount of proteinase-K-resistant α-syn aggregates was also significantly lowered in the striatum of VX-765 treated PLP-SYN compared to placebo PLP-SYN mice (-22%, P<0.05) (Fig. 1*J-L)* but not in the cortex (P=0.23) (Fig. 2*I-K*).

VX-765 protects tyrosine hydroxylase (TH) neurons in the substantia nigra pars compacta (SNc) of PLP-SYN mice.

Oligodendroglial α-syn overexpression in PLP-SYN mice induces a loss of TH positive neurons in the SNc (Fernagut *et al.*, 2014b; Stefanova *et al.*, 2005a). Accordingly, post-hoc analysis of stereological counts of dopaminergic neurons in the SNc revealed a significant loss of TH positive neurons (-38%, $p<0.05$) in placebo PLP-SYN mice compared to placebo WT mice (significant effect of treatment ($P<0.05$) and interaction between genotype and treatment ($P<0.05$, Fig. 3*A*-*C*)). More importantly, VX-765 treatment reduced dopaminergic neuron loss in the SNc of PLP-SYN mice, as demonstrated by a 40% difference in TH positive neuron counts compared to placebo-treated PLP-SYN mice ($p<0.05$, Fig. 3*A*-*C*), a result further confirmed by counting the number of Nissl stained neurons in the SNc (data not shown).

Discussion

In the current study, PLP-SYN mice, a transgenic mouse model of MSA, and age-matched WT mice were treated for a period of 11 weeks with VX-765 or placebo. VX-765 prevented motor deficits in PLP-SYN mice compared to placebo controls. More importantly, VX-765 was also able to limit the progressive toxicity of α-syn aggregation by reducing its load in the striatum of PLP-SYN mice. Not only did VX-765 reduce truncated α-syn, it also decreased its monomeric and oligomeric forms. Finally, VX-765 showed neuroprotective effects by preserving TH positive neurons in the SNc of PLP-SYN mice.

Transgenic models have been developed to support studies on the underlying mechanisms of MSA pathogenesis and preclinical drug screening. These models are based on overexpression of α-syn in oligodendrocytes and replicate several aspects of MSA pathology (Fernagut and Tison, 2012; Stefanova *et al.*, 2005b). The PLP-SYN mouse model used in this study displays motor deficits, neuroinflammation and loss of TH positive neurons in the SNc in addition to the presence of α-syn inclusions in oligodendrocytes (Stefanova *et al.*, 2005a; Stefanova *et al.*, 2007). We here show that these mice also show C-terminal truncated α-syn.

Intracellular α-syn inclusions are the pathological hallmark of several neurodegenerative disorders known as synucleinopathies that include dementia with Lewy bodies (DLB), Parkinson's disease (PD) and MSA (Spillantini and Goedert, 2000). Most of the work done to assess α-syn toxicity and to describe the relationships between α-syn burden, spreading and disease severity has however been done in PD models (Recasens and Dehay, 2014; Recasens *et al.*, 2014). The precise mechanism by which α-syn aggregate formation leads to neurodegeneration remains unclear (Conway *et al.*, 2000; Recasens and Dehay, 2014). Recent research efforts have focused on limiting α-syn induced neurodegeneration by inhibiting α-syn oligomerization and aggregation (Bieschke *et al.*, 2010; Levin *et al.*, 2014; Masliah *et al.*, 2011; Myohanen *et al.*, 2012; Savolainen *et al.*, 2014). Several studies have

shown that C-terminal truncated α-syn is prone to form fibrils (Crowther *et al.*, 1998; Dufty *et al.*, 2007; Murray *et al.*, 2003; Serpell *et al.*, 2000). In turn, α-syn fibrillization is toxic when overexpressed in animal models of PD (Periquet *et al.*, 2007; Ulusoy *et al.*, 2010). More importantly, C-terminal truncation elicits the production of toxic α-syn aggregates and promotes neurodegeneration (Daher *et al.*, 2009; Diepenbroek *et al.*, 2014; Dufty *et al.*, 2007; Games *et al.*, 2014; Hoyer *et al.*, 2004; Li *et al.*, 2005b; Liu *et al.*, 2005; Masliah *et al.*, 2011; Michell *et al.*, 2007; Mishizen-Eberz *et al.*, 2003; Murray *et al.*, 2003; Periquet *et al.*, 2007; Tofaris *et al.*, 2006; Tsigelny *et al.*, 2007; Ulusoy *et al.*, 2010; Winner *et al.*, 2011). Some studies have shown that C-terminally truncated α-syn is found in GCIs in MSA (Gai *et al.*, 1999; Tong *et al.*, 2010) as well as in Lewy bodies of PD and DLB patient brains (Baba *et al.*, 1998; Campbell *et al.*, 2001; Dufty *et al.*, 2007; Li *et al.*, 2005b; Liu *et al.*, 2005). Several proteases such as calpain, matrix metalloproteases, cathepsin D and plasmin have been implicated in α-syn truncation subsequently resulting in increased levels of protein aggregates; however, none has been established as a major producer of C-terminally truncated α-syn *in vivo*, especially in response to inflammation (Choi *et al.*, 2011; Dufty *et al.*, 2007; Kim *et al.*, 2012; Liu *et al.*, 2005; Mishizen-Eberz *et al.*, 2003; Sung *et al.*, 2005).

VX-765 is a prodrug that *in vivo* produces a potent and selective inhibitor of caspase-1, an inflammatory protease that has recently been shown to cleave α-syn in its disordered C-terminal region following residue Asp 121 (Wang *et al.*, 2015). This same study showed that VX-765 decreases C-terminal truncation and aggregate formation *in vitro*. Here, we demonstrate the ability of VX-765 to mitigate MSA-like neuropathology together with a concomitant reduction in C-terminal truncation and aggregation of α-syn as well as dopaminergic neurodegeneration in PLP-SYN mice.

Recent efforts targeting α-syn truncation have shown that the overexpression of a calpain-specific inhibitor reduces α-syn aggregation and other neuropathological features in the [A30P]α-syn-Thy-1 PD mouse model (Diepenbroek *et al.*, 2014), while immunotherapy directed against the C-terminal region of α-syn proved to be beneficial in the mThy1-α-syn PD mouse model (Games *et al.*, 2014) and the transgenic DLB mouse model using the PDGF β promoter (Masliah *et al.*, 2011). These studies have shown that targeting α-syn truncation *in vivo* decreases α-syn aggregation and neurotoxicity. Interestingly, Games *et al.* (2014) reported that decreasing α-syn truncation and the resultant effects could well be explained by blocking α-syn propagation from neurons.

We cannot rule out the possibility that the preservation of dopaminergic neurons reported here might also involve caspase-1 dependent mechanisms other than the inhibition of α-syn truncation and the resultant decrease in oligomeric species.

VX-765 treatment also reduced monomeric α-syn in oligodendrocytes. This might be due to the decrease in truncated and oligomeric α-syn load, which allowed the clearance systems in oligodendrocytes to better handle the overexpressed monomeric α-syn. Truncated and oligomeric α-syn are both products of monomeric α-syn modification (Baba *et al.*, 1998; Conway *et al.*, 2000; Fauvet *et al.*, 2012; Lashuel *et al.*, 2013; Murray *et al.*, 2003). Thus, a marked decrease in both forms might well be secondary to the decrease in monomeric α-syn. This might not be the case with VX-765 treatment since it cancelled the correlation between truncated and oligomeric α-syn observed in placebo treated mice.

We here present an *in vivo* proof of concept of the ability of the caspase-1 inhibitor prodrug VX-765 to mitigate α-syn pathology and to mediate neuroprotection in a MSA mouse model. Our results show that VX-765, a drug that was well tolerated in a phase II trial in patients with epilepsy (Vertex, 2011), is a promising candidate to achieve disease modification in synucleinopathies by limiting α-syn accumulation.

Material and methods

Animals

Mice expressing human wild-type α-syn in oligodendrocytes under the control of the proteolipid promoter (PLP-SYN) were previously generated on a C57BL/6 background (Kahle et al., 2002). PLP-SYN (n=16) and WT littermates (n=16) aged 6 weeks at the beginning of the treatment period were randomly allocated into two groups, placebo (8 WT, 8 PLP-SYN) and VX-765 (8 WT and 8 PLP-SYN). After 11 weeks of daily treatment, motor behaviour was tested before killing the animals. Brain tissue was further processed for histopathological and biochemical analysis. All experiments 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. Mice were maintained in a temperature- and humidity-controlled room on a 12:12 light-dark cycle with food and water *ad libitum*.

Pharmacological treatment

Mice were treated via gavage (VetTech solutions Ltd, Dosing Catheter: 4.5fg, length 60mm) once a day with VX-765 (MedKoo Biosciences), which was prepared daily and dissolved in deionized water containing 0.5% methylcellulose and 0.1%Tween-80 at a dose of 100 mg/kg. The same solution without VX-765 was administered to the placebo group. VX-765 is an orally absorbed prodrug of VRT-043198, a potent and selective inhibitor of caspases in the ICE/caspase-1 subfamily of cysteine proteases. VX-765 is converted to the cell permeable inhibitor VRT-043198 *in vivo* by the action of plasma and liver esterases. Although brain

penetrance of the active drug is modest, it has been shown to inhibit caspase-1 in mouse brain at the doses employed here (Maroso *et al.*, 2011). This dose is lower than the dose that was well-tolerated over a six-week period in a phase II clinical trial in patients with epilepsy (Vertex, 2011).

Behavioral test

Motor coordination and balance were assessed with a modified version of the traversing beam task that was adapted from a previously described method (Fleming *et al.*, 2004). This test measures the ability to traverse a narrow beam to reach a goal box. The beam consists of four narrowing plexiglas segments placed horizontally 50 cm above the floor. During training, three trials were performed using the beam. Mice then underwent the test where a grid was added on top of the beam. Mice were allowed to perform three consecutive trials. The number of sideslips was recorded on each trial and the mean number of sideslips during a three-trial session was kept as the variable (Fleming *et al.*, 2004).

Tissue processing

At the end of the 11-week treatment period, mice were anesthetized with pentobarbital (100 mg/kg i.p) and intracardially perfused with 0.9% saline. Brains were quickly removed and cut in half between the two hemispheres. The right hemisphere was frozen directly for biochemical analysis while the left hemisphere was post-fixed for 5 days in 4% PFA, then cryoprotected in 30% sucrose in 0.1M PBS, frozen on powdered dry ice and stored at -80°C.

Immunoblotting

For western blot analysis, patches were taken from the motor cortex and striatum of PLP-SYN and WT mice. Tissue extracts were lysed in buffer containing 25mM Tris HCL (pH 6.8), 1% SDS, 250mM DTT, 7.5% glycerol and 0.05% bromophenol blue. To measure oligomeric forms of α-syn, 30 μg of protein were loaded per lane, run on 4–15% gradient gels (Bio-rad Laboratories) and transferred onto nitrocellulose membranes (Millipore). After washing in TBS, membranes were blocked for 1 hour in TBS and 0.1% Tween-20 (TBST) containing 5% milk at room temperature and subsequently incubated overnight at 4°C with human-specific antibodies Syn-211 (1:1000, Thermo Fisher Scientific) diluted in the blocking buffer. After washing with TBST, membranes were probed with corresponding secondary antibody (1:2000, Jackson laboratories), visualized with enhanced chemiluminescence and analysed using the ChemiDoc gel imaging system (BioRad). To assess truncated forms of α-syn, the same protocol was used but proteins were run on 18% SDS/PAGE gels and incubated with

human-specific antibody Syn-204 (1:500, Abcam). Proteins were normalized to actin (1:2000, Sigma), used as a loading control.

Histopathological analysis

40 µm free-floating coronal sections were collected for histopathological analysis. To assess the solubility of α-syn inclusions in oligodendrocytes, sections from PLP-SYN mice were first incubated with proteinase-K at 10 µg/ml (Sigma-Aldrich) for 10 minutes at room temperature as previously described (Fernagut *et al.*, 2007). Sections were then washed in 0.1M PBS (pH = 7.4) 3 X 10 minutes and incubated with 0.3% hydrogen peroxide for 10 minutes to block endogenous peroxidases. After washing, sample sections were processed for alpha-synuclein immunohistochemistry. Sections were first incubated with mouse on mouse blocking reagent for 1 hour (M.O.M kit, Vector laboratories). Sections were then incubated overnight at room temperature with the primary antibody against α-syn (clone LB509, invitrogen Laboratories, 1:200) diluted in M.O.M diluent (M.O.M kit, Vector laboratories). After washing in PBS, sections were incubated with the goat anti-mouse (1:250, M.O.M kit, Vector Laboratories) at room temperature for 1 hour. The avidin-biotin complex method was used to detect the secondary antibody (ABC elite kit, Vector laboratories) and the reaction product was visualized by 3,3'-diaminobenzidine tetrachloride (DAB, Sigma). Adjacent sections were processed for α-syn immunohistochemistry without proteinase-K pre-treatment.

For TH immunostaining, every fourth section was processed for tyrosine hydroxylase immunohistochemistry. The same protocol was used as the one previously mentioned for synuclein staining without proteinase-K pre-treatment but with the addition of the other groups, WT mice. After washing in PBS, sections were incubated for 1 hour with mouse on mouse blocking reagent (M.O.M kit, Vector laboratories), then incubated overnight at room temperature with the primary antibody: mouse anti TH (Millipore, 1:10000).

Quantification

The distribution of α-syn-immunopositive inclusions was assessed in the cortex and striatum of PLP-SYN mice. Regions of interest were delineated at 5x objective according to the Watson and Paxinos mouse brain atlas and quantified at 40x objective. Two adjacent sections were quantified per level and per mouse.

For SNc TH-counts, stereological sampling was performed using the Mercator Pro V6.5 software (Explora Nova) coupled to a Leica DM-6000B microscope with a motorized XYZ stage (Märzhäuser). Following delineation of the SNc at 5x objective as described previously (Fernagut *et al.*, 2007), counting was performed at 63x objective. Guard zones of 1.5 µm

ensured the exclusion of lost profiles on the top and bottom of the section sampled.

Statistical analysis

Behavioral data were analysed using a Mann-Whitney test between WT and PLP-SYN mice. Histopathological data for TH and Nissl staining were analyzed using two-way ANOVA with genotype and treatment as independent variables. ANOVAs were followed by post hoc t-tests corrected for multiple comparison by the method of Bonferroni whenever appropriate. Biochemical and histopathological analysis of α-syn inclusions between placebo and VX-765-treated PLP-SYN mice were performed using Mann-Whitney test. Statistical analyses were performed with Graphpad Prism 6.0. For all statistical tests, the level of significance was set at $p < 0.05$. All data are expressed as mean ± SEM.

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Figures

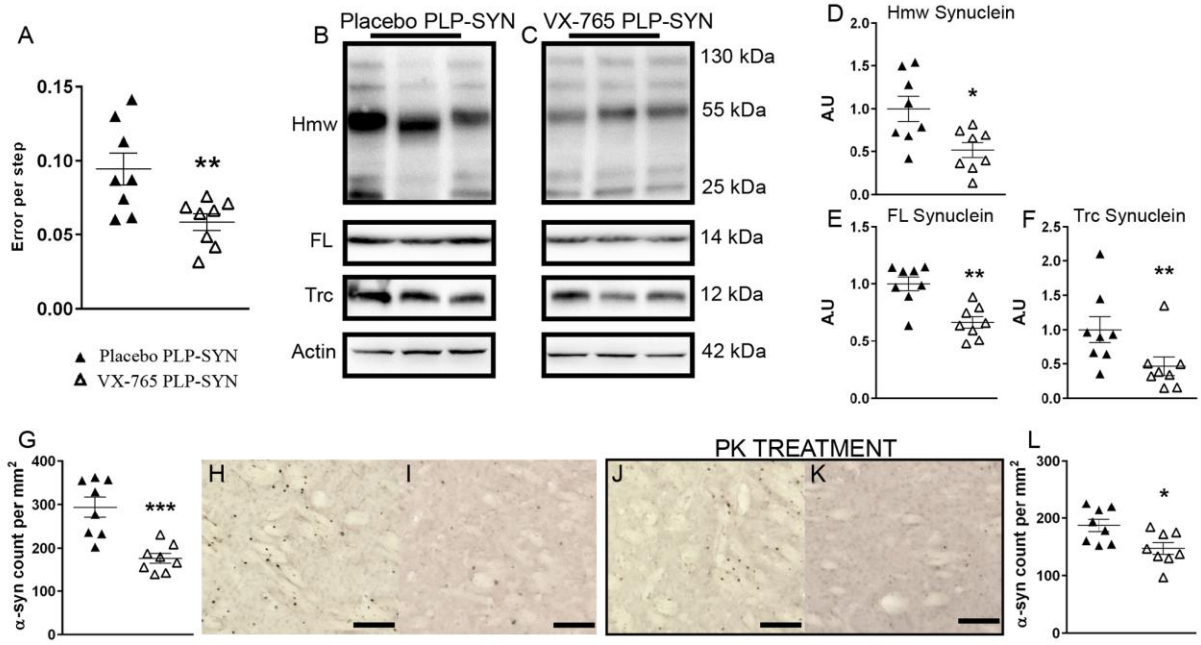

Fig. 1. VX-765 treatment reversed α-syn induced pathology in PLP-SYN mice by decreasing α-syn load in the striatum and rescuing motor performance. (*A*) Placebo treated PLP-SYN mice produced more errors per step compared to VX-765 treated PLP-SYN mice in the challenging beam test. (*B-F*) Representative immunoblot levels of oligomeric (*D*), monomeric (*E*) and truncated (*F*) α-syn in placebo (*B*) and VX-765 (*C*) treated PLP-SYN mice. (*G-L*) Immunohistochemical analysis of α-syn load (*G-I*) and insolubility (*J-L*) in the striatum of placebo (*H, J*) and VX-765 (*I, K*) treated PLP-SYN mice. In all panels, n=8 per experimental group. Error bars indicate standard error. *p<0.05, **p<0.01, ***p<0.001. FL=full length, Hmv=high molecular weight, PK=proteinase-K, Trc=truncated.

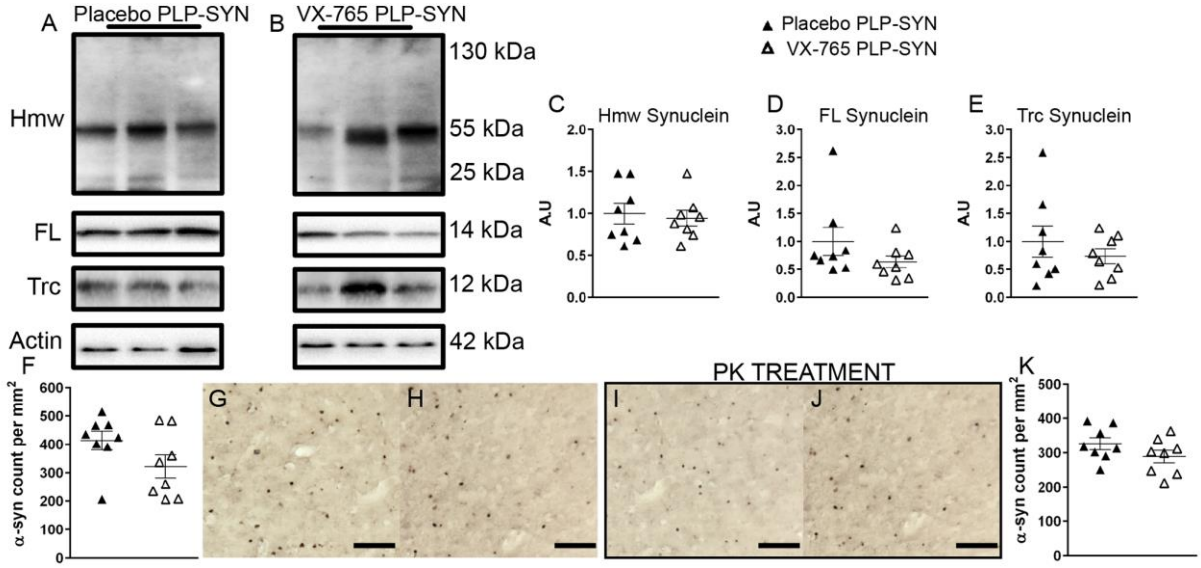

Fig. 2. (*A*-*E*) Representative immunoblot levels of oligomeric (*C*), monomeric (*D*) and truncated (*E*) α-syn in the cortex showing no significant difference between placebo (*A*) and VX-765 (*B*) treated PLP-SYN mice. (*F*-*K*) α-syn immunohistochemistry assessing the load (*F*-*H*) and insolubility of (*I*-*K*) α-syn in placebo (*G*, *I*) and VX-765 (*H*, *J*) treated PLP-SYN mice. In all panels, n=8 per experimental group. Error bars indicate standard error. FL=full length, Hmv=high molecular weight, PK=proteinase-K, Trc=truncated.

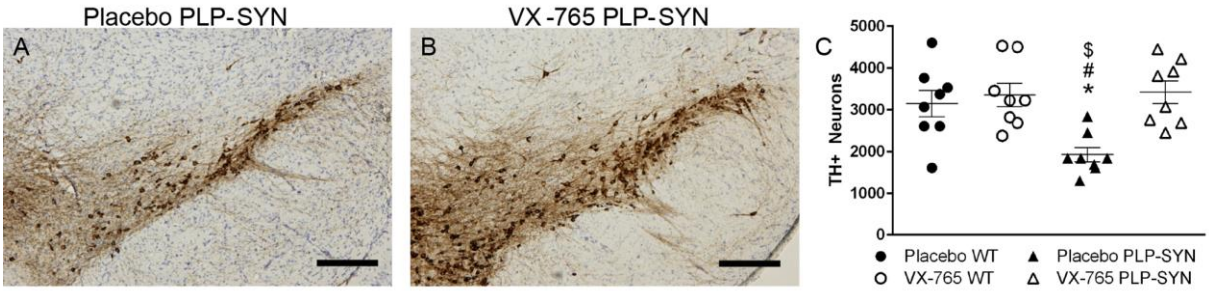

Fig. 3. PLP-SYN mice treated with VX-765 showed no loss of tyrosine hydroxylase (TH) positive neurons in the substantia nigra. (*A*, *B*) Representative nigral sections from placebo (*A*) and VX-765 (*B*) treated PLP-SYN mice. (*C*) Statistical analysis of TH+ stereological counting in the nigra showing a loss in TH+ neurons in the nigra of placebo treated PLP-SYN mice compared to VX-765 treated PLP-SYN mice, placebo and VX-765 treated WT mice. In all panels, n=8 per experimental group. Error bars indicate standard error. *$p<0.05$ Placebo PLP-SYN vs VX-765 PLP-SYN, #$p<0.05$ Placebo PLP-SYN vs VX-765 WT, \$$p<0.05$ Placebo PLP-SYN vs Placebo WT.

Article 3:

Region-specific alterations of matrix metalloproteinase activity in multiple system atrophy

Fares Bassil, Arnaud Monvoisin, Marie-Helene Canron, Anne Vital, Wassilios G. Meissner, François Tison, MD, Pierre-Olivier Fernagut*

(Movement Disorders – sous presse)

Les métalloprotéinase matricielles (MMP) sont des endopeptidases impliquées dans le remodelage de la matrice extracellulaire, la démyélinisation et la perméabilité de la barrière hémato-encéphalique. L'expression de ces enzymes peut être augmentée lors de processus inflammatoires et plusieurs études indiquent une implication des MMP dans divers processus pathologiques, tels que la sclérose en plaques, la maladie de Parkinson et la maladie d'Alzheimer. L'AMS étant caractérisée par une forte neuro-inflammation, associée à un dysfonctionnement de la barrière hémato-encéphalique et à une dégradation de la myéline, cette étude visait à évaluer les modifications potentielles de plusieurs MMP. Nous avons étudié l'expression et l'activité des MMP-1, -2, -3 et -9 dans du tissue cérébral de patients AMS par rapport à des sujets sains. L'utilisation de la zymographie et l'immunohistolochimie nous on permis de mesurer l'activité et d'évaluer la distribution de ces MMP dans le putamen et le cortex frontal, deux régions affectées différemment dans l'AMS. Nos expériences on démontré que l'expression des MMP-1, -2, -3 est augmentée dans les neurones et la glies, principalement dans le putamen des patients AMS. Par ailleurs, nous avons mis en évidence une augmentation de l'activité de la MMP-2 dans le putamen. Nous avons ensuite démontré par double immunofluorescence que MMP-1, -2, -3 sont exprimées dans les astrocytes et/ou la microglie, et que la MMP-2 est colocalisée avec l'α-synucléine dans des inclusions cytoplasmiques oligodendrogliales caractéristiques de l'AMS. L'ensemble de ces résultats indique que l'augmentation d'expression et/ou d'activité de plusieurs MMP pourraient contribuer à la physiopathologie de l'AMS en favorisant par exemple la démyélinisation et/ou la perméabilité de la barrière hémato encéphalique.

Region-specific alterations of matrix metalloproteinase activity in multiple system atrophy

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Abstract

Background: Multiple system atrophy (MSA) is a sporadic progressive neurodegenerative disorder characterized by a variable combination of parkinsonism, cerebellar ataxia and autonomic dysfunction. The pathological hallmark of MSA is the accumulation of α-synuclein aggregates in the cytoplasm of oligodendrocytes along with neuronal loss and neuroinflammation, as well as blood brain barrier dysfunction and myelin deterioration. Matrix metalloproteinases are zinc-dependent endopeptidases involved in the remodelling of the extracellular matrix, demyelination and blood brain barrier permeability. Several lines of evidence indicate a role for these enzymes in various pathological processes including stroke, multiple sclerosis, Parkinson's and Alzheimer's disease. *Methods:* This study aimed to assess potential alterations of matrix metalloproteinase-1, -2, -3 and -9 expression or activity in MSA postmortem brain tissue. *Results:* Gelatin zymography revealed increased matrix metalloproteinase-2 activity in the putamen but not in the frontal cortex of MSA patients relative to controls. Immunohistochemistry revealed increased number of glial cells positive for matrix metalloproteinase-1, -2, and -3 in the putamen and frontal cortex of MSA patients. Double immunofluorescence revealed that matrix metalloproteinase-2 and -3 were expressed in astrocytes and microglia. Only matrix metalloproteinase -2 colocalized with α-synuclein in oligodendroglial cytoplasmic inclusions. *Conclusion:* These results demonstrate widespread alterations of matrix metalloproteinases expression in MSA and a pattern of increased matrix metalloproteinase-2 expression and activity affecting preferentially a brain region severely affected (putamen) over a relatively spared one (frontal cortex). Elevated matrix metalloproteinase expression may thus contribute to the disease process in MSA by promoting blood brain barrier dysfunction and/or myelin degradation.

Introduction

Multiple system atrophy (MSA) is a fatal neurodegenerative disorder characterized by a variable combination of autonomic dysfunction, cerebellar ataxia and parkinsonism.(Jellinger and Lantos, 2010; Wenning *et al.*, 2008) Considered as a primary oligodendrogliopathy, the cytopathological hallmark of the disorder is the accumulation of α-synuclein (α-syn) protein aggregates in oligodendrocytes forming glial cytoplasmic inclusions (GCIs). (Papp *et al.*, 1989) MSA is also characterized by secondary neuronal loss and myelin alteration, (Ahmed *et al.*, 2012; Horimoto *et al.*, 2000; Matsuo *et al.*, 1998) as well as blood brain barrier (BBB) dysfunction, neuroinflammation and microglial activation, and oxidative stress.(Abdo *et al.*, 2004; Gerhard *et al.*, 2003; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Lee *et al.*, 2013; Shibata *et al.*, 2010; Song *et al.*, 2011)

As the mechanisms underlying secondary neuronal and myelin alteration in MSA are still poorly understood, several lines of evidence suggest a potential involvement of matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases known for their capacity to degrade several components of the extracellular matrix and basement membranes.(Nagase and Woessner, 1999; Yong, 2005; Yong *et al.*, 2001) In addition to their involvement in many physiological processes requiring extracellular remodelling such as cell differentiation and migration or angiogenesis, MMPs may also contribute to various pathological processes. Indeed MMPs are enriched in microglia and their increased expression upon microglial activation can possibly foster the neuroinflammatory process.(Gottschall and Yu, 1995; Gottschall *et al.*, 1995; Nuttall *et al.*, 2007) Neuroinflammation and microglial activation are also known to be main initiators of BBB dysfunction in several neurodegenerative disorders.(Bruck *et al.*, 1997; de Vries *et al.*, 1997; Gerhard *et al.*, 2003; Marques *et al.*, 2013) In addition, several MMPs can cleave α-syn in its C-terminal domain (Sung *et al.*, 2005), such proteolytic truncation enhances its toxicity and aggregation.(Levin *et al.*, 2009; Li *et al.*, 2005b) Increasing evidence indicates that MMPs are involved in the pathogenesis of several proteinopathies including Alzheimer's disease (AD) (Asahina *et al.*, 2001; Lorenzl *et al.*, 2003; Peress *et al.*, 1995), amyotrophic lateral sclerosis (ALS) (Fang *et al.*, 2009; Kiaei *et al.*, 2007; Lim *et al.*, 1996; Lorenzl *et al.*, 2006; Yushchenko *et al.*, 2000) and Parkinson's disease (PD).[34,35] Furthermore, MMPs can also degrade myelin (Chandler *et al.*, 1995; Kieseier *et al.*, 1999) as in multiple sclerosis, and in its animal model of experimental allergic encephalomyelitis.(Avolio *et al.*, 2003; Benesova *et al.*, 2009; Kieseier *et al.*, 1999; Yong *et al.*, 2001)

Considering the interplay between neuroinflammation and MMPs, their ability to cleave α-syn, and their demonstrated role in several neurodegenerative disorders, it is thus tempting to speculate that MMPs may be involved in the pathogenesis of MSA. In this present

study, we aimed to investigate alterations of MMPs in MSA brains by measuring using zymography the activity of MMP-2 and MMP-9, two well-known and characterized proteases, which have shown altered expression in neurodegenerative disorders.(Fang *et al.*, 2010; Lim *et al.*, 1996; Mroczko *et al.*, 2013; Yushchenko *et al.*, 2000) We also analysed the pattern and extent of MMP-1, MMP-2 and MMP-3 expression in controls and MSA patients by immunohistochemistry.

Materials and Methods

Patient Samples

Human brain samples were obtained from the Queen Square Brain Bank and the French national brain repository (Comité Protection des Personnes N° CEBH 2009/03; Ministère Enseignement Supérieur et Recherche: DC-2008-337). The present study was declared and approved by the ethics committee ("Comité de Protection des Personnes du Sud-Ouest et Outre Mer III") of Bordeaux University Hospital. Patient characteristics are given in **Table 1**.

Zymography

Tissues were lysed using a lysis buffer (50 mM Tris HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100) complemented with complete protease inhibitors (Roche). A constant buffer/tissue ratio of 1 ml/200 mg was used to ensure equal sample loading. Homogenized extracts were clarified by centrifugation at 12000g at 4°C for 20 min. 25 µl of sample were mixed with 13 µl of 3X loading buffer (30% glycerol, 185 mM Tris-HCl, pH6.8, 6% SDS, 0.01% bromophenol blue) and loaded on SDS-PAGE gel containing 2 mg/ml gelatin. Electrophoresis was performed at 150 V for 90 min. After electrophoresis, gels were rinsed twice with 2.5% Triton X-100 for 1 hr and incubated for 3 days in incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl2, 2 µM ZnCl2 and 0.05% Brij 35). Gelatin activity was visualized by staining gels with 0.5% Coomassie blue R250 in 4% (vol/vol) and 10% (vol/vol) acetic acid for 1 hr and destained with the same solution without Coomassie blue. Densitometric quantitation of MMP activity was performed using Scion Image software. Values were normalized to the control sample in each gel.

Histopathological analysis

Morphological details were assessed on routine hematoxylin and eosin-stained formalin fixed paraffin embedded samples. Immunohistochemical studies were processed on human formalin fixed, paraffin embedded samples with anti-MMP1 (ab52631), anti-MMP2 (ab37150) and anti-MMP3 (ab52915) (MMP-1, MMP-3: rabbit monoclonal antibodies, MMP-2: rabbit

polyclonal antibody, 1:100; Abcam, Cambridge, England). For double immunofluorescence studies, each of the anti-MMP antibodies were coupled to antibodies directed against markers of astrocytes, i.e. Glial fibrillary acidic protein (GFAP, mouse monoclonal antibody, 1:500; Millipore, France) combined with S100β (mouse monoclonal antibody, 1:1000; Abcam, Cambridge, England), microglial marker anti-Iba1 (monoclonal mouse antibody clone [B32.1], 1/1000; Abcam, Cambridge, England) and anti α-synuclein clone LB509 (monoclonal mouse antibody, 1:100; Invitrogen/life technologies, France).

Immunoperoxidase labelling

4 µm thick coronal sections were deparaffinized in toluene and rehydrated in graded series of ethanol prior to be pressure-cooked in EDTA buffer, pH 9.0 for antigen retrieval. After cooling the sections were washed and blocked with 5% normal goat serum containing 0.05% tween in PBS for 30 min at room temperature followed by overnight incubation with primary antibody. Control sections were incubated without primary antibody. Subsequently, sections were transferred in 3% H2O2 in PBS for 10 min to quench endogenous peroxidase activity and treated with a ready-to-use goat anti rabbit EnVision-HRP enzyme conjugate (Dako, Trappes, France) for 40 min. Immunoreactions were revealed using the highly sensitive diaminobenzidine plus (DAB+) (Dako, Trappes, France) as substrate chromogen. Finally sections were counterstained with Mayer's hemalum, dehydrated and mounted in Eukitt. Pictures were taken with a Leica microscope (Leica DM6000B) at x40 magnification. For densitometric measurements, the same protocol was used but no counterstaining was done.

Immunofluorescent labelling

Double immunofluorescence was performed to localize MMP-1, MMP-2, MMP-3 and α-synuclein, Iba-1 or GFAP/S100β. Following antigen retrieval with EDTA buffer and blocking with 5% normal goat serum containing 2% BSA in PBS, sections were incubated overnight at 4 °C with a mixture of the two primary antibodies. Secondary antibodies were Alexa Fluor 488 labeled goat anti-mouse for α-synuclein, GFAP/S100β, Iba-1 and Alexa Fluor 568 labeled goat anti-rabbit for MMP-1, MMP-2 and MMP-3 (both from Invitrogen SARL, Cergy Pontoise, France). Nuclei were stained using 4,6-diamidino-2-phenylindole (DAPI) (D9542, Sigma-Aldrich, St Quentin Fallavier, France).

To lower the intensity of lipofuscin auto-fluorescence, slides were incubated for 10 min in 0.1% Sudan Black B (Sigma-Aldrich, St Quentin Fallavier, France) in 70% ethanol. After thorough washing in PBS, slides were mounted in permafluor mounting medium (Thermo scientific). Immunofluorescence was visualized by using a Zeiss Axioplan 2 epifluorescent microscope at x40 and x63 magnification.

Quantitative analysis

Analysis of the number of immunopositive cells was done using a computerized image analysis system (Mercator V6.50, Explora Nova) linked to a Leica microscope type DM 6000B. Quantitative analysis was carried out on the whole structure area and results are expressed as a proportion of immunopositive cells over total cells per mm^2. For colocalization analysis, 6 images were taken randomly from each patient and image analysis was done using Image J colocalization threshold plugin. 6 images were taken randomly in the striatum and the cortex of MSA patients providing about 80 GCIs per patient to be quantified. A threshold was applied to all images in green (α-synuclein) and another was used for the red (MMP) filter to assess colocalization. For densitometric analysis of MMP-1, MMP-2, and MMP-3 in the putamen and cortex, images were scanned at x20 magnification with Hamamatsu Nanozoomer 2.0HT and quantification was performed with the offline version of a computerized image analysis system (Mercator V6.50, Explora Nova). To determine the density of staining, rectangular frames were placed on the studied structures and a staining threshold was established and used to measure all slides. Results are expressed as the surface area stained with respect to the surface area selected. To minimize the inherent variability in the immunochemical procedure, sections from all cases were processed simultaneously for a given structure.

Comparison of the distribution of MMP-1, MMP-2, and MMP-3 staining between MSA and healthy patients was performed using Student *t* test. Statistical analyses were performed with Graphpad Prism 6.0 (GraphPad, San Diego, CA, U.S.A). Data are presented as mean ± SEM. For all statistical tests, the level of significance was set at $p< 0.05$.

Results

Increased MMP-2 activity in the putamen of MSA patients

Analysis of gelatin zymograms revealed an increased MMP-2 activity in the putamen of MSA patients relative to healthy controls (+38%, $p<0.01$, **Fig. 1A, C**), without significant modification in the frontal cortex (**Fig. 1B, C)**. No difference in MMP-9 activity was found in the frontal cortex and the putamen (**Fig. 1A, B, C**). We further studied the pattern and extent of MMP-2 tissue distribution and expression in the putamen and frontal cortex of MSA patients with respect to healthy controls by immunohistochemistry. In addition, we also assessed the pattern and extent of MMP-1 and MMP-3 expression.

Densitometry

Densitometric analyses showed that the overall levels of MMP-1 and MMP-2 but not MMP-3 expression (including its extracellular distribution) were significantly increased in the putamen of MSA patients compared to healthy controls (MMP-1: +54%, $p<0.05$, **Fig. 1D, E**;

MMP-2: +87%, p<0.01, **Fig. 1G, H**). In the frontal cortex, MMP-1 and MMP-3 expression levels were significantly increased compared to controls (MMP-1: +67%, p<0.01, **Fig. 1D, F**; MMP-3: +88%, p<0.01, **Fig. 1J, L**). No difference in MMP-2 expression levels was found in the frontal cortex of MSA patients compared to controls (p=0.3, **Fig. 1I)**. This result is consistent with increased MMP-2 activity and extends the results of zymography by showing increased MMP-2 expression in the putamen of MSA patients.

MMP tissue distribution

Histopathological analysis – Neuronal loss in MSA

Using hematoxylin and eosin staining, we quantified the number of neurons in putamen and cortex of control and MSA patients. As expected, there was significant neuronal loss in the putamen (76%, p<0.001, controls: 33.15 ± 7.69, MSA: 8.05 ± 0.98 neurons/mm^2) and to a lesser extent in frontal cortex (40%, p<0.05, controls: 35.16 ± 4.10, MSA: 20.68 ± 3.31 neurons/mm^2) of MSA patients with respect to healthy controls. Neurons of control cases were lightly stained for MMP-1, MMP-2 and MMP-3, while remaining putaminal neurons in MSA showed a marked staining (**Fig. 2A, H, O**). A significant increase in the proportion of MMP-1 and MMP-3 but not MMP-2 positive neurons was found in the putamen of MSA patients compared to controls (MMP-1 +100%, p<0.01; MMP-3 +69%, p<0.01, **Fig. 2A, B, H, I, O, P**). In the frontal cortex, the proportion of MMP-1 and MMP-3, but not MMP-2 positive neurons was significantly increased in MSA patients compared to healthy controls (MMP-1: +90%, p<0.0001, MMP-3: +69%, p<0.01, **Fig. 2C, J, Q**).

MMP positive glial cells are increased in MSA

MMP-1, -2 and -3 immunostaining was increased in the putamen and frontal cortex of MSA patients with respect to healthy controls (**Fig. 2**). Minimal MMP-1, MMP-2 and MMP-3 immunostaining intensity was found in glia of healthy controls while marked immunoreactivity was detected in MSA (**Fig. 2D, K, N, R**).

A significant increase in the proportion of glial cells immunopositive for MMP-1, MMP-2, and MMP-3 was found in the putamen of MSA patients (MMP-1: +94%, p<0.05; MMP-2: +80.5%, p<0.01; MMP-3: +67.3%, p<0.01, **Fig. 2D, E, K, L, N, R, S**). Interestingly, immunopositive glial cells had a star shaped morphology or a cone/hat like structure, a similar morphology to astrocytes, microglia and GCIs (**Fig. 2D, K, N, R**). In addition, MMP-2 positive cells were also found in white matter bundles of MSA patients **(Fig. 2N)** but not in controls. Similarly, in the frontal cortex, MMP-1, MMP-2 and MMP-3 positive glial cell proportions were significantly increased in MSA patients relative to healthy controls (MMP-1: +93%, p<0.001; MMP-2: +46%, p<0.05; MMP-3: +78%, p<0.01, **Fig. 2F, M, T**).

Expression of MMP-1, MMP-2 and MMP-3 in astrocytes and microglia in MSA

To further investigate the glial origin of MMP, sections from the putamen of MSA patients and controls were immunostained for MMP-1, MMP-2 or MMP-3, coupled to GFAP or Iba-1. Consistent with immunohistochemical results, MMP immunofluorescence was lower in controls compared to MSA patients (data not shown). Neuroinflammation was evident in MSA patients as shown by marked GFAP and Iba-1 immunostaining **(Fig. 3)**. MMPs were found in microglia and astrocytes in both controls (data not shown) and MSA patients (**Fig. 3A-F**). Specifically, in MSA patients, increased GFAP (**Fig. 3A, B, C**) and Iba-1 (**Fig. 3D, E, F**) immunostaining was accompanied by an increase in MMP-2 and MMP-3 staining in microglia and astrocytes (**Fig. 3B, C, E, F**), while MMP-1 immunostaining was increased only in astrocytes (**Fig. 3A**).

MMP-2 is present in GCIs

Sections from the putamen and frontal cortex of MSA patients and healthy controls were immunostained for MMP-1, MMP-2 or MMP-3, and α-syn (**Fig. 4**). Interestingly, double immunofluorescence demonstrated that only MMP-2 colocalized with α-syn in GCIs (**Fig. 4A, B, C**). Quantification revealed that MMP-2 is present in 100% of α-syn-positive GCIs.

Discussion

In the present study, MMP-2 activity, tissue distribution and expression of MMP-1, MMP-2, and MMP-3, as respectively assessed by zymography and immunohistochemistry, were significantly altered in MSA patients. MSA patients showed increased MMP-1 and MMP-2 in the putamen, and increased MMP-1 and MMP-3 in the frontal cortex. MMP-3 and MMP-2 also showed a trend to an increase in the putamen and cortex respectively. Moreover, increased MMP-1 and MMP-2 in the putamen suggest a disease-specific pattern of alterations in MSA since studies in PD and ALS showed reduced MMP-2 activity while MMP-1 levels were unaffected.(Lim *et al.*, 1996; Lorenzl *et al.*, 2002) Finally, we show that MMP-2 was present in GCIs, unlike MMP-1 and MMP-3. MMP-2 may be a key component for oligodendroglial dysfunction and myelin sheath breakdown in MSA.[4,39,42-44]

Immunohistochemistry allowed characterizing and localizing the source of this increase, which mainly comes from non-neuronal cells in MSA patient brains, presumably astrocytes and microglia, as well as cells present in white matter bundles (i.e. oligodendrocytes). However, surviving neurons in MSA also showed increased staining intensity for MMP-1, MMP-2 and MMP-3 compared to a faint staining in healthy patients.

Increased non-neuronal staining for MMP-1, MMP-2 and MMP-3 prompted us to further investigate their expression in glial cells. MMP-2 and MMP-3 were largely found colocalized with the astrocytic marker GFAP and the microglial marker Iba-1, a finding consistent with previous studies showing their expression in these cell types.[34,45-47] Interestingly, immunofluorescence imaging also showed that only MMP-2 co-localized with α-syn in GCIs. In addition, extracellular MMP-1 and MMP-2 immunoreactivities were markedly increased in MSA putamen compared to controls, suggesting an increased secretion from producing cells. As of late, increasing evidence shows that MMPs are involved in the clearance of aggregation-prone proteins such as beta-amyloid.(Liao and Van Nostrand, 2010; Roher *et al.*, 1994; Yin *et al.*, 2006) In addition, studies have focused on the relation and implication of MMPs in amyloid beta (Aβ) aggregation in AD (Mroczko *et al.*, 2013; Ou-Yang and Van Nostrand, 2013) and that Aβ is a potent stimulant of MMP production.(Deb and Gottschall, 1996) Moreover, membrane type 1-MMP (MT1-MMP), a physiological activator of MMP-2 can degrade Aβ in vitro and in-situ, while also being expressed in astrocytes around Aβ rich regions in a mouse model of AD.(Li *et al.*, 2011; Liao and Van Nostrand, 2010) Interestingly, α-syn has been shown to induce MT1-MMP expression in vitro and in a PD mouse model.(Kim *et al.*, 2009a) Several MMPs including MMP-1, MMP-2 and MMP-3 have also been demonstrated to mediate C-terminal cleavage of α-syn.(Sung *et al.*, 2005) Although MMP-3 has already been found to co-localize with α-syn in Lewy bodies in PD (Choi *et al.*, 2011), to our knowledge no study has assessed the presence of MMP in GCIs. Here, the presence of MMP-2 in all α-syn-positive GCIs suggests that increased MMP-2 activity could participate to inclusion formation by increasing α-syn truncation, therefore promoting its aggregation on the long haul.

The mechanisms underlying increased MMP-2 activity and increased MMP-1, MMP-2 and MMP-3 expression in MSA patients remain unknown, yet MMPs have been found to be activated by cytokines, reactive oxygen species, nitric oxide and other neuroinflammatory triggers; factors that are commonly increased in MSA.(Abdo *et al.*, 2004; Gerhard *et al.*, 2003; Ishizawa *et al.*, 2004; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Shibata *et al.*, 2010) MMP-2, and to a lesser extent MMP-3 and MMP-1 are expressed in neutrophils, macrophages and resident glia of the brain. [17,18,51,56] Interestingly, type IV collagen, the main substrate of MMP-2 and MMP-9, is decreased in MSA patients.(Miller *et al.*, 2007) Our results thus suggest that the loss of type IV collagen might be caused by the increased MMP-2 activity.

Alterations in MMP-9 expression have been previously found in neurodegenerative disorders such as an increase in MMP-9 activity in AD (Lorenzl *et al.*, 2003) and ALS patients (Lim *et al.*, 1996) whereas in PD no significant difference was found.(Lorenzl *et al.*,

2002) Similarly in this study, we found no significant alteration of MMP-9 activity in MSA, suggesting that MMP-9 may not be altered in synucleinopathies.

MMPs have been shown to degrade the BBB and release proinflammatory cytokines, and are believed to contribute to neurodegenerative conditions such as stroke and traumatic brain injury.(Dev *et al.*, 2010; Grossetete *et al.*, 2009; Kieseier *et al.*, 2006; Shubayev *et al.*, 2006; Truettner *et al.*, 2005) Even though BBB weakness in MSA is evident and correlates with disease severity and progression (Lee *et al.*, 2013; Song *et al.*, 2011), the potential implication of MMPs in BBB dysfunction in MSA will deserve further investigations.

MMPs are important regulators of synaptic plasticity, axon outgrowth and myelin turnover (Agrawal *et al.*, 2008) yet are involved in the pathophysiology of several neurodegenerative disorders. Here, the increased MMPs expression and activity occurring preferentially in a brain region severely damaged in MSA such as the putamen suggests that MMPs may contribute to the degenerative process of MSA. Although MMPs may have both beneficial and detrimental effects depending on the phase of a given disorder (Yong, 2005) in the pathological context of MSA, the loss of myelin and type IV collagen, together with prominent neuroinflammation and BBB dysfunction all suggest that MMP-1, MMP-2 and MMP-3 may play detrimental roles. An urgent need for disease modifying therapies is needed in MSA as its etiologic cause is still unknown and the only existing symptomatic treatments provide only little or temporary benefit (Flabeau *et al.*, 2010). Even though inhibition of MMPs is suggested as an early and brief therapeutic option in acute degenerative conditions such as stroke or spinal cord injury, (Yong, 2005) a better understanding of their involvement in chronic neurodegenerative disorders is mandatory before considering modulating MMPs activity as a future target for disease modification.

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Table and figures

Patient		Type	Age	Sex	Postmortem Delay	Disease Duration
P1	P78/06	Control	78	M	<48h	-
P2	P17/07	Control	38	M	<48h	-
P3	P45/04	Control	78	F	<24h	-
P4	P94/05	Control	71	M	<24h	-
P5	P15/05	Control	57	M	<48h	-
P6	P23/07	Control	76	M	<24h	-
P7	N13-199	Control	23	M	48	-
P8	N13-212	Control	54	F	48	-
P9	N13-54	Control	35	M	48	-
P10	P24/00	MSA-P	64	M	<48h	9
P11	P06/99	MSA-P	75	M	13	7
P12	P33/99	MSA-P	57	F	20	4
P13	P67/00	MSA-P	60	M	<48h	5
P14	P70/06	MSA-P	50	M	<24h	7
P15	P68/00	MSA-P	54	M	<24h	5
P16	N10-569	MSA-C	73	F	24	3
P17	N10-794	MSA-P	57	F	24	2
P18	N11-982	MSA-P	57	F	7	7
P19	N11-441	MSA-C	59	M	24	8
P20	N10-1157	MSA-P	83	F	12	6
P21	N07-1159	MSA-P	71	F	24	3
P22	N02-99	MSA-P	72	M	48	6

Table 1: Demographic and neuropathological characteristics of cases used in this study.

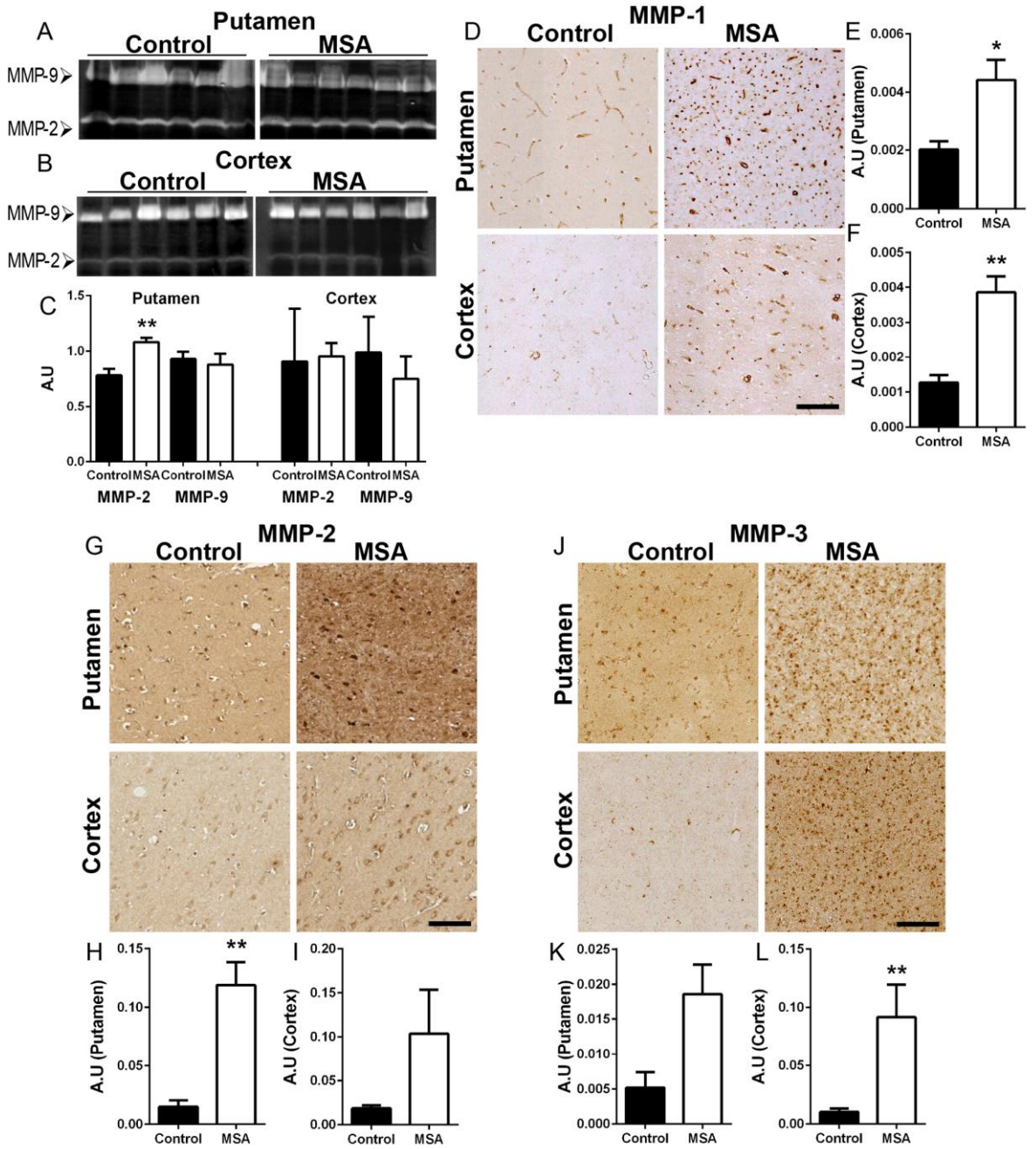

Figure 1: Gelatin zymography showing MMP-9 and MMP-2 enzymatic activity in the putamen (**A**) and the cortex (**B**). A significant increase in MMP-2 activity is shown in the putamen (**A, C**) **, p<0.01 (A.U: arbitrary units). Representative images of MMP-1, MMP-2, and MMP-3 immunostaining from control and MSA putamen and frontal cortex (**D, G, J**). Significant increase in MMP-1 **(D, E)** and MMP-2 **(G, H)** but not MMP-3 **(J, K)** immunoreactivity in the putamen of MSA patients. MMP-1 **(D, F)** and MMP-3 **(J, L)** immunoreactivity is significantly increased in MSA patients while no significant difference in MMP-2 immunoreactivity was found in the cortex (**G**, **I**) * p<0.05; ** p<0.01. Scale bar = 50 µm (A.U: arbitrary units).

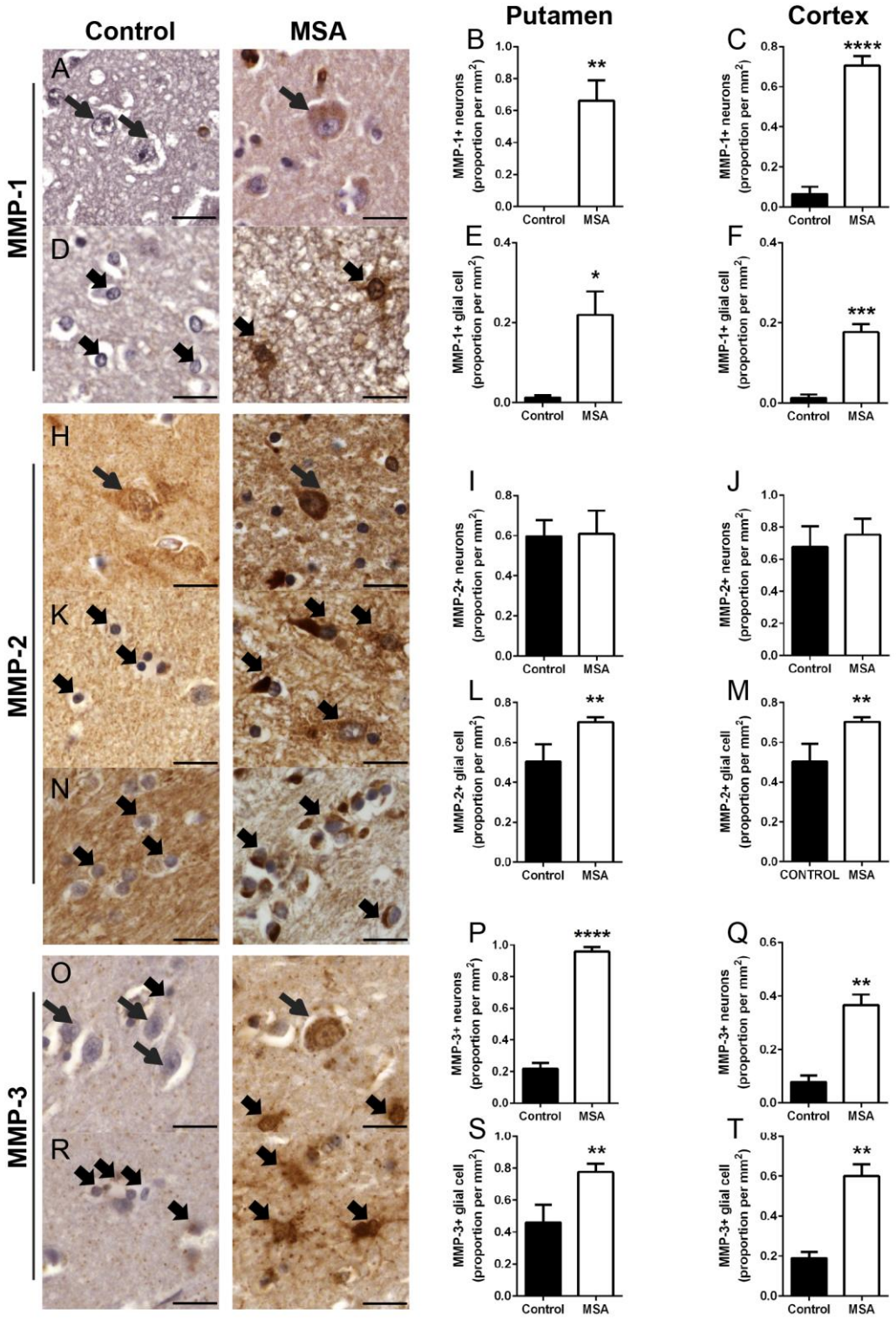

Figure 2: Representative images of MMP-1, MMP-2, and MMP-3 immunostaining in the putamen of MSA and controls (**A, D, H, K, N, O, R**). The proportion of MMP-1 and MMP-3 positive neurons and glial cells is significantly increased in the putamen (**B, E, P, S**) and cortex (**C, F, Q, T**) of MSA patients. No significant difference in the proportion of MMP-2 positive neurons was found (**I, J**), while the proportion of MMP-2 positive glial cells was increased in the putamen and frontal cortex of MSA patients (**L, M**). * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$. Short condensed arrow: glia, long thin arrow: Neurons. Scale bar = 20 μm .

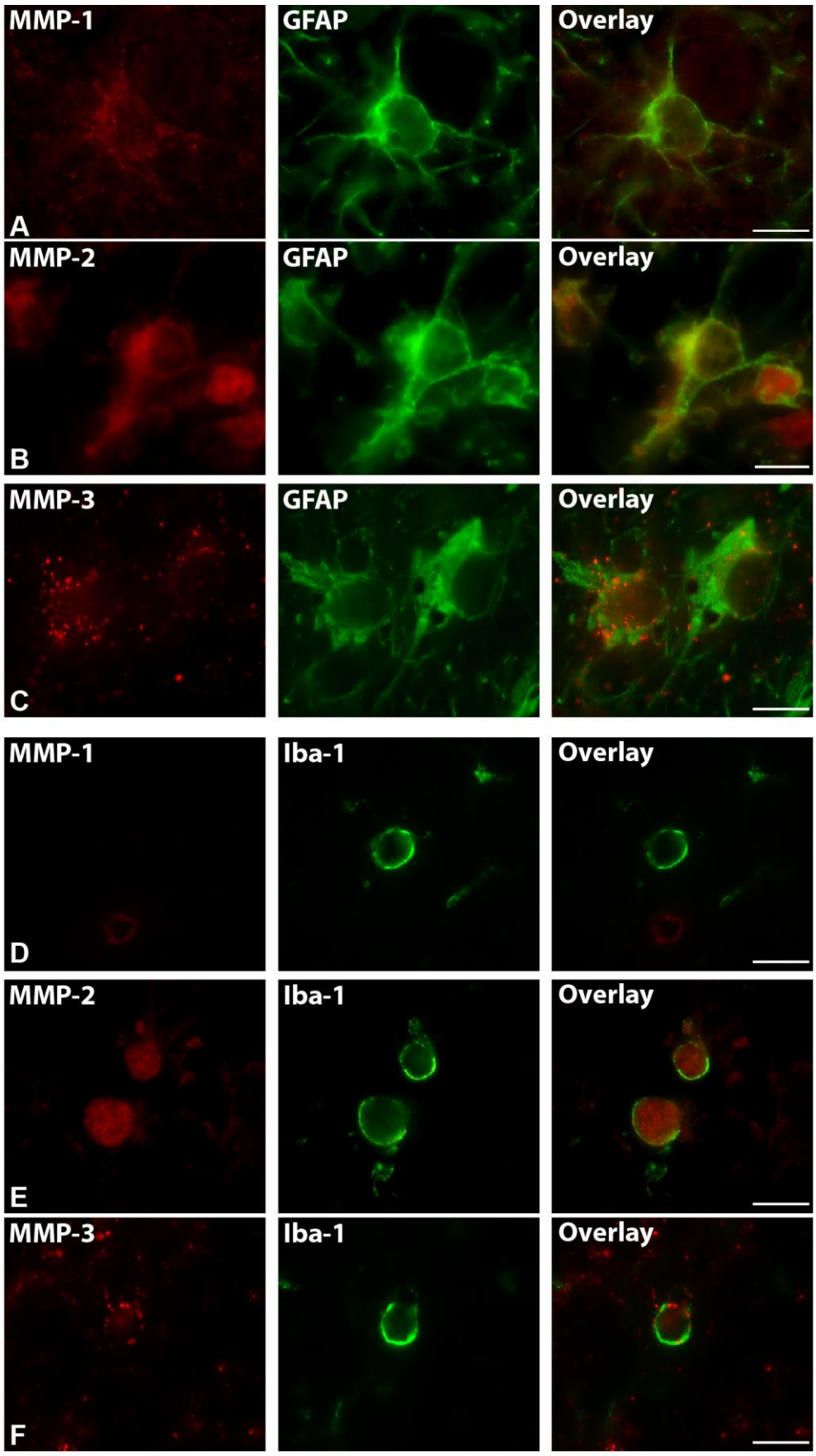

Figure 3: Double immunofluorescence from the putamen of MSA patients showing MMP-1, MMP-2, and MMP-3 in astrocytes (**A, B, C)** as well as MMP-2 and MMP-3 in microglia (**D, E, F**). Microglial cells show no staining for MMP-1 (**D**) Scale bar = 10 µm.

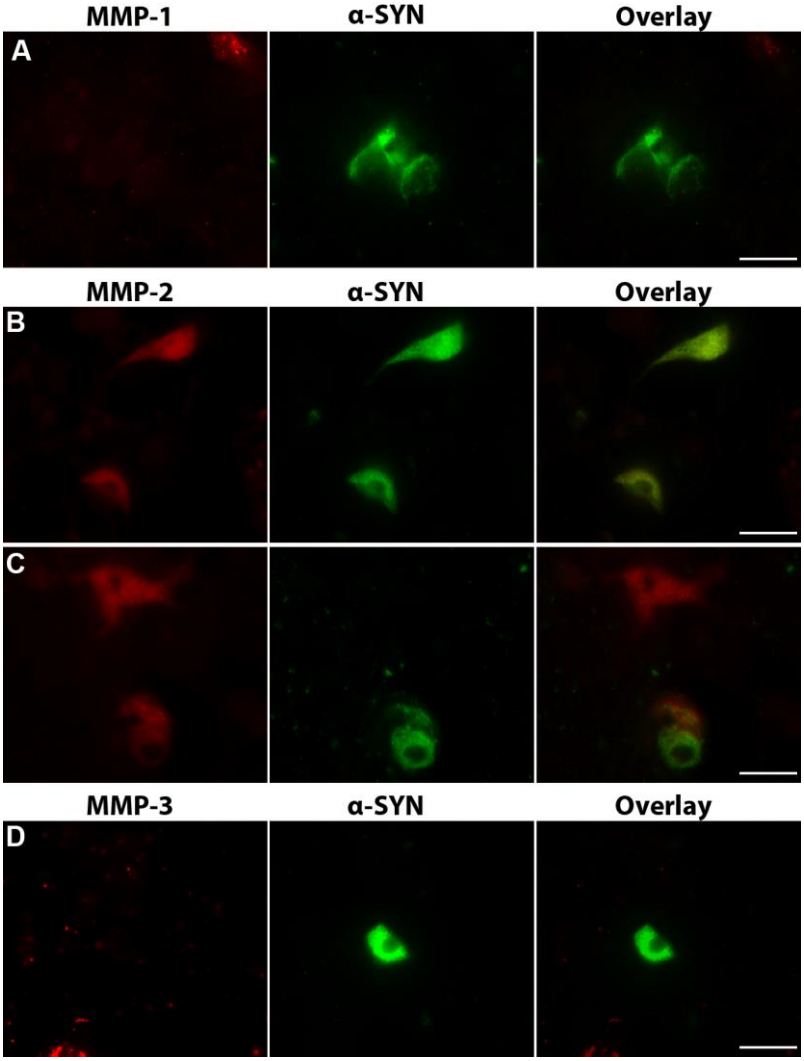

Figure 4: Double immunofluorescence from MSA patient brains showing the absence of MMP-1 and MMP-3 staining in GCIs (**A, D**) compared to MMP-2 positive staining in GCIs (**B, C**). Scale bar = 10 µm.

Discussion

V- Discussion

This work is part of a translational approach targeting synucleinopathies with an emphasis on MSA. One main objective of this approach was to assess insulin resistance in the brain of preclinical animal models and postmortem human brain tissue of MSA and PD patients in view of developing therapeutic strategies. In line with this translational approach, we also investigated the effect of reducing α-syn C-terminal truncation in transgenic mice of MSA as well as MMP activity and distribution in the brain of MSA patients to determine whether they are implicated in the disease pathology.

1. General result statement

The first part of this work aimed at characterizing insulin signalling and insulin resistance in the brain of MSA and PD patients. We here show that insulin resistance is present in different cell types in the putamen of MSA and PD patients. Specifically, the expression of the insulin resistance marker insulin receptor substrate-1 (IRS-1pS312) was increased in neurons of PD and MSA patients compared to healthy controls. We also report that microglia and oligodendrocytes show altered insulin/IGF-1 signalling. Similar to neurons, oligodendrocytes are insulin resistant in MSA patients as indicated by the increase in IRS-1pS312. Finally, our results confirm the absence of a significant loss of oligodendrocytes in the putamen of MSA patients.

α-syn is the main component of GCIs and LBs in MSA and PD. Therefore, we developed an approach aimed at limiting α-syn aggregation and α-syn induced cell death by reducing α-syn truncation. As previously mentioned (section I.3.a), α-syn truncation is hypothesized to be a promoter/enhancer of α-syn oligomerization and aggregation. PLP-SYN mice were treated over a period of 11 weeks with placebo or the caspase-1 inhibitor VX-765 an, the latter was recently shown by our collaborators to prevent cleavage of α-syn at its C-terminal. We here show that VX-765 treatment in PLP-SYN mice prevented motor deficits, limited α-syn truncation and decreased the amount of monomeric and oligomeric α-syn. VX-765 was also able to limit the progressive toxicity of α-syn by reducing its load in the striatum of PLP-SYN mice. Finally, VX-765 showed neuroprotective effects by preserving TH positive neurons in the SN of PLP-SYN mice. In conclusion, our results suggest that VX-765, a drug that was well tolerated in a six-week-long phase 2 trial in patients with epilepsy, is a promising candidate to achieve disease modification in synucleinopathies by limiting α-syn accumulation.

Studies have implicated altered MMP levels and functions in α-syn C-terminal truncation, BBB dysfunction, neuroinflammation and myelin alterations in several neurodegenerative disorders. This prompted us to characterize MMP expression in the brain of MSA patients. Our work allowed us to assess the activity, distribution pattern and localization of MMP-1, MMP-2, MMP-3 and MMP-9 in two distinct structures, the putamen and the frontal cortex of MSA patients. The results of this study showed a distinct pattern of expression and activity of MMPs that differentiates MSA from other neurodegenerative disorders such as AD, PD and ALS. Not only did we show increased MMP-1, MMP-2, MMP-3 distribution in different cells of the putamen and cortex, but we were also able to localize the source of this increase in different subtypes of glial cells. More importantly, we showed for the first time the presence of MMP-2 in GCIs of MSA patients and confirmed the absence of MMP-3 in GCIs unlike their presence in PD LBs.

Altogether, the results point to altered mechanisms such as insulin/IGF-1 signalling, MMPs, C-terminal α-syn truncation and subsequent neurodegeneration in MSA and PD while providing further support on the difference between MSA and PD pathogenesis. Our results also point to novel therapeutic approaches using drugs that are validated in clinical trials as safe and tolerable inviting the use of these drugs in synucleinopathies.

2. Insulin/IGF-1 signalling in MSA and PD

Accumulating evidence point to altered insulin/IGF-1 signalling and brain insulin resistance in synucleinopathies and AD (Aviles-Olmos *et al.*, 2013b; Bomfim *et al.*, 2012; Moloney *et al.*, 2010; Talbot *et al.*, 2012; Tong *et al.*, 2009). Here we demonstrate altered insulin signalling in synucleinopathies resulting in insulin resistance in different cell types in the putamen of PD and MSA patients. Specifically, expression of the insulin resistance marker IRS-1pS312 was increased in neurons of PD and MSA patients compared to healthy controls. Moreover, in MSA patients, increased IRS-1pS312 staining intensity was also present in oligodendrocytes, while microglia showed decreased insulin resistance.

a) Insulin signalling in PD and MSA neurons

Even though local production of insulin/IGF-1 exists in the brain, peripheral Insulin/IGF-1 remains the primary source in the brain (Jafferali *et al.*, 2000; Schechter *et al.*, 1990; Schechter *et al.*, 1992; Suh *et al.*, 2013; Torres-Aleman, 2010). Several cohort studies have reported increased serum IGF-1 levels in PD patients compared to controls, while IGF-1 serum levels are increased in MSA patients compared to PD and controls (Godau *et al.*, 2010; Godau *et al.*, 2011; Numao *et al.*, 2013; Pellecchia *et al.*, 2010; Picillo *et al.*, 2013a).

Similarly, several studies have reported altered central and peripheral insulin/IGF-1 levels in AD compared to controls (Johansson *et al.*, 2013; Salehi *et al.*, 2008; Tham *et al.*, 1993; Vardy *et al.*, 2007). It remains to be understood how these results reflect and contribute to altered brain metabolism or compensatory mechanisms.

We here assessed insulin resistance in different subsets of cells in the putamen of controls, as well as in PD and MSA patients. We show that IRS-1 serine phosphorylation in PD patients was intermediate between MSA and controls corresponding to the extent of alteration in the putamen. As a result of neuronal loss (Salvesen *et al.*, 2015; Sato *et al.*, 2007), MSA patients had lower IRS-1pS312/S616 positive neuronal counts compared to PD and healthy patients, while neurons in PD and surviving neurons in MSA showed increased IRS-1pS312 staining intensity. MSA neurons also showed a trend to increased IRS-pS616 staining intensity compared to PD and controls.

Insulin/IGF-1 signalling plays a pivotal role in neuronal function and survival in the brain since it modulates the activity of several prosurvival or proapoptotic effectors such as FoxO, GSK-3β, caspases and Bcl-2 (Bassil *et al.*, 2014). Insulin/IGF-1 signalling is a repressor of FoxO activity in the brain, while increased FoxO activity has been linked to apoptosis through activation of FasL promoter and Bim (Barthelemy *et al.*, 2004; Dijkers *et al.*, 2000; Matsuzaki *et al.*, 2003). Moreover, insulin/IGF-1 signalling is essential for axonal growth, regeneration and protein synthesis through the activation of mTOR and inhibition of GSK-3β (Delcommenne *et al.*, 1998; Dupraz *et al.*, 2013; Leibinger *et al.*, 2012; Yang *et al.*, 2011).

Insulin resistance is the inability of cells to use or bind insulin/IGF-1 efficiently, which in turn leads to decreased signalling and modulation of downstream targets. Insulin resistance in neurons of MSA and PD patients may contribute to neuronal dysfunction by decreasing the activity of prosurvival activity effectors such as Bcl-2 and mTOR and gene expression in neurons via decreased CREB activity (Chen *et al.*, 2009; Chu *et al.*, 2009; Dehay *et al.*, 2010; Golpich *et al.*, 2015; Kawamoto *et al.*, 2014; Kragh *et al.*, 2013; Levy *et al.*, 2009; Nakamura *et al.*, 2001; Nakamura *et al.*, 1998). Moreover, altered insulin/IGF-1 signalling in MSA and PD patients may lead to decreased repression of proapoptotic effectors such FoxO and caspases leading to cell death. Our results indicate that despite severe neuronal loss in MSA, surviving neurons show similar levels of insulin resistance compared to PD neurons indicating that insulin resistance might not be the primary factor for neuronal death. This would suggest that other contributing sources of insulin resistance could account for the differential neuronal vulnerability observed between PD and MSA. We here also noted a trend to increased IRS-

1pS616 staining intensity in MSA neurons compared to PD and controls. This trend might reflect different regulatory mechanisms that are altered in MSA with respect to PD and controls.

Insulin resistance as assessed by phosphorylation of IRS-1 on serine residues 312, 616 and 636 is increased in the hippocampus in preclinical models of AD and postmortem brain tissue of AD patients (Bomfim *et al.*, 2012; Moloney *et al.*, 2010; Talbot *et al.*, 2012). Yarchoan *et al.* (2014) reported an increased area with IRS-1pS616 staining in the hippocampus and midfrontal gyrus cortex of AD and tauopathies compared to synucleinopathies and controls. Together with the findings of Yarchoan et al., our results suggest that synucleinopathies and tauopathies have distinct, disease-specific regional patterns of insulin resistance.

b) Insulin/IGF-1 signalling in glial cells

Neuronal loss was accompanied by an increased number of microglia and astrocytes in the putamen of MSA as previously described (Gerhard *et al.*, 2003; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Salvesen *et al.*, 2015; Shibata *et al.*, 2010).

To our knowledge, this is the first study assessing insulin resistance in glial cells. Studies have shown that insulin/IGF-1 signalling is required for astrocyte anti-oxidative function, proliferation, glutamate transporter expression, glycogen levels and neuroprotection by decreasing oxidative stress (Genis *et al.*, 2014; Heni *et al.*, 2011; Muhic *et al.*, 2015). Interestingly, the main energy source in the brain, glycogen, is almost exclusively found and regulated by insulin/IGF-1 signalling in astrocytes (Brown and Ransom, 2007; Muhic *et al.*, 2015). Moreover, activated astrocytes produce less IGF-1 compared to naïve astrocytes, which might also contribute to decreased IGF-1 availability in the brain (Muhic *et al.*, 2015). In this study, increased IRS-1pS312 and IRS-1pS616 cell counts were partly due to an increased number of astrocytes that stained positive for both markers in the putamen of MSA patients. However, our data did not reveal significant differences in astrocytic insulin resistance between groups.

The number of microglial cells that stained for IRS-1pS312 and IRS-1pS616 were increased in the putamen of MSA patients compared to healthy controls and PD patients. Moreover, microglial cells in MSA patients showed decreased IRS-1pS312 staining intensity compared to PD and healthy controls. Microglia are implicated in the production of neuronal growth factors such as IGF-1 (Beilharz *et al.*, 1998; Butovsky *et al.*, 2006; Suh *et al.*, 2013). Interestingly, the PI3-K/Akt pathway has been implicated in the inflammatory response of

microglia in inflammatory conditions (Saponaro *et al.*, 2012). More specifically, the activation of the PI3-K/Akt pathway mediates the transition of microglia from a pro-inflammatory role to an anti-inflammatory (Tarassishin *et al.*, 2011). Hence, decreased IRS-1pS312 in microglia of MSA patients suggest that microglial cells in MSA might be in an anti-inflammatory state in late stages of the disease.

Oligodendrocyte cell counts were unchanged in the putamen of MSA patients, while oligodendrocytes of MSA patients were insulin resistant compared to PD and healthy controls. Widespread myelin degeneration in MSA is believed to be due to loss of function in oligodendrocytes with consecutive demyelination instead of oligodendrocyte cell death (Ahmed *et al.*, 2013; Song *et al.*, 2007; Wenning *et al.*, 2008; Yoshida, 2007). As mentioned in the introduction, oligodendrocytes are of particular importance in MSA since they host GCIs due to the accumulation and formation of α-syn aggregates in their cytosol (Papp *et al.*, 1989; Spillantini *et al.*, 1998b; Wakabayashi and Takahashi, 2006).

Whether insulin resistance precedes the aggregation of α-syn or is a result of α-syn inclusions in oligodendrocytes remains an unanswered question. Indeed, α-syn aggregates in oligodendrocytes impair oligodendrocyte functioning (Ettle *et al.*, 2014; May *et al.*, 2014). *In vitro* studies support the second hypothesis since transient overexpression of α-syn in human neuroblastoma cells alters insulin/IGF-1 signalling and induces insulin resistance via the activation of Src that inhibits protein phophastase 2A activity, by phosphorylating it at Y307, and mTORC1-mediated phosphorylation of IRS-1 on serine residues (Boura-Halfon and Zick, 2009; Gao *et al.*, 2015; Harrington *et al.*, 2005; White, 2006; Yang *et al.*, 2013; Zick, 2005).

Insulin/IGF-1 signalling plays a prominent role in oligodendrocyte survival, proliferation, differentiation and functioning (Carson *et al.*, 1993; Chesik *et al.*, 2007; De Paula *et al.*, 2014; Goddard *et al.*, 1999; Zeger *et al.*, 2007). Studies have shown that insulin/IGF-1 signalling acts as a myelin proliferation and maturation factor in several demyelinating disorders (Liu *et al.*, 1995; Mason *et al.*, 2000; Yao *et al.*, 1995), while myelin loss, fragmentation and alteration occurs in MSA (Ishizawa *et al.*, 2008; Matsuo *et al.*, 1998; Papp *et al.*, 1989; Papp and Lantos, 1994; Song *et al.*, 2007). In this line, it has been shown that mRNA and protein levels of MBP, a main constituent of myelin, are decreased in the brain of MSA patients pointing to a possible decrease in MBP synthesis (Salvesen *et al.*, 2015; Song *et al.*, 2007). Interestingly, IGF-1 has been shown to play a pivotal role in myelin synthesis by increasing MBP, PLP and CNPase mRNA levels, all known to be important and pivotal for myelin formation (Mozell and McMorris, 1991; Yao *et al.*, 1995, 1996). MSA patients also exhibit decreased levels of myelin associated lipids that are main constituents of

myelin sheath and are implicated in myelin stability (Don *et al.*, 2014; O'Brien and Sampson, 1965) while IGF-1 has been shown to stimulate *de-novo* fatty acid biosynthesis via PI3-K/Akt activation (Liang *et al.*, 2007).

Oligodendrocytes have several roles in supporting neuronal functioning via the expression of neurotrophic factors such as glial derived neurotrophic factor (GDNF) and IGF-1 (Dai *et al.*, 2003; Du and Dreyfus, 2002; Wilkins *et al.*, 2001; Wilkins *et al.*, 2003). Early oligodendroglial dysfunction may include altered insulin/IGF-1 signalling and insulin resistance contributing to abnormal oligodendrocyte functioning and myelin pallor. As a result, oligodendroglial trophic support to neurons may also be compromised in MSA and contribute to degeneration of neurons that also show insulin resistance. Interestingly, GDNF has been shown to be decreased in the brain of MSA patients (Ubhi *et al.*, 2011). Decreased insulin/IGF-1 signalling in oligodendrocytes may also contribute to the impaired maturation of oligodendrocytes progenitors occurring in MSA (May *et al.*, 2014). Indeed, IGF-1 and PI3-Kinase/Akt activation promote the differentiation of oligodendrocyte progenitors and myelinisation (De Paula *et al.*, 2014).

We here show that abnormal IRS-1 phosphorylation at serine residues is present in PD and MSA neurons and also in MSA oligodendrocytes. These results implicate insulin resistance in abnormal neuron and glial cell functioning in the putamen of MSA patients. Abnormal insulin/IGF-1 signalling in oligodendrocytes may be involved in impaired oligodendrocyte maturation and functioning, thus promoting secondary neurodegeneration occurring in the putamen of MSA patients.

3. Targeting insulin/IGF-1 as disease modification strategies

Administration of insulin and/or IGF-1 can mitigate disease severity in preclinical models of AD. *In vitro* studies have also shown beneficial effects of IGF-1 on α-syn or dopamine-induced toxicity and 1-methyl-4-phenylpyridinium ion exposure, an active metabolite of MPTP, while IGF-1 rescued dopaminergic neurons from programmed cell death (Kao, 2009; Offen *et al.*, 2001; Sun *et al.*, 2010; Zawada *et al.*, 1996). Similarly IGF-1 administration to *in-vivo* models of PD prevented the loss of TH-positive neurons in the SN and reversed motor behavior abnormalities (Ebert *et al.*, 2008; Guan *et al.*, 2000; Krishnamurthi *et al.*, 2004; Quesada *et al.*, 2008). Nevertheless, the positive effects of insulin and IGF-1 treatment in preclinical studies failed to translate into clinical trials. Two randomized double-blind placebo-controlled clinical trials administering recombinant human IGF to amyotrophic lateral sclerosis (ALS) patients showed little or no effect on disease progression even though IGF-1

was found to be neuroprotective in an ALS transgenic mouse model (Borasio *et al.*, 1998; Dodge *et al.*, 2008; Kaspar *et al.*, 2003; Lai *et al.*, 1997).

Glucagon like peptide (GLP-1), an insulinotropic hormone, activates the same effectors as insulin and IGF-1 through PI3-K and MAPK pathways (Baggio and Drucker, 2007; Li *et al.*, 2005a; Perry *et al.*, 2002). GLP-1 is expressed in neurons and has positive effects on cell proliferation, neurogenesis and apoptosis (Bassil *et al.*, 2014). Synthetic GLP-1 analogues are resistant to dipeptidyl peptidase-4 (DPP-4), a GLP-1 degrading enzyme, thus having longer half-lives than GLP-1 itself (Martin *et al.*, 2011b). Several GLP-1 analogues are FDA approved for the treatment of diabetes and some were evaluated in clinical trials for treating neurodegenerative disorders (Bassil *et al.*, 2014). GLP-1 analogues pass the BBB similar to GLP-1 and bind to the GLP-1 receptor (GLP-1R). These agonists have shown positive effects on behavior and surrogate markers of neurodegeneration in preclinical models of AD and PD (Bassil *et al.*, 2014). In this line, exendin-4 improves motor performance and rescues TH positive neurons from 6-OHDA-induced cell death (Bertilsson *et al.*, 2008; Harkavyi *et al.*, 2008). Similarly, exendin-4 decreased MPTP-induced loss of nigral neurons and striatal dopaminergic fibers, decreased proinflammatory markers and improved motor function in a mouse model of PD (Kim *et al.*, 2009b; Li *et al.*, 2009). In AD, exendin-4 reversed Aβ-induced neurodegeneration and insulin resistance in *in vitro* and improved cognition in *in-vivo* models (Bomfim *et al.*, 2012; Li *et al.*, 2010). Another GLP-1 analogue, liraglutide, was shown to decrease Aβ pathology and microglial activation, to increase insulin degrading enzyme levels, to improve measures of synaptic plasticity in hippocampal neurons together with cognition and to promote cell proliferation and differentiation into neurons in an AD model (McClean *et al.*, 2010; McClean and Holscher, 2013; McClean *et al.*, 2011; Parthsarathy and Holscher, 2013).

Successful studies in preclinical models of PD paved the way for a small open-label clinical trial assessing the effects of exendin-4 in 45 PD patients who were followed for 14 months. Motor and cognitive outcomes were significantly better in patients receiving exendin-4 compared to placebo (Aviles-Olmos *et al.*, 2013a; Aviles-Olmos *et al.*, 2014). The results of this preliminary open-label trial have set the grounds for a randomized, double blind, placebo-controlled study (EXENATIDE-PD trial, NCT01971242) in 60 PD patients that has started enrollment in December 2013. This study compares the effects of exendin-4 (2mg subcutaneously given once a week) with placebo. Similar to the open-labeled pilot study, the primary outcome is to compare the effectiveness of exendin-4 with placebo on Unified Parkinson's Disease Rating Scale (UPDRS) motor scores in the defined OFF-medication

condition at 60 weeks. Secondary outcomes include safety and health-related quality of life. The completion of this study is expected for March 2016.

DPP-4 inhibitors are an alternative for the treatment of insulin resistance as they increase endogenous GLP-1 levels. DPP-4 inhibitors have low BBB permeability, i.e. their function is primarily to increase peripheral amounts of GLP-1. Sitagliptin and Saxagliptin are two DPP-4 inhibitors that have shown promising effects in preclinical models of AD (D'Amico *et al.*, 2010; Kosaraju *et al.*, 2013). Compared to GLP-1 analogues, insulin and IGF-1, DPP-4 have been poorly studied in preclinical models of neurodegeneration mainly due to the success obtained with GLP-1 analogues.

None of the preclinical studies that used insulin, IGF-1 or GLP-1 analogues measured insulin resistance in the brain of PD animal models or the ability to GLP-1 analogues to reverse insulin resistance. Moreover, these studies were all done on toxin-based models that lack α-syn aggregates. This prompted us to the assessment of insulin/IGF-1 signalling and insulin resistance in the transgenic PLP-SYN mouse model. In preliminary experiments, we have assessed insulin resistance in brain tissue of PLP-SYN mice by measuring the levels of IRS-1pS312 and IRS-1pS616. Our results show a significant increase in both insulin resistance markers in the striatum of 9 months old PLP-SYN transgenic mice. Based on these findings, we hypothesize that exendin-4 has neuroprotective effects in MSA transgenic mice by rescuing TH neurons in the SNc from α-syn induced cell death.

4. Therapeutic strategies targeting α-synuclein in synucleinopathies

C-terminal truncation has been identified as an enhancer/promoter of α-syn oligomerization and fibrillization (Hoyer *et al.*, 2004; Li *et al.*, 2005b; Liu *et al.*, 2005; Ulusoy *et al.*, 2010). Some studies have shown that C-terminally truncated α-syn is found in GCIs in MSA (Gai *et al.*, 1999; Tong *et al.*, 2010) as well as in LBs of PD and DLB patient brains (Baba *et al.*, 1998; Li *et al.*, 2005b; Liu *et al.*, 2005; Murray *et al.*, 2003).

Inhibiting α-syn truncation may alter the disease course in MSA (and other synucleinopathies) by decreasing α-syn oligomerization and aggregation. Hitherto, the inflammatory protease caspase-1 cleaves α-syn at Asp121, promoting its aggregation into amyloid fibrils similar to those previously found both *in vitro* and *in vivo* (Wang *et al.*, 2015). VX-765 also known as Belnacasan ($C_{24}H_{33}ClN_4O_6$) is an orally active, well-tolerated, brain-penetrant prodrug that is hydrolyzed by esterases *in vivo* to produce a potent and selective caspase-1 inhibitor (Boxer *et al.*, 2010; Wannamaker *et al.*, 2007). It was initially developed

for the treatment of inflammatory diseases such as psoriasis and rheumatoid arthritis and later also tested as possible therapeutic for epilepsy (Vertex, 2011).

We here showed that VX-765 was able to decrease α-syn truncation and aggregation and to rescue SNc TH neurons from α-syn-induced toxicity similar to the *in vitro* study (Wang *et al.*, 2015). Not only did VX-765 decrease both truncated and oligomeric forms of α-syn but also monomeric forms of α-syn. A marked decrease in both truncated and oligomeric forms of α-syn might well be secondary to the decrease in monomeric forms since both are products of monomeric α-syn modification (Baba *et al.*, 1998; Fauvet *et al.*, 2012; Lashuel *et al.*, 2013; Murray *et al.*, 2003). This might not be the case with VX-765 treatment since it cancelled the correlation between truncated and oligomeric α-syn observed in placebo treated mice. The decrease in monomeric α-syn in oligodendrocytes might well be due to the decrease in both truncated and oligomeric forms of α-syn which allowed the clearance systems in oligodendrocytes to better handle overexpressed α-syn.

Several studies have shown that targeting α-syn truncation *in vivo* decreases α-syn aggregation and neurotoxicity. Overexpression of a calpain-specific inhibitor in the [A30P]α-syn-Thy-1 PD mouse model reduced α-syn aggregation and other neuropathological features (Diepenbroek *et al.*, 2014). Interestingly, immunotherapy directed against the C-terminal region of α-syn proved to be beneficial in a transgenic DLB mouse model using the PDGF β promoter (Masliah *et al.*, 2011) and in the mThy1-α-syn PD mouse model where it reduced the amount of C-terminal truncated α-syn and blocked the propagation of α-syn from neurons (Games *et al.*, 2014). Other immunization strategies exist to decrease the α-syn load using antibodies directed against the protein itself. Studies have shown neuroprotection after passive (based on the use of antibodies against the protein) or active (vaccination-based approach using full-length protein or short peptides) immunization in mouse models based on the overexpression of α-syn or the injection of α-syn preformed fibrils (Bae *et al.*, 2012; Mandler *et al.*, 2014; Masliah *et al.*, 2011; Tran *et al.*, 2014). Successful results in preclinical studies have led pharmaceutical companies to start clinical trials based on the use of PRX002, a monoclonal antibody directed against α-syn (NCT02095171, NCT02157714). Moreover, another study based on active immunization with Affitope PD01 was found safe in a first pilot study in 32 PD patients (NCT01568099).

Other approaches targeting post-translational modifications of α-syn species, such as phosphorylation, nitration and oxidization may be viable therapeutic strategies. Preventing phosphorylation at S129 prevents neurotoxicity, while increasing the numbers of large inclusion bodies in transgenic flies (Chen and Feany, 2005). Moreover, Lee and colleagues

reported that pharmacological activation PP2A dephosphorylates α-syn and decreases the α-syn burden in a transgenic mouse model of PD (Lee *et al.*, 2011).

Several aggregation inhibitors were reported to efficiently provide neuroprotection by decreasing the formation of α-syn oligomers; these include epigallocatechin-3-gallate, anle138b (3-(1,3-benzodioxol-5-yl)-5-(3- bromophenyl)-1*H*-pyrazole), CLR01 and a prolyl oligopeptidase inhibitor, KYP2047 (Bieschke *et al.*, 2010; Myohanen *et al.*, 2012; Prabhudesai *et al.*, 2012; Savolainen *et al.*, 2014; Wagner *et al.*, 2013).

Finally, increasing autophagy might be a therapeutic approach to enhance α-syn degradation. mTOR dependent and independent autophagy enhancers have provided neuroprotection in several models of PD (Dehay *et al.*, 2010; Malagelada *et al.*, 2010; Sarkar *et al.*, 2007) and have shown to exert part of their proautophagy actions by enhancing lysosomal activation and autophagosome clearance. Similarly, viral-vector–mediated expression of autophagy regulators, such as transcription factor EB, a master regulator of lysosomal biogenesis, Beclin-1 or LAMP-2A, have been shown to reduce α-syn accumulation and synaptic pathology in rodent models of PD, including a rat model based on the overexpression of human α-syn or in a transgenic mouse model of PD (Decressac *et al.*, 2013; Spencer *et al.*, 2009; Xilouri *et al.*, 2013).

The work presented here is an *in vivo* proof of concept of the ability of the caspase-1 inhibitor prodrug VX-765 to alleviate α-syn pathology and to mediate neuroprotection in a MSA mouse model. We here show that VX-765, a drug that was well tolerated in a phase II trial in patients with epilepsy (Vertex, 2011), may be a promising candidate to achieve disease modification in synucleinopathies by limiting α-syn accumulation.

5. MMP distribution and alteration in MSA

Studies have shown the presence of proteases other than caspase-1 in LBs of PD patients. Several proteases such as calpain, plasmin, cathepsin D and MMPs have been implicated in α-syn truncation resulting in increased levels of protein aggregates (Choi *et al.*, 2011; Dufty *et al.*, 2007; Kim *et al.*, 2012; Levin *et al.*, 2009; Liu *et al.*, 2005; Mishizen-Eberz *et al.*, 2003; Sung *et al.*, 2005). Moreover, proteases have also shown the capacity to degrade several components of the extracellular matrix and basement membranes. Several lines of evidence suggest a potential involvement of MMPs in synucleinopathies (Choi *et al.*, 2011; Levin *et al.*, 2009; Nagase and Woessner, 1999; Yong, 2005; Yong *et al.*, 2001; Yong *et al.*, 2007), especially in MSA since it is characterized by neuronal loss, myelin alteration, BBB dysfunction, neuroinflammation, microglial activation, and oxidative stress (Abdo *et al.*,

2004; Ahmed *et al.*, 2012; Gerhard *et al.*, 2003; Horimoto *et al.*, 2000; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Lee *et al.*, 2013; Matsuo *et al.*, 1998; Shibata *et al.*, 2010; Song *et al.*, 2011). MMPs are enriched in glial cells and their increased expression upon glial activation can possibly foster the neuroinflammatory process (Bruck *et al.*, 1997; de Vries *et al.*, 1997; Gerhard *et al.*, 2003; Gottschall and Yu, 1995; Gottschall *et al.*, 1995; Marques *et al.*, 2013; Nuttall *et al.*, 2007). MMPs are altered and co-localize with α-syn in LBs in PD (Choi *et al.*, 2011), yet no studies have assessed the activity or the presence of MMPs in MSA.

Increased MMP expression and activity was evident in the putamen and cortex of MSA patients compared to healthy controls. Zymography allowed us to assess MMP-2 and MMP-9 activity in MSA patients compared to healthy controls. MMP-2 activity was significantly increased in the putamen but not the cortex of MSA patients compared to healthy controls while MMP-9 activity remained unchanged in both regions. Alterations in MMP-9 expression have been previously found in neurodegenerative disorders such as an increase in MMP-9 activity in AD and ALS patients whereas in PD no significant difference was found (Lim *et al.*, 1996; Lorenzl *et al.*, 2002; Lorenzl *et al.*, 2003). MMP-9 is upregulated in sick and surviving neurons in neurodegenerative disorders (Estus *et al.*, 1994; Lorenzl *et al.*, 2002). The absence of significant changes in MMP-9 activity levels in our work might be due to neuronal loss observed in the striatum and cortex of MSA patients. The expression and activity of MMP-9 might be more robust in an earlier phase of disease where neuronal loss is less prominent, thus one could expect to find a significant increase in MMP-9 levels in early stages.

We then measured staining density of MMP-1, MMP-2 and MMP-3 in the putamen and cortex of MSA patients. Extracellular and intracellular levels of MMP-1, MMP-2 and MMP-3 were increased in the putamen and cortex in MSA patients compared to controls, suggesting increased production and secretion of these MMPs primarily by glial cells. Immunohistochemistry allowed the characterization and localization of the sources of this increase, which mainly came from non-neuronal cells in MSA patient brains, presumably astrocytes and microglia, as well as cells present in white matter bundles (i.e. oligodendrocytes). More importantly, surviving neurons in MSA also showed increased MMP-1, -2, -3 staining intensity compared to a faint staining in healthy patients. Increased non-neuronal staining for MMP-1, MMP-2 and MMP-3 prompted us to further investigate their expression in glial cells. MMP-2 and MMP-3 were largely found co-localized with the astrocytic marker GFAP and the microglial marker Iba-1, a finding consistent with previous studies showing their expression in these cell types (Crocker *et al.*, 2006; Lorenzl *et al.*, 2002; Yamada *et al.*, 1995; Yin *et al.*, 2006). Double immunofluorescence imaging also showed that

only MMP-2 co-localized with α-syn in GCIs. In addition, extracellular MMP-1 and MMP-2 immunoreactivities were markedly increased in MSA putamen compared to controls, suggesting an increased secretion from producing cells.

Several MMPs including MMP-1, MMP-2 and MMP-3 have also been demonstrated to mediate C-terminal cleavage of α-syn (Sung *et al.*, 2005). Although MMP-3 has already been found to co-localize with α-syn in LBs in PD (Choi *et al.*, 2011), to our knowledge no study has assessed the presence of MMPs in GCIs. Here, the presence of MMP-2 in all α-syn-positive GCIs suggests that increased MMP-2 activity could participate in inclusion formation by increasing α-syn truncation, therefore promoting its aggregation on the long haul. We here point to a potential process of α-syn aggregate formation that might differently regulate GCIs and LBs formation.

As of late, increasing evidence shows that MMPs are involved in the clearance of aggregation-prone proteins such as Aβ (Liao and Van Nostrand, 2010; Roher *et al.*, 1994; Yin *et al.*, 2006). In addition, studies have focused on the relation and implication of MMPs in Aβ aggregation in AD (Mroczko *et al.*, 2013; Ou-Yang and Van Nostrand, 2013) and that Aβ is a potent stimulant of MMP production (Deb and Gottschall, 1996). Moreover, membrane type 1-MMP (MT1-MMP), a physiological activator of MMP-2 can degrade Aβ *in vitro* and in-situ, while also being expressed in astrocytes around Aβ rich regions in a mouse model of AD (Li *et al.*, 2011; Liao and Van Nostrand, 2010). Interestingly, α-syn has been shown to induce MT1-MMP expression *in vitro* and in a PD mouse model (Kim *et al.*, 2009a).

The mechanisms underlying increased MMP-2 activity and increased MMP-1, MMP-2 and MMP-3 expression in MSA patients remain unknown, yet MMPs are activated by cytokines, ROS, nitric oxide and other neuroinflammatory triggers; factors that are believed to contribute to the neurodegenerative process in MSA (Abdo *et al.*, 2004; Gerhard *et al.*, 2003; Ishizawa *et al.*, 2004; Kaufman *et al.*, 2013; Kikuchi *et al.*, 2002; Shibata *et al.*, 2010). MMP-2, and to a lesser extent MMP-3 and MMP-1 are expressed in neutrophils, macrophages and resident glia of the brain (Deb and Gottschall, 1996; Gottschall and Yu, 1995; Ihara *et al.*, 2001; Nuttall *et al.*, 2007). Interestingly, type-IV collagen, the main substrate of MMP-2 and MMP-9, is decreased in MSA patients (Miller *et al.*, 2007). Thus, the loss of type IV collagen might be caused by increased MMP-2 activity. MMPs degrade the BBB and release proinflammatory cytokines (Dev *et al.*, 2010; Grossetete *et al.*, 2009; Kieseier *et al.*, 2006; Shubayev *et al.*, 2006; Truettner *et al.*, 2005). Even though BBB weakness in MSA is evident and correlates with disease severity and progression (Lee *et al.*, 2013; Song *et al.*, 2011), the potential implication of MMPs in BBB dysfunction in MSA remains to be investigated.

6. Limitations

There are limitations to using postmortem brain tissue for characterizing signalling cascades and identifying molecular targets for drug development. A pure disease effect on neuropathologic traits is difficult to identify due to many factors. Human postmortem studies show several confounding factors such as aging, treatment induced alterations and end-stage disease. These limitations are inherent to postmortem human brain studies. In addition, human postmortem studies fail to answer the question of whether changes observed are a cause or a consequence of neuronal death. Postmortem interval and brain tissue temperature may further impact phosphorylation (Li *et al.*, 2003; Li *et al.*, 2004; Li *et al.*, 2005c).

Phosphorylation of IRS-1 on tyrosine residue is required for insulin/IGF-1-stimulated responses while the phosphorylation of IRS-1 on serine residues is known to block insulin/IGF-1 signalling. We measured here two well-characterized insulin resistance markers, IRS-1pS312 and IRS-1pS616, but we are aware of additional serine/tyrosine phosphorylation sites on IRS-1 that are implicated in insulin signalling and insulin resistance.

We also acknowledge the limited number of available subjects for our postmortem study. In this view, a larger sample size might have allowed to detect significant effects where only trends were observed. However, it is difficult to achieve a large sample size because of limited postmortem brain availability, especially in MSA because of the rareness of the disorder. On the other hand, the strength of our work comes from the extensive cellular characterization.

No available animal model perfectly replicates MSA and PD etiopathogenesis. So far, toxic models have shown the ability to recapitulate several aspects of neurodegeneration that are found in the brain of MSA and PD patients but failed to reproduce the hallmark of these disorders i.e. intracellular protein aggregates. On the other hand, genetic models of MSA and PD are based on the overexpression of intracellular α-syn aggregates. These models show robust and widespread overexpression of α-syn throughout the brain including structures that are not or modestly implicated in human disease pathology such as the cortex, the amygdala and the hippocampus. However, beyond their ability to overexpress α-syn and α-syn aggregates, all transgenic models fail to reproduce the profound neurodegeneration that is found in PD patients and to a higher extent in MSA patients.

7. Perspectives

Future studies should characterize insulin resistance in other regions in the brain such as the SN and cerebellar structures implicated in the neurodegenerative process in MSA and PD. Moreover, structures that are relatively spared in MSA are interesting to look at as they may inform about the contribution of insulin resistance early in the disease. Future studies should also include a wider group of patients and assess groups from both subtypes of MSA to determine if there is a difference in insulin resistance between both subtypes. Future work should compare insulin resistance in different structures between synucleinopathies and other neurodegenerative disorders to determine if insulin resistance is region-dependent or a general disease mechanism.

Phosphorylation of IRS-1 is a dynamic process occurring in the brain of mammals controlled by several factors including activation of the insulin/IGF-1 cascade by activating ligands, the amount of IGF-1 binding protein (IGFBP) and activity of phosphatases and kinases. Studies assessing insulin resistance in the brain are relatively recent and several questions remain unanswered especially in synucleinopathies. To address these questions, future studies should:

1. Assess insulin and IGF-1 concentration in the brain, which would give further insight on the bio-availability of insulin/IGF-1 in the brain and its effects on central insulin/IGF-1 signalling. Moreover, it would also be interesting to assess the relationships between peripheral and central insulin and IGF-1 levels in search of potential biomarkers for MSA and PD.

2. Weigh the contribution of regulatory proteins that are known to be implicated in the regulation of peripheral and central insulin/IGF-1 levels in synucleinopathies. IGFBP regulates free IGF-1 amounts in the brain by binding to it and inhibiting the activation of the receptors or conversely increasing the half-life of IGF-1 in the brain.

3. Assess insulin/IGF-1 signalling in the brain by measuring the receptors and downstream effectors in MSA and PD patients. Several studies have assessed insulin/IGF-1 signalling in AD patient brains compared to healthy controls implicating upstream activators of the pathway in insulin resistance and showing altered insulin receptor expression in the brain of AD patients (Moloney *et al.*, 2010; Rivera *et al.*, 2005; Steen *et al.*, 2005; Talbot *et al.*, 2012; Tong *et al.*, 2009; Zhao *et al.*, 2008). Similarly, insulin receptor and IGF-1R gene expression levels were decreased in the brain of PD and DLB patients (Tong *et al.*, 2009). Control over insulin/IGF-1

signalling can be achieved by a negative feedback regulation loop whereby downstream effectors inhibit IRS-1.

4. Measure the expression and activity of kinases and phosphatases in the brain of MSA and PD patients compared to controls. IRS-1 activation and inhibition is dynamically regulated by the activity of kinases and phosphatases. For instance, reductions in insulin-induced signalling in AD patient brains is due to increased activity of kinases such as ERK2, GSK-3, IKK, JNK, mTOR and PKCζ (Talbot *et al.*, 2012).

5. Target IRS-1 tyrosine phosphorylation as direct markers of insulin/IGF-1 signalling activity. We have shown altered IRS-1 serine phosphorylation in synucleinopathies. IRS-1 tyrosine phosphorylation has been shown to mediate the activation of insulin/IGF-1 signalling.

6. Whether insulin resistance precedes the aggregation of α-syn or is the result of α-syn inclusions in oligodendrocytes remains an unanswered question. Future *in vitro* studies should target this question by overexpressing α-syn in oligodendrocytes and measuring insulin resistance markers. Moreover, it would be interesting to induce insulin resistance in oligodendrocytes and oligodendrocytes expressing hα-syn to determine if insulin resistance is primary or secondary to α-syn overexpression.

Part of the translational approach in our work involves the use of exendin-4, a GLP-1 analogue, to modulate insulin/IGF-1 signalling in PLP-SYN mice in view of disease modification. Preliminary results show the presence of insulin resistance in the brain of PLP-SYN mice. We are currently testing the therapeutic efficacy of exendin-4 treatment in PLP-SYN mice. Briefly, 6-week old PLP-SYN mice and aged matched wild-type (WT) littermates are being treated with 2 doses of exendin-4 or placebo for a period of 9 weeks. Study endpoints will be the therapeutic efficacy of exendin-4 to reverse insulin resistance, decrease α-syn burden and the resultant α-syn induced neurodegeneration. If successful, the preclinical proof of concept study in PLP-SYN mice might pave the way for translating an innovative treatment with putative disease-modifying properties to MSA patients.

We also showed differential expression of MMPs in MSA patients compared to other neurodegenerative disorders, especially PD. Moreover, we here showed that GCIs did not stain positive for MMP-3, previously shown to be present in LBs, but only for MMP-2. It is yet to be known the respective contribution of MMP-2 in α-syn truncation and whether MMP-2 cleaved α-syn fragments harbor a different mechanism than other MMPs, which may lead to GCI formation. Future research should determine MMP-2 α-syn cleavage sites *in vitro* and

compare them to those produced by MMP-3. On that point and if additional cleavage sites of α-syn are found, studies should assess the propensity of the newly characterized truncated α-syn forms to promote oligomerization *in vitro* and *in vivo* compared to previously characterized α-syn truncated species.

A better understanding of the involvement of MMPs in the neurodegenerative process in MSA is necessary before considering them as targets for disease modification. MMP inhibitors were already tested in phase 2 and phase 3 clinical trials in patients with late stage brain, breast, colon, pancreas, ovarian, renal or stomach cancer (Coussens *et al.*, 2002; Zucker and Cao, 2009). Hitherto, they failed to show a significant effect on survival (Coussens *et al.*, 2002).

8. Conclusion

Several findings arise from this work and contribute to the understanding of MSA pathogenesis:

1. Insulin resistance in oligodendrocytes of MSA patients might contribute to the dysfunction of oligodendrocytes and the subsequent loss of neurons.

2. Insulin resistance in neurons is unlikely to cause cell death on its own but might induce neuronal dysfunction due to the importance of neurotrophic and neuroprotective properties of IGF-1 in the brain.

3. Increased MMP expression and activity provides additional evidence implicating glial cells and neuroinflammation in MSA pathogenesis and shows the uniqueness of MSA in MMP alterations compared to other neurodegenerative disorders including PD. We here also point to an important difference between LBs and GCIs as the latter do not express the same MMPs, which might point to different pathological mechanisms implicated in aggregate formation in each disorder.

4. Targeting α-syn truncation with a caspase-1 inhibitor reduces the amount of α-syn aggregates and protects TH neurons in MSA transgenic mice.

Overall, we here show several pathophysiological features unique of MSA compared to other synucleinopathies. More importantly, our data show several key alterations occurring in oligodendrocytes, further supporting the concept of MSA as an oligodendrogliopathy. At this stage, we propose VX-765, a candidate drug for disease modification in synucleinopathies

whereas the work on postmortem human brain tissue points to anti-diabetics as candidate drugs in MSA.

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VI- References

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