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Establishment of Primary Culture Models of Multiple System Atrophy Based on Expression of α-Synuclein in Oligodendrocytes: Analysis of α-Synuclein Aggregation and Associated Pathologies

vorgelegt von

Gwenaëlle Fillon

aus Dreux, Frankreich

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1. Gutachter : Prof. Dr. Thomas Cremer
2. Gutachter : Prof. Dr. Stefan Jentsch
   Sondergutachter: Prof. Dr. Christian Haass

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A mon grand-père, Amand,
dont l'amour et la maladie m'ont amenée très jeune à vouloir
consacrer ma carrière aux neurosciences

A mes parents, Annick et François,
que je chéris par dessus tout

Experience is not what happens to you;
it is what you do with what happens to you.

– Aldous Huxley

SUMMARY

Multiple system atrophy (MSA) is a neurodegenerative syndrome characterized by (oligodendro)glial cytoplasmic inclusions (GCIs) composed of α-synuclein. I have developed cell culture models of MSA based on overexpression of human α-synuclein in primary mouse oligodendrocytes. In oligodendrocytes derived from (PLP)- α-synuclein transgenic mice, elevation of α-synuclein levels by proteasome inhibition induced GCI formation and enhanced apoptosis. The same effects were observed in wild-type oligodendrocytes transduced with a lentiviral vector encoding α-synuclein. In contrast, lenti-β-synuclein failed to yield inclusions, and even prevented aggregation and cytotoxicity of α-synuclein. Selective caspase inhibitors blocking the intrinsic (mitochondrial) apoptosis pathway and the extrinsic pathway reduced αSYN-mediated oligodendrocyte cell death. α-synuclein overexpressing oligodendrocytes strongly expressed the pro-apoptotic Fas receptor and were specifically sensitized to Fas-mediated apoptosis. In MSA brain, Fas was expressed on oligodendrocytes with GCIs. Thus, induction of α-synuclein leads to GCI formation and may contribute to oligodendrocyte dysfunction and cell death in MSA.

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Table of Abbreviations

AR = Adrenegic Receptor
bFGF = basic Fibroblast Growth Factor
bHLH = basic Helix-Loop-Helix Protein
BS = Bottenstein-Sato medium
CNP = 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase
CNS = Central Nervous System
DA=Dopamine
DAT=DopamineTransporter
DLB = Dementia with Lewy Bodies
DMEM = Dulbecco's Modified Eagle Medium
DMSO = DiMethyl SulfOxide
eGFP = enhanced Green Fluorescent Protein
FCS = Fetal Calf Serum
GalC = Galactocerebroside
GCI = Glial Cytoplasmic Inclusion
GFP: Green Fluorescent Protein
HNE = 4-Hydroxy-2-NonEnal
hsp = heat shock protein
IgG = Immunoglobulin G
kDa = kilo Dalton
LB = Lewy Body
LN = Lewy Neurites
LTR = Long Terminal Repeat
MAG = Myelin-Associated Glycoprotein
MBP = Myelin Basic Protein
MOG = Myelin Oligodendrocyte oligoprotein
MPP^+ = 1-Methyl-4-PhenylPyridinium ion
MPTP = 1-Methyl 4-Phenyl 1,2,3,6-TetrahydroPyridine
mRNA = messenger Ribonucleic Acid
MSA = Multiple System Atrophy
MSA-C = Multiple System Atrophy with predominant cerebellar ataxia's symptoms
MSA-P = Multiple System Atrophy with predominant Parkinsonism symptoms
NAC = non amyloid component
NBIA-1 = Neurodegeneration with Brain Iron Accumulation type 1
NCI = Neuronal Cytoplasmic Inclusion
NGF = Nerve Growth Factor
NNI = Neuronal Nuclear Inclusion
OPC = Oligodendrocyte Precursor Cells
OPCA = OlivoPontoCerebellar Atrophy
PBS = Phosphate Buffered Saline
PD = Parkinson's Disease
PDGF = Platelet Derived Growth Factor
PDGFR = Platelet-Derived Growth Factor Receptor
PGK = PhosphoGlycerate Kinase
PLD2 = Phospholipase D2

PLP = ProteoLipid Protein
PVDF = polyvinylidene difluoride
RT = room temperature
SDS = Sodium Dodecyl Sulfate
SDS-PAGE = Sodium DodecylSulfate Polyacrylamide Gel
Shh = Sonic hedgehog
TBS = Tris Buffered Saline
TNFα = Tumor Necrosis Factor-α
TRAIL = TNF-Related Aptosis-Inducing Ligand
UPS = Ubiquitin Proteasome System
WPRE = Woodchuck Hepatitis Posttranscriptional Regulatory Element

Chapter 1: GENERAL INTRODUCTION

1 STRUCTURE AND PHYSIOLOGICAL FUNCTIONS OF α-SYNUCLEIN

1.1 The synuclein protein family

a α-synuclein

In 1988, a novel protein was isolated from the electric lobe of the Pacific ray, *Torpedo californica*, and from rat brain (Maroteaux et al., 1988). Due to its distribution on portions of the nuclear membrane and its presence in high concentrations in presynaptic nerve terminals, this protein was named α-syn (synapse) -nuclein (nucleus); however, localization of mammalian synucleins to the nucleus was not confirmed by subsequent studies.

Human α-synuclein is a 140 amino acid protein that is ubiquitously expressed at high levels in all regions of the brain where it is estimated to account for up to 0.1 % of total brain proteins (Clayton and George, 1999; George, 2002). α-synuclein is present most abundantly in presynaptic terminals (George et al., 1995; Iwai et al., 1995) from neurons of various regions of the central nervous system (CNS) including the neocortex, hippocampus, dentate gyrus, olfactory bulb, thalamus and cerebellum, and also in the amygdala and nucleus accumbens (George et al., 1995; Iwai et al., 1995; Jakes et al., 1994; Wersinger et al., 2004). α-synuclein is also detected in perikarya within several brainstem structures, including raphe, hypoglossal, and arcuate nuclei (Giasson et al., 2001), in olfactory receptor neurons and basal cells of the olfactory epithelium (Duda et al., 1999).

Although biochemical analysis revealed that α-synuclein is predominantly localized to the cytoplasm of presynaptic terminals (Hurtig et al., 2000; Iwai et al., 1995; Jakes et al., 1994; Masliah et al., 1996; Petersen et al., 1999), a small fraction of α-synuclein may be

associated with vesicular membranes (George et al., 1995; Irizarry et al., 1996; Jensen et al., 1998). α-synuclein is enriched in synaptosomal preparations (but is not found in highly purified synaptic vesicle fractions) (George et al., 1995; Irizarry et al., 1996; Kahle et al., 2002b). Immunogold electron microscopy showed that α-synuclein is localized to the inner face of plasma membranes in close proximity, but loosely associated with, synaptic vesicles at axonal termini (Clayton and George, 1998; Iwai et al., 1995; Jenco et al., 1998a; Maroteaux et al., 1988). Thus α-synuclein exists in both cytoplasmic and membrane bound forms, most likely in a dynamic equilibrium (Kahle et al., 2000; Nuscher et al., 2004).

b β-synuclein

The second member of the synuclein protein family, initially named phosphoneuroprotein-14 (Nakajo et al., 1993), was identified in bovine brain. Because of its high homology with α-synuclein at the amino acid sequence level (62%), this protein was named β-synuclein (Jakes et al., 1994). β-synuclein, the most conserved of the synuclein proteins, is 134 amino acids long and contains five repeats of the EKTKEGV consensus sequence (Figure 1).

Like α-synuclein, β-synuclein is predominantly expressed in neuronal axon termini of CNS neurons (Jakes et al., 1994; Nakajo et al., 1990; Nakajo et al., 1994) but its distribution throughout the brain is more even than that of α-synuclein (Abeliovich et al., 2000; Nakajo et al., 1994). Similarities in sequence and predominant localization in presynaptic terminals may suggest that α- and β-synuclein share similar functions.

c γ-synuclein

Another synuclein homologue, originally called breast cancer-specific gene-1 protein, was identified in 1997 in metastatic brain cancer tissue (Ji et al., 1997). Due to its significant sequence homology with α-synuclein (55%), this protein was named γ-synuclein (Lavedan et al., 1998b) and is also refered to as persyn (Ninkina et al., 1998) or synoretin (Surguchov et al., 1999).

γ-synuclein is 127 amino acids long and like α-synuclein, it contains six repeats of the EKTKEGV consensus sequence. γ-synuclein is expressed in CNS as well as in the spinal cord, but it is most abundant in the peripheral nervous system including neurons of the dorsal root ganglia and trigeminal ganglia (Buchman et al., 1998; Lavedan et al., 1998a). Whereas α-synuclein and β-synuclein are concentrated in synaptic vesicles, with little staining in cell bodies and dendrites, γ-synuclein is distributed throughout the neuronal cytosol (Buchman et al., 1998).

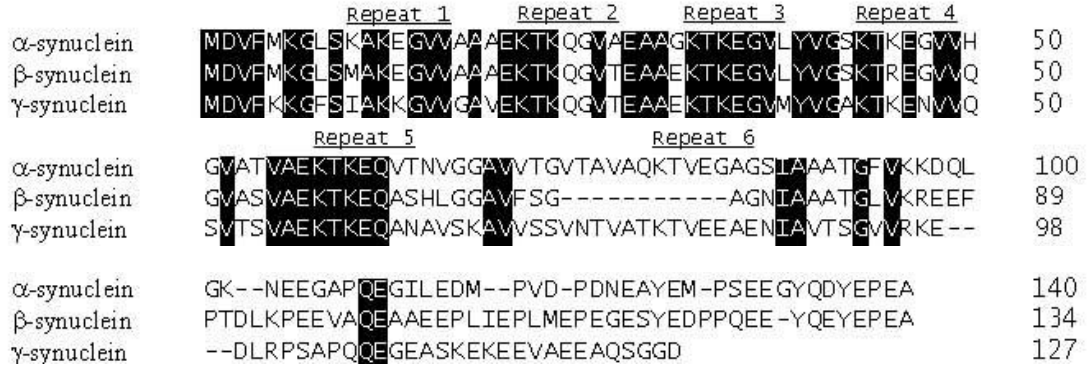


Figure 1: Amino acid sequence alignment of the human synuclein proteins. The imperfect EKTKEGV repeats are identified. The black background highlights amino acid residues conserved between α- and β-synucleins (Jakes et al., 1994), and γ-synuclein (Ji et al., 1997). The very high conservation between species for EKTKEGV consensus sequence suggests that the repeats have arisen from the duplication of a single domain within an ancestral synuclein gene.

1.2 Structural properties of α-synuclein

α-Synuclein consists of three major regions (figure 2):

(1) The highly conserved N-terminal region contains 60 amino acid residues and four repeats, with each repeat containing an imperfectly conserved KTKEGV motif (Clayton and George, 1998; George, 2002). These repeats form amphipathic α-helices similar to apolipoprotein class A2 molecules through which membrane binding becomes stabilized (Davidson et al., 1998; Eliezer et al., 2001; Perrin et al., 2000).

(2) Residues 61–95 form the hydrophobic and amyloidogenic central region, referred to as the non-amyloid component (NAC) of the amyloid precursor protein (see part 2.3), which includes two additional EKTKEGV repeats. This domain is the building block of α-synuclein aggregation (Giasson et al., 2001; Kahle et al., 2002a), with critical residues for the fibrillization of the protein being residues 66 to 74 (Du et al., 2003). β-synuclein lacks 11 amino acids in the region corresponding to NAC (Figure 1). β-synuclein is a non-amyloidogenic homolog of α-synuclein and may act as a physiological inhibitor of α-synuclein aggregation (see part 2.1; (Hashimoto et al., 2001).

(3) The C-terminal acidic tail (residues 95-140) is less well conserved and rich in glutamate and aspartate residues. This domain remains free and unfolded upon membrane binding, potentially providing a site for interactions with binding partners (Eliezer et al., 2001) (see part 2.1). Post-translational modifications such as phosphorylation can occur at serine 129 (Fujiwara et al., 2002; Okochi et al., 2000) and/or Tyr125 (Ellis et al., 2000).

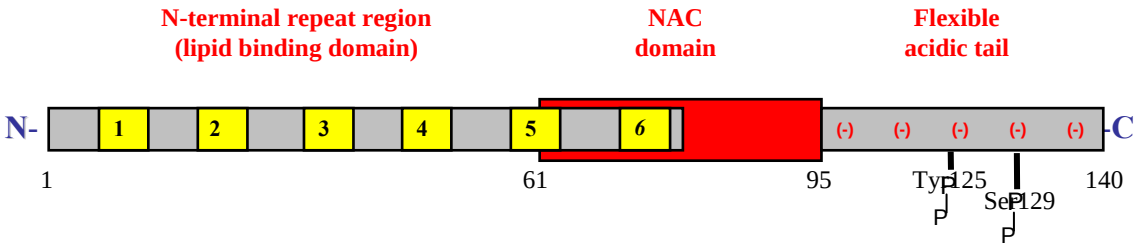


Figure 2: Schematic diagram of α-synuclein structure

The structure of α-synuclein allows the molecule to exhibit different conformations depending on its interacting environment (Uversky, 2003). This protein is natively unfolded in solution (Weinreb et al., 1996), adopts an α-helical conformation upon binding to lipid vesicles (Davidson et al., 1998), or forms β-pleated sheets in its aggregated form, suggesting highly dynamic structural changes depending upon the local cellular milieu. Nuclear magnetic resonance and electron paramagnetic resonance spectroscopy analysis indicates that binding of α-synuclein to lipids induces a reorganization of the repeats into 3 complete helical turns (Bussell and Eliezer, 2003; Jao et al., 2004).

1.3 Functions of α-synuclein

As mentioned above, α-synuclein exists physiologically in both soluble and membrane-bound states, in natively unfolded and α-helical conformation, respectively. The physiological function of α-synuclein appears to require its translocation between these subcellular compartments and interconversion between the 2 conformations. However, the general functions of α-synuclein under physiological conditions remain unclear and many roles for this protein have been proposed.

a α-Synuclein effects on synapse organization and on synaptic vesicle maintenance

Much research has been carried out in humans, mice, and chickens to study the role of α-synuclein in neuronal development/synaptic plasticity (Eells, 2003; Sidhu et al., 2004b) and synaptic vesicle formation (Lotharius et al., 2002; Lotharius and Brundin, 2002). α-synuclein is initially present in the perikarya of mature neurons and is translocated to axon terminals between 15 and 18 weeks of gestation (Galvin et al., 2001; Murphy et al., 2000). Therefore, α-synuclein does not appear to play a role in initial synapse formation since it is expressed later in development and localizes to synapses after they are formed (Murphy et al., 2000; Withers et al., 1997). Abeliovich et al. developed mice homozygously deleted for α-synuclein by targeted gene disruption. These mice were viable and fertile; they exhibited no morphological deficits and possessed a normal complement of neuronal cell bodies, fibers, and synapses (Abeliovich et al., 2000; Cabin et al., 2002), providing further evidence that α-synuclein is not necessary for synaptic development. However, these mice exhibited significant impairments in synaptic response to tetanic stimulation, suggesting that α-synuclein may regulate synaptic vesicle mobilization at nerve terminals (Abeliovich et al., 2000).

b α-Synuclein effects on synaptic vesicle recruitment

Converging observations suggest that a primary function for α-synuclein may be the physiological maintenance of synaptic homeostasis and plasticity (George et al., 1995; Iwai et al., 1995; Jakes et al., 1994; Sidhu et al., 2004b). α-synuclein has been suggested to regulate axonal transport of synaptic vesicles (Jensen and Gai, 2001; Sidhu et al., 2004b), by interacting with several proteins that either bind to, or are part of the cytoskeleton, such as tubulin, tau, microtubule-associated proteins (Payton et al., 2001; Sidhu et al., 2004b). α-synuclein effect on synaptic vesicle cycling may be partly mediated through the ability of its N-terminal repeat region (residues 7-87), to physically interact with, and inhibit, the

activity of phospholipase D2 (PLD2) (Ahn et al., 2002; Jenco et al., 1998a), an enzyme localized primarily along the plasma membrane (Colley et al., 1997). Activation of PLD2 in the plasma and endosomal membranes, regulates the recycling of synaptic vesicles at or near the plasma membrane or endosomal compartments in response to external stimuli, and is instrumental in vesicle formation, through production of phosphatidic acid which recruits adaptor molecules, which, in turn, trigger the building of vesicles from donor membranes (Lotharius et al., 2002; Lotharius and Brundin, 2002). The regulatory effect of α-synuclein on synaptic vesicles recycling may be tightly regulated by various serine/threonine or tyrosine protein kinases (Lotharius and Brundin, 2002; Sidhu et al., 2004b). Hence, by inhibiting PLD2, α-synuclein may play a role in the control of synaptic vesicle cycling (Jenco et al., 1998b). Phosphorylation of membrane-bound α-synuclein by G-protein coupled receptor kinases, lowers its ability to inhibit PLD2 activity (Lotharius and Brundin, 2002). Binding of phosphorylated α-synuclein to phospholipids is reduced (Sidhu et al., 2004b) and as a consequence monomeric α-synuclein is released into the cytoplasm (Leng et al., 2001; Pronin et al., 2000). Thus, through reduction of its tonic inhibition of PLD2, phosphorylated α-synuclein might promote vesicle recycling during periods of high neuronal activity and favor synaptic plasticity, whereas non-phosphorylated α-synuclein may suppress synaptic vesicle formation during periods of low neuronal activity (Sidhu et al., 2004b).

The modulation of vesicle recycling by α-synuclein may also be partly mediated through its putative ability to transfer fatty acids to sites of synaptic vesicle formation (i.e. early endosomes) and/or regulate the turnover or local organization of polyunsaturated fatty acid acyl groups implicated in clathrin-mediated endocytosis and, therefore, in vesicles

recycling (Lotharius et al., 2002; Lotharius and Brundin, 2002). These fatty acid-binding protein properties of α-synuclein rely on two observations:

- its N-terminal lipid (and vesicles) binding domain (residues 7-87; figure 2) bears significant homology with the lipid-binding class A apolipoproteins A2 and C (George, 2002; Goedert, 2001; Maries et al., 2003), proteins implicated in lipid transport (Sharon et al., 2001).
- short amino acyl stretches in α-synuclein N- and C-termini share more than 55 to 67% identity with a cytosolic fatty acid-binding motif of fatty acid-binding proteins (Sharon et al., 2001).

c α-Synuclein effects on neurotransmission

Many studies suggest the important role of α-synuclein in the regulation of neurotransmission and in the physiological maintenance of dopamine (DA) homeostasis in dopaminergic neurons of the substantia nigra pars compacta (Abeliovich et al., 2000; Lotharius et al., 2002; Lotharius and Brundin, 2002; Perez et al., 2002; Sidhu et al., 2004b).

Although α-synuclein knock-out mice display normal brain development and neuronal architecture, some small functional abnormalities in the dopaminergic system, reflected by a reduction in striatal DA and an attenuation of DA-dependent locomotor response to amphetamine, have been reported (Abeliovich et al., 2000). DA release and reuptake was studied in striatal brain slices of these mice. After stimulation of α-synuclein knock-out mouse striatal brain slices, nigrostriatal terminals displayed a standard pattern of DA release and reuptake in response to simple electrical stimulation. However, they exhibited an increased DA release with paired electrical stimuli, as compared to wild-type mouse brain slices, suggesting that α-synuclein is a negative regulator of DA neurotransmission

(Abeliovich et al., 2000). In a second study, ultrastructural examination of synapses of α-synuclein knock-out mice also showed a reduction in the reserve-resting pool of synaptic vesicles in the hippocampus (Cabin et al., 2002). These data were consistent with previous *in vitro* data (Murphy et al., 2000). After lowering the amount of α-synuclein in cultured rat hippocampal neurons using antisense oligonucleotides, Murphy et al. (2000) detected a decrease in the number of resting-reserve synaptic vesicles, suggesting that α-synuclein may be required for the genesis and/or maintenance of a subset of presynaptic vesicles, those in the 'reserve' or 'resting' pools (Cabin et al., 2002).

A more specific link to DA neurotransmission was established by the findings that α-synuclein binds to tyrosine hydroxylase, the rate-limiting enzyme for DA synthesis (Perez et al., 2002) and to dopamine transporter (DAT) (Lee et al., 2002; Wersinger and Sidhu, 2005), thereby controlling the extravesicular cytoplasmic levels of DA (Sidhu et al., 2004b). Disruption of this function of α-synuclein can result in abnormal intracellular and extracellular DA content, which upon autoxidation and enzymatic metabolization can generate reactive oxygen species, ultimately leading to cell death. α-Synuclein interacts directly with the DAT (Lee et al., 2001a) through its NAC domain (Wersinger and Sidhu, 2003). It was recently demonstrated that in the presence of α-synuclein, DAT is dynamically trafficked away from the plasma membrane into the cytoplasm (Wersinger et al., 2003; Wersinger and Sidhu, 2003). These findings suggest that α-synuclein may act to tether the DAT to a cytoplasmic compartment, thereby keeping it away from the cell surface.

Studies linking 1-methyl-4-phenylpyridinium ion (MPP^+), a neurotoxin that inhibits mitochondrial complex I, and α-synuclein effects further implicate a role of α-synuclein in the regulation of DAT. MPP^+, whose intracellular transport within neurons occurs

specifically only through the DAT in an energy-dependent manner, reversed the inhibitory effects of α-synuclein on DAT (Wersinger et al., 2003). The presence of α-synuclein enhances the vulnerability of cells to MPP^+ exposure (Kanda et al., 2000), whereas α-synuclein null-mice are essentially resistant to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced degeneration of dopaminergic neurons (Dauer et al., 2002). Schluter et al. generated α-synuclein-deficient mice by homologous recombination. Upon acute MPTP challenge, α-synuclein knockout mice were partly protected from chronic depletion of nigrostriatal DA when compared with littermates of the same genetic background (Schluter et al., 2003).

d α-Synuclein and neuroprotection

Several studies indicate that α-synuclein may have a neuroprotective effect by modulating the expression levels or activity of anti-apoptotic molecules and decreasing the expression/activity of pro-apoptotic molecules after exposure to certain neurotoxins, oxidative stress, and glutamate-induced excitotoxicity (da Costa et al., 2000; da Costa et al., 2003; Hashimoto et al., 2002; Lee et al., 2001b). The anti-apoptotic effect of α-synuclein seems to be mediated by a stimulation of the PI3/Akt signaling pathway (Seo et al., 2002), followed by an increase in the expression levels of the anti-apoptotic protein Bcl-2 (Seo et al., 2002), and an inhibition of the activity of proapoptotic proteins (da Costa et al., 2000; Hashimoto et al., 2002; Lee et al., 2001b). Although it is conceivable that neuroprotection may be a putative physiological function for this protein that may be lost under pathological conditions (Sidhu et al., 2004a), targeted disruption of α-synuclein gene did not interfere with programmed cell death (Abeliovich et al., 2000). Further, α-synuclein knock-out mice display striking resistance to MPTP induced degeneration of dopaminergic neurons (Dauer et al., 2002). Contrary to predictions from in vitro data, this

resistance was not due to abnormalities of the DAT, which appeared to function normally in the null mice.

e α-Synuclein: a molecular chaperone protein

Conformational analysis of human α-synuclein has demonstrated that this protein is natively unfolded, leading to the speculation that it could be involved in protein–protein interactions (Weinreb et al., 1996). Its variable carboxyl terminal acidic tail (residues 95-140) suggests a chaperone-like activity for α-synuclein (Uversky, 2003). Furthermore, this protein binds to and shares 40% sequence homology with the cytoplasmic chaperone 14-3-3 (Ostrerova et al., 1999), lending more support to the hypothesis that α-synuclein may also function as a molecular chaperone, thus displaying pleiotropic effects in cells (Souza et al., 2000). It was shown *in vitro* that recombinant alpha-synuclein has a chaperone-like function against thermal and chemical stress (Kim et al., 2000).

2 ROLE OF α-SYNUCLEIN IN NEURODEGENERATIVE DISEASES

α-synuclein is the major building block of pathological inclusions that characterize many neurodegenerative disorders, including Parkinson's disease (PD), dementia with Lewy bodies (DLB), neurodegeneration with brain iron accumulation type 1 (NBIA-1) and multiple system atrophy (MSA), collectively termed α-synucleinopathies (Trojanowski and Lee, 2002). Pathological aggregates specifically contain α-synuclein but not β-and γ-synucleins. The factors leading to aggregation of α-synuclein are of critical importance as potential mechanisms of pathogenesis.

2.1 α-Synuclein aggregation and toxicity

α-synuclein was demonstrated to self-aggregate into protofibrils, small α-synuclein oligomers which precede the development of larger fibrillary aggregates, and amyloid like fibrillar conformations (Conway et al., 1998; Conway et al., 2000a; Conway et al., 2000b; Giasson et al., 1999; Paik et al., 1998; Serpell et al., 2000). Polymerization is associated with a concomitant change in secondary structure from random coil to anti-parallel β-sheet structure (Narhi et al., 1999) consistent with the thioflavin S reactivity of these fibrils (Hashimoto et al., 1998; Narhi et al., 1999). β-synuclein is not only devoid of pathological aggregation capacity, but even suppresses α-synuclein aggregation (Park and Lansbury, 2003). Overexpression of β-synuclein in α-synuclein transgenic mice reduces the number of α-synuclein inclusions (Hashimoto et al., 2004). In DLB patients (see part 2.3.b), increased α-synuclein expression combined with decreased β-synuclein expression was

detected (Rockenstein et al., 2001). Thus the balance between both synucleins *in vivo* might be an important factor regulating α-synuclein aggregation.

The formation of α-synuclein fibrils is greatly accelerated *in vitro* above a critical concentration of purified recombinant α-synuclein (Conway et al., 2000a; Giasson et al., 1999; Hashimoto et al., 1998; Serpell et al., 2000) and by increased expression levels of α-synuclein in transgenic animals (Giasson et al., 2002; Lee and Lee, 2002; Neumann et al., 2002). However, since α-synuclein is ubiquitiously expressed in the brain it is unlikely that high expression levels of α-synuclein are the sole criteria to cause protofibril and/or α-synuclein aggregation in synucleinopathies (see part 2.3 for definition). In fact, many other factors can also influence the fibrillization of α-synuclein. Catecholamines, including DA, can form covalent adducts with α-synuclein and thereby increase the number of α-synuclein protofibrils (Conway et al., 2001). This might explain the vulnerability of dopaminergic neurons of the substantia nigra pars compacta in PD.

The specific vulnerability of certain cell types to degenerate in α-synucleinopathies may be linked to additional factors such as the presence of metals which have also been implicated in α-synuclein aggregation. It was shown that ferrous iron stimulates α-synuclein aggregation in different cell lines (Ostrerova-Golts et al., 2000), and the concomitant presence of hydrogen peroxide accelerates the formation of insoluble amyloid-like α-synuclein aggregates by means of the Fenton reaction (Hashimoto et al., 1998). Free radicals such as free iron or iron-centered radicals (Youdim, 2003) accelerate and stabilize the formation of α-synuclein protofibrils (Lee and Lee, 2002; Volles and Lansbury, 2003) by inhibiting the conversion of soluble protofibrils into insoluble fibrils (Conway et al., 2001; Li et al., 2004). This is particularly interesting regarding the high concentrations of iron in human substantia nigra pars compacta and in oligodendrocytes, which display α-

synuclein aggregates and signs of degeneration in PD and MSA respectively (see part 2.3.a and 3.1).

Post-translational modifications such as phosphorylation at Ser129 has been also shown to induce or accelerate α-synuclein aggregation, both *in vivo* and *in vitro* (Chen et al., 2005; Fujiwara et al., 2002; Smith et al., 2005). Only 4% of α-synuclein is Ser129 phosphorylated under steady state physiological conditions, contrasting with the massively disproportionate concentration of phosphorylated α-synuclein (90%) in proteinaceous inclusions characteristic of specific neurodegenerative diseases (respectively part 2.3 and 3.1). This findings suggest that extensive phosphorylation at Ser129 of α-synuclein in the brain is a highly pathological event. However, these correlative studies did not answer the question whether Ser129-phosphorylation is a cause or rather a secondary effect of α-synuclein fibrillization *in vivo*.

Furthermore, α-synuclein monomer containing a tissue transglutaminase crosslinked intramolecular bond was extracted from PD substantia nigra pars compacta (Andringa et al., 2004). The presence of this abnormal internal bond may impair the ability of α-synuclein to interconvert between its α-helical configuration when bound to membrane and its unstructured cytoplasmic form, a process that appears to be necessary for its normal function. Concentration of this isoform increases with disease progression and might serve as a nucleation site that could initiate α-synuclein filament assembly.

Converging evidence that α-synuclein can function as nucleation site and promote aggregation under certain conditions has led several groups to investigate the direct effects of α-synuclein on cell viability. It is believed that the α-synuclein toxicity is associated with its oligomerization into soluble protofibrils. Protofibrils have been shown to penetrate membranes and form pore-like channels through which contents of vesicle membranes can

leak (Figure 3), elevating intracellular levels of potential cytotoxins such as calcium and DA (Conway et al., 1998; Conway et al., 2000b; Conway et al., 2001; Lashuel et al., 2002a; Rochet et al., 2000; Volles et al., 2001).

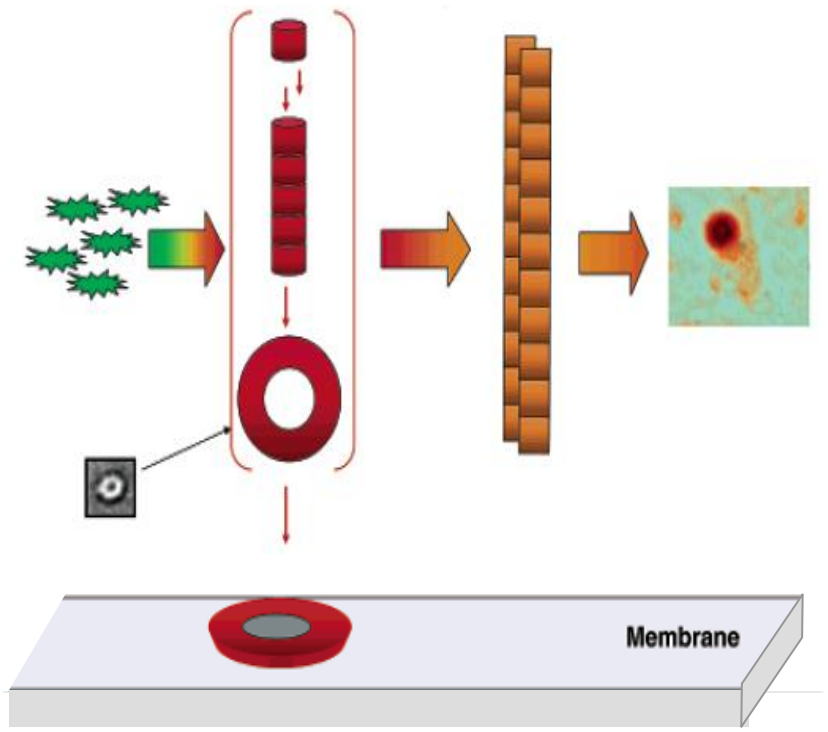


Figure 3: Putative model for pathological α-synuclein inclusion formation. Disordered monomeric (green starburst) α-synuclein oligomerizes to form a heterogeneous population of *β*-sheet rich protofibrils (red), which includes pore-like structures (electron micrograph, image-averaged, 10-12nm outer diameter, from (Lashuel et al., 2002b). Insertion of the protofibril pore into a membrane, which is thought to account for protofibril permeabilizing activity, is shown schematically. The protofibril population dissipates as amyloid fibrils (orange rods) are formed. Eventually, the fibrils coalesce to form a unique cytosolic proteinaceous inclusion. A Lewy body (see part 2.3) is shown in the tissue section at the far right. [Modified from (Volles and Lansbury, 2003)]

Recently, it has been suggested that α-synuclein fibrils may in fact be neuroprotective as a cellular response to sequester the toxic protofibrils (Caughey and Lansbury, 2003) and α-synuclein aggregates are formed as a compensatory attempt to sequester neurotoxic molecules (Tanaka et al., 2004). However, ultimately, the accumulation of a large volume

of non degradable protein aggregates would be predicted to crowd cellular machinery and impair cellular transport, thereby inducing cell death.

In addition it is conceivable that formation of protofibrils and aggregation of α-synuclein during the pathological process of α-synucleinopathies reduces the bioavailability of the physiological form of α-synuclein and may dampen the "anti-apoptotic" and neuroprotective effects of physiological concentrations of α-synuclein.

2.2 Possible role of proteasome inhibition on α-synuclein fibril formation

Understanding of the normal cellular α-synuclein processing may give clues about potential reasons for α-synuclein aggregation and subsequent toxicity (Figure 4). The ubiquitin proteasome system (UPS) is the primary pathway for degrading and clearing proteins that have been damaged or are no longer needed (Friguet et al., 2000). To enter this pathway, proteins are tagged with ubiquitin and then enter the large multiprotein proteasome complex, where they are broken into peptide fragments (Weissman, 2001). The UPS appears to be the major machinery responsible for degradation of α-synuclein, although direct catabolism by the proteasome 20S core (Tofaris et al., 2001) and autophagy (Cuervo et al., 2004; Webb et al., 2003) has been reported. Prior to the discovery that α-synuclein was the dominant component of cytosolic proteinaceous inclusions found in many neurodegenerative diseases, ubiquitin antigenicity was the most reliable immunohistochemical marker of these inclusions (Gai et al., 2000; Lennox et al., 1989). In addition to ubiquitin, which polymerizes on lysine residues of substrate proteins targeted for proteasomal catabolism, numerous enzymes and proteins related to the machinery of protein degradation have been identified in α-synuclein fibrous cytoplasmic inclusions,

including ubiquitin carboxyl-terminal hydrolase, a deubiquitinating enzyme, chymotrypsin A and proteasome subunits (Lowe et al., 1990). The common appearance of components of the UPS machinery in α-synuclein aggregates suggests an unsuccessful attempt of degradation by the proteasome (Gai et al., 2000; Sharma et al., 2001). Finally, studies have shown that PD patients have significantly reduced levels of proteasomal subunits in the substantia nigra pars compacta compared to age-matched healthy controls, and proteasomal function is impaired in idiopathic PD, further strengthening the links between UPS dysfunction and α-synuclein mismetabolism (McNaught et al., 2003; McNaught and Jenner, 2001; McNaught and Olanow, 2003).

The level of protofibrils and aggregated forms of α-synuclein is likely to be modulated through the ubiquitin-proteasome pathway (Giasson and Lee, 2003), although a causal link between a decrease in proteasomal degradation and high amounts of α-synuclein aggregates has not yet been demonstrated *in vivo*. The UPS is clearly saturable and excess aggregated protein leads to saturation of proteolysis systems, inducing feed-forward toxicity (Bence et al., 2001). In fact, α-synuclein overexpression potentiates proteasome-inhibitor mediated apoptosis (Tanaka et al., 2001) and its aggregation is enhanced by proteasome inhibition (Dawson and Dawson, 2003; Maries et al., 2003; Rideout et al., 2001).

α-synuclein could also inhibit the proteasome through a direct interaction. It was shown that both monomeric and aggregated α-synuclein impair proteasome function (Stefanis et al., 2001) by binding directly to Tat-binding protein 1, a component of the 19S regulatory subunit of the proteasome (Ghee et al., 2000). In the latter study, fibrillar α-synuclein had a much stronger inhibitory effect on the activity of the proteasome compared to the monomeric protein. Taken together, these data imply that impairment of the ubiquitin

proteasome pathway may contribute to the pathophysiology of the neurodegenerative diseases defined by the mismetabolism of α-synuclein.

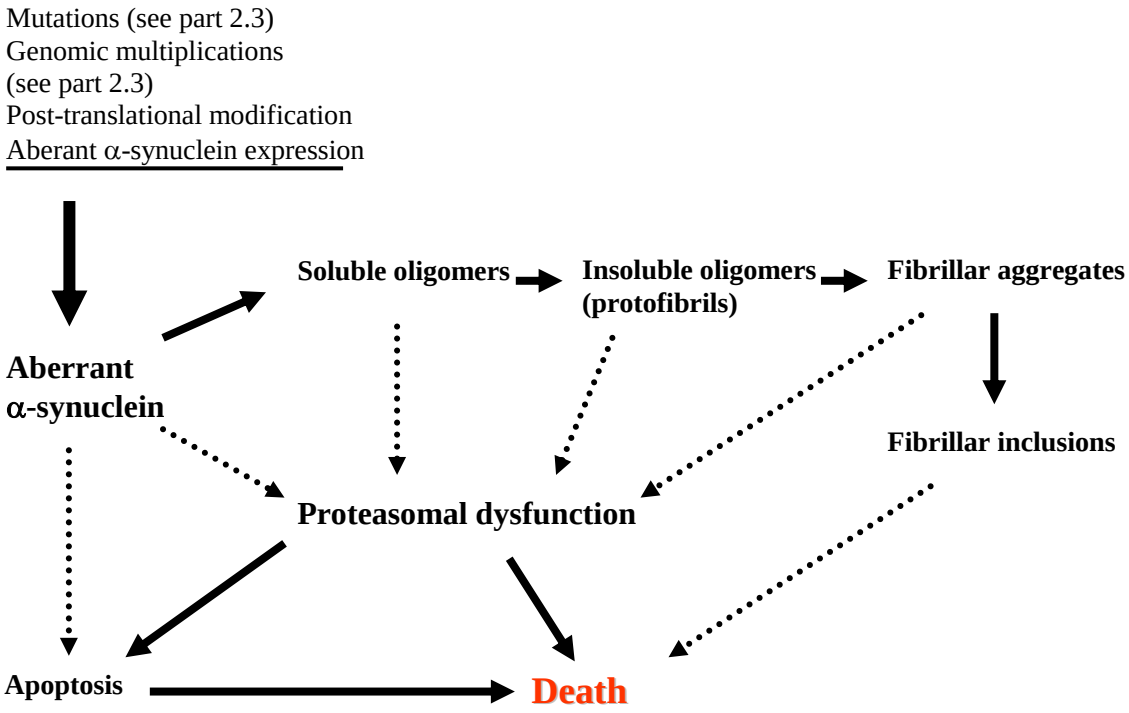


Figure 4: Postulated mechanisms of α-synuclein mediated toxicity. The presence of cytosolic aggregates in α-synucleinopathies is caused by a failure to properly dispose of α-synuclein. α-synuclein protofibrils and intracellular protein aggregates have been shown to impair the proteasome potentially leading to enhanced accumulation. Solid arrows indicate more established pathways, whereas dotted arrows indicate largely hypothetical mechanisms.

2.3 α-Synuclein aggregation in neuronal and glial diseases: α-synucleinopathies

α-synuclein was first associated with a neurodegenerative disease when a fragment of α-synuclein corresponding to the NAC domain was detected in amyloid plaques in Alzheimer's disease patient brains (Iwai et al., 1996; Masliah et al., 1996; Takeda et al., 1998a; Takeda et al., 1998b; Uéda et al., 1993). This finding stimulated great interest among researchers in a potential role for this protein in Alzheimer's disease pathogenesis. However, further research failed to confirm that the NAC peptide is an integral component of senile plaques (Bayer et al., 1999; Culvenor et al., 1999).

The discovery of α-synuclein in Lewy bodies in PD was followed quickly by its detection in cellular inclusions in several other neurodegenerative diseases including DLB (Baba et al., 1998; Spillantini et al., 1998a; Spillantini et al., 1998b), Hallervorden-Spatz syndrome, now known NBIA-1 (Arawaka et al., 1998; Saito et al., 2000), and MSA (Arawaka et al., 1998; Arima et al., 1998b; Fujiwara et al., 2002; Gai et al., 1999; Saito et al., 2000; Tu et al., 1998; Wakabayashi et al., 1998). Collectively, these neurodegenerative diseases that share α-synuclein pathology as a primary feature have come to be known as α-synucleinopathies (Arawaka et al., 1998; Arima et al., 1998b; Fujiwara et al., 2002; Saito et al., 2000; Spillantini et al., 1998b; Tu et al., 1998; Wakabayashi et al., 1998)

a Parkinson's disease (PD)

PD is a progressive neurodegenerative disease mainly affecting people from 65 years old and over (Lim et al., 2002). The primary pathology of PD is degeneration of the dopaminergic neurons of the substantia nigra pars compacta (Lang and Lozano, 1998a; Lang and Lozano, 1998b), leading to slowed movement, rigidity, rest tremor and gait

disturbances. As the disease progresses, many patients develop cognitive dysfunction, which includes dementia (Lim et al., 2002). In contrast to other neurodegenerative disorders, there is relatively efficient symptomatic therapy for PD. This mainly consists of DA replacement and surgical therapy (deep brain stimulation) that relieves most motor symptoms. However, there is no proven therapy to prevent cell death or restore sick neurons to a normal state. Dopaminergic neurons contain intracytoplasmic inclusions called Lewy bodies (LBs), which contain mainly α-synuclein fibrils (~10nm) (Spillantini et al., 1998b). It is believed that α-synuclein, in patients with PD, undergoes a conformational change acquiring a predominantly β-pleated sheet structure that facilitates polymerization of α-synuclein into amyloid fibrils (McNaught and Jenner, 2001). Dystrophic ubiquitin-positive neurites associated with PD pathology, known as Lewy neurites (LNs), are also α-synuclein positive (Braak et al., 1999; Spillantini et al., 1998b; Spillantini et al., 1997). Immunoelectron microscopy studies of the ultrastructure of synuclein in LBs, have demonstrated that LBs consisted of α-synuclein forming radially filaments surrounding an unstructured α-synuclein core.

Genetic studies have also highlighted the significant contribution of α-synuclein to the etiology of PD. α-synuclein is a key genetic factor implicated in the pathogenesis of PD, as three substitution mutations in the gene encoding α-synuclein appear to be linked to hereditary PD (Krüger et al., 1998; Polymeropoulos et al., 1997; Zarranz et al., 2004).

However, α-synuclein mutations causing disease have only been found in a small number of kindreds afflicted with familial PD and analysis of large number of patients with idiopathic PD (Chan et al., 1998) or other forms of inherited PD (Scott et al., 1999) failed to reveal mutations in α-synuclein gene. The extreme rarity of α-synuclein mutations has therefore prompted investigators to examinate polymorphisms that may be linked to PD.

There is some evidence that a dinucleotide repeat polymorphism located at a significant distance upstream in the promoter region of the α-synuclein gene may be associated with increased risk of PD in Caucasians (Farrer et al., 2001; Kruger et al., 1999). Similar observations concerning the variability of these repeats and allelic length as a risk for PD have been obtained by independent studies in an Asian population (Tan et al., 2000). Another study showed that this region may play a role in transcriptional regulation of α-synuclein (Chiba-Falek and Nussbaum, 2001); thus α-synuclein may also play a role in PD pathogenesis through alterations in its transcriptional control.

The recent identification of genomic triplication in α-synuclein gene suggests that overexpression of α-synuclein causes pathology in neurons. Singleton and colleagues reported a triplication in a region of chromosome 4 that includes the α-synuclein locus and an estimated 17 other genes (Singleton et al., 2003). Carriers of this triplication are predicted to have four functional copies of α-synuclein, arguing for an increased gene dosage as a cause for PD. α-synuclein locus duplication has also been reported to cause PD phenotype which, by contrast with α-synuclein triplication families, closely resembles idiopathic PD with late age-of-onset, slow progression, and no prominent cognitive decline nor dementia (Ibanez et al., 2004).

b Dementia with Lewy Bodies (DLB)

DLB is considered to be the second most common form of neurodegenerative dementia after Alzheimer's disease (McKeith et al., 1996). The age of onset is typically between the ages of 60 to 80 and males may be a greater risk. The average length of duration is around 6 years but is variable. The disease typically has a rapid, fluctuating progressive course. Scientists continue to search for a specific course of therapy for people with DLB. Treatment is symptomatic, often involving the use of medication to control the

parkinsonian and psychiatric symptoms. However, antiparkinsonian drugs that may help to reduce tremor and loss of muscle movement may actually worsen such symptoms as hallucinations and delusions. Recently, several clinical trials using cholinesterase inhibitors have shown its efficacy in the symptomatic treatment of cognitive impairment in DLB patients (Aarsland et al., 2004; Werber and Rabey, 2001).

Pathologically, DLB patient brains contain a large number of α-synuclein-positive LBs in neurons from the substantia nigra pars compacta like in PD and in the cerebral cortex, which is less common in PD (McKeith et al., 1996; Spillantini et al., 1997). The number of LBs correlates with the severity of the dementia (Hurtig et al., 2000). In addition to LB pathology, LNs are fairly abundant in the striatum of DLB brains, and these inclusions may contribute to the parkinsonian symptoms in DLB (Duda et al., 2002). Because LBs and LNs in DLB are so similar to those found in PD and are also mostly composed of filamentous α-synuclein protein (Baba et al., 1998; Crowther et al., 2000; Spillantini et al., 1998b; Spillantini et al., 1997), PD and DLB may share a common disease mechanism.

c Neurodegeneration with Brain Iron Accumulation Type 1 (NBIA-1)

NBIA-1 is a rare familial and sporadic neurodegenerative disorder (Jellinger, 1973). Disease onset is typically in late adolescence or early adulthood and the disease is unremitting in its progression (Jellinger, 1973). The clinical symptoms include motor disabilities and rigidity, dementia and dystonia (Jellinger, 1973). The pathology in NBIA-1 consists of cerebral atrophy and lesioning in the globus pallidus; however, an intense accumulation of (Jankovic et al., 1985) in the substantia nigra pars compacta and globus pallidus is the prominent pathological hallmark of NBIA-1 (Dooling et al., 1974). Axonal spheroids, LB-like lesions, and glial inclusions composed of insoluble, fibrillar α-synuclein are also present in brain of NBIA-1 patients (Galvin et al., 2000). These inclusions are

found mostly throughout the cortex, subcortical regions, and brain stem (Arawaka et al., 1998; Saito et al., 2000; Tu et al., 1998).

3 MULTIPLE SYSTEM ATROPHY (MSA)

3.1 Clinical characteristics and etiology of MSA

In 1900, Dejerine and Thomas described 2 patients with a degenerative disorder leading to progressive cerebellar dysfunction, parkinsonism and prominent dysautonomia. Upon postmortem examination of these brains, neurodegeneration was found in the inferior olives, pons and cerebellum, and the disease was termed olivopontocerebellar atrophy (OPCA). In 1960, Shy and Drager described a neurological syndrome (Shy-Drager syndrome) of orthostatic hypotension in patients who also displayed parkinsonian features. Following this report, Van de Eecken, Adams, and van Bogaert reported more cases with the features of OPCA and Shy-Drager syndrome associated with striationigral degeneration and atrophy of the caudate nucleus and putamen; this disorder was named striatonigral degeneration (Adams et al., 1964). In 1969, Graham and Oppenheimer noted that the clinical and pathologic findings of OPCA, striationigral degeneration, and Shy-Drager syndrome overlapped significantly although reflecting degeneration of separate neuronal subsystems. They proposed the term multiple system atrophy (MSA) to describe patients with these disorders. Since then, the discovery of the specific pathological lesions unique to MSA, known as glial cytoplasmic inclusions (GCIs; Figure 5), has confirmed that these diseases are clinical forms of the same syndrome (Papp et al., 1989a). The lumping concept of MSA was detailed further in the clinical paper of Quinn which brought attention to the disease by introducing a first set of diagnosis criteria (Quinn, 1989).

The median age of onset is 55 years, but ranges from 33 to 76 years of age and affects more men than women (Wenning et al., 1995). The symptoms of MSA progress faster than those of PD with an average patient survival of 6 to 7 years (Wenning et al., 2004). Approximately 0.31 % of people over 65 years old suffer of MSA (Trenkwalder et al., 1995). Assuming that there exist worldwide 600 million people older than 65, we extrapolate that 1.7 million may suffer from MSA. However, the true prevalence of MSA has been underestimated, since many patients remain undiagnosed or misdiagnosed. In fact, MSA is a difficult diagnosis (especially early in the clinical course) and is clinically commonly confounded with PD at the initial stage of illness. Approximately 10% of patients diagnosed in life as having PD are found at autopsy to have MSA (Hughes et al., 1992). MSA is suggested when (1) disability progresses rapidly, (2) patients are poorly responsive to levodopa, (3) autonomic symptoms, ranging from impotence to urinary incontinence to orthostatic hypotension (drop in blood pressure when patient stands upright), are pronounced, (4) rigidity and bradykinesia are out of proportion to tremor and (5) severe dementia is absent. Mild dementia is not uncommon, but severe dementia is atypical in MSA (Wenning et al., 1997b).

Recently, two subtypes of MSA have been proposed (Gilman et al., 1999). Parkinsonism predominant (MSA-P; 80% of MSA cases) and cerebellar ataxia predominant (MSA-C; 20% of MSA cases) forms of the disease can be distinguished. The different symptoms can be explained in part by the distinct brain regions affected: striatonigral degeneration in MSA-P and olivopontocerebellar atrophy in MSA-C. However, the factors determining the regional specificity of neurodegeneration in MSA-P and MSA-C remain to be elucidated.

The cause of MSA remains unknown and no current therapy can reverse or halt progression of the disease. A genetic component seems unlikely and screening studies for candidate genes revealed no risk factors (Bandmann et al., 1997; Nicholl et al., 1999). Autoimmune mechanisms and environmental toxins have been suggested to be involved in MSA pathogenesis (Hanna et al., 1999), but evidence for these etiologies is weak

3.2 Neuropathological features of MSA

Histopathological findings include moderate depigmentation of the substantia nigra pars compacta and locus ceruleus, and demonstrate neuronal and oligodendroglial cell loss as well as gliosis in striatum (mainly putamen), substantia nigra pars compacta, inferior olives, pons, cerebellum in addition to the intermediolateral cell columns and Onuf's nucleus in the spinal cord (Wenning et al., 1997a). Microvacuolation within the involved neuronal systems has also been reported. These findings are present to a lesser extent even in regions where atrophy is not noted.

Initially, it was assumed that MSA was caused by gray matter damage. Neuronal inclusions have been observed in both the cytoplasm and nucleus of neurons in patients with MSA (Papp and Lantos, 1992). However, the discovery in 1989 by Papp and colleagues of GCIs being the hallmark lesion in MSA brains suggests that damage is primarily in the white matter (Figure 5) (Papp et al., 1989b). GCIs are triangular shaped argyrophilic inclusions and, like LBs and LNs, are composed mostly of insoluble filamentous polymers of α-synuclein protein (Dickson et al., 1999; Duda et al., 2000; Duda et al., 2002; Gai et al., 2003; Gai et al., 1998; Tu et al., 1998). Post-translational modifications, including phosphorylation at Ser129 (Fujiwara et al., 2002) and oxidative

nitration (Giasson et al., 2000) are prominently visualized in GCIs of MSA patients (Fujiwara et al., 2002). α-synuclein mRNA has not been detected by in situ hybridization in oligodendrocytes from normal human adult brain (Solano et al., 2000b); however, it is transiently expressed in cultured oligodendrocytes and increased to peak levels at 2 or 3 days (Richter-Landsberg et al., 2000). It is not understood how in MSA α-synuclein gets into oligodendrocytes (by uptake from damaged fiber tracts or by *de novo* synthesis).

The distribution of GCIs correlates with the subtypes of MSA, with putaminal lesions prevalent in patients with the MSA-P subtype and pontine lesions prevalent in patients with the MSA-C subtype (Arima et al., 1992; Duda et al., 2000; Papp et al., 1989b; Papp and Lantos, 1994b). Furthermore there is significant correlation between the frequency of GCIs and the severity of neuronal cell loss and disease duration (Ozawa et al., 2002).

Although GCIs resemble LBs, their shape, size, ultrastructure, imunocytochemical profile, and regional distribution are quite distinctive. GCIs are composed of loosely aggregated 20- to 30-nm tubular filaments intensely labeled with α-synuclein oligomers forming the central core fibrils of the glial inclusions (Gai et al., 2003). Immunoelectron microscopy studies have demonstrated that α-synuclein filaments in GCIs are wider in diameter than those of LBs and LNs and are sometimes found in a twisted formation (Crowther et al., 2000; Spillantini et al., 1998a). The composition of GCIs is slightly different from LBs. The latter mainly contain α-synuclein along with some ubiquitin and neurofilament subunits; GCIs of MSA mainly contain α-synuclein in addition to αB-crystallin, ubiquitin, microtubule-associated proteins and α- and β-tubulin, but lack neurofilament subunits (Arima et al., 1998b; Gai et al., 1999; Tamaoka et al., 1995). GCIs frequently contain markers of cellular stress such as heat shock protein 70 (Namba et al., 1991), which is induced by various forms of cell stress.

Increasing evidence indicates that the formation of GCIs leads to cellular dysfunction. It has been proposed that GCIs often entrap cytoplasmic organelles (eg, mitochondria, secretory vesicles) and disrupt normal protein and organelle trafficking within affected cells (Arima et al., 1998a; Tu et al., 1998). These chronic alterations may lead to glial cell dysfunction and death and may impair trophic function between oligodendrocytes and axons, thereby causing neuronal damage.

It was shown recently that extensive myelin abnormalities occur in the white matter of the involved regions in MSA. Matsuo and colleagues reported extensive myelin degeneration in MSA brains (Matsuo et al., 1998) using antibodies recognizing degenerating myelin but not normal appearing myelin (Matsuo et al., 1997). The presence of unusual myelin basic protein epitopes in MSA, in both affected and unaffected brain regions, substantiates the notion of widespread oligodendroglial dysfunction in MSA and highlights white matter disease as an integral component. The dysmyelination observed in MSA results from dysfunction of the oligodendrocytes, the cells which produce and maintain myelin in the CNS (see part 4.1).

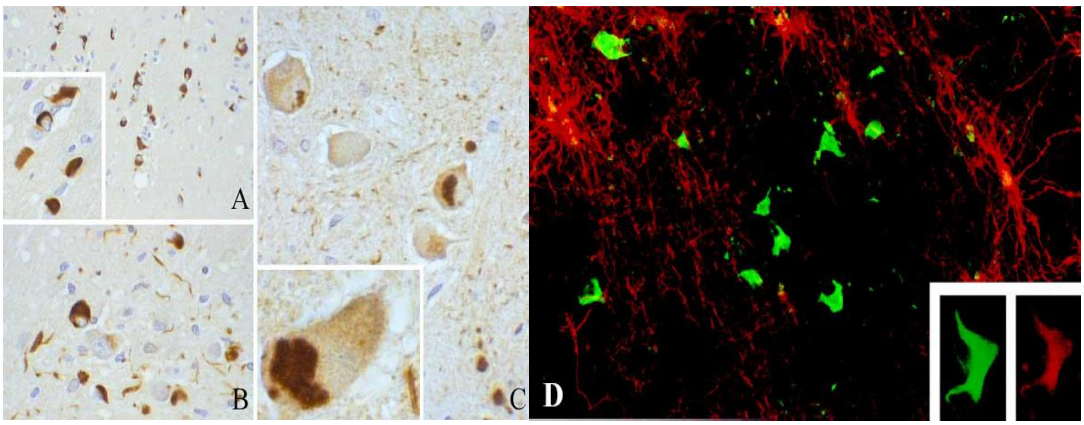


Figure 5: α-synuclein pathology in multiple system atrophy. Immunostaining for α-synuclein in paraffin sections (**A, B,** and **C**) in MSA. Note many GCI in white matter in basal ganglia (**A** and **inset**). In the pons (**B** and **C**) was a mixture of glial and neuronal inclusions. The neuronal inclusions were cytosolic and pleomorphic (**C** and **inset**). **D:** Double immunostaining of Vibratome sections with GFAP

(rhodamine filter, red) and α-synuclein (fluorescein filter, green). Note the absence of colocalization of the two signals. **Inset** shows a GCI that is double labeled with α-synuclein (fluorescein filter, green) and C4d (rhodamine filter, red) [from Dickson, 1999]

3.3 Animal models of MSA

The adverse consequences of α-synucleinopathy in MSA are not understood to date. In order to shed light on the molecular pathology of α-synuclein in these diseases, several animal models have been generated (Fillon and Kahle, 2005; Stefanova et al., 2005c). Animal models of MSA are urgently required as test-bed for the evaluation of novel therapeutic interventions in the disorder. Initial attemps to mimic MSA pathology in rodents relied on the destruction of brain regions that degenerate in human MSA-P (Wenning et al., 1999). The nigral and striatal "double-lesion" rat model is based on sequential injection of 6-hydroxydopamine and quinolinic acid into the medial forebrain bundle and ipsilateral striatum, respectively ("double toxin-double lesion" approach). Intrastriatal injections of 3-nitropropionic acid and MPP^+ in rodents result in secondary excitotoxic striatal lesions and subtotal neuronal degeneration of substantia nigra pars compacta, thus producing MSA-P like pathology by a simplified "single toxin-double lesion" approach. However, such models do not show the neuropathological hallmarks of MSA. Thus, chemical lesion models are of limited use for the investigation of MSA etiopathogenesis. To gain further insight into the molecular mechanisms of MSA, transgenic mouse models are currently being developed.

An early transgenic model of glial α-synucleinopathy relied on imperfect neuronal specificity of the including platelet-derived growth factor β (PDGFβ) promoter. Some transgenic α-synuclein expression was detected in glial cells of (PDGFβ)-α-synuclein mice, but α-synuclein fibrillization could not be demonstrated (Rockenstein et al., 2002).

In situ hybridization with transgene-specific probes directly demonstrated that the glial α-synuclein protein was expressed from glial mRNA (Rockenstein et al., 2002). Glial α-synuclein was not observed in mice that expressed transgenic α-synuclein under the control of the highly neuron-specific Thy1 promoter (Kahle et al., 2000; Rockenstein et al., 2002; van der Putten et al., 2000). These mouse model data do not support the hypothesis that the neuronal protein α-synuclein transmigrates from axons into oligodendrocytes in MSA patients.

Specific, long-lasting expression of transgenic α-synuclein in oligodendrocytes throughout the brain was achieved with a myelin proteolipid protein (PLP) promoter (Kahle et al., 2002b). Unlike the disease-irrelevant control green fluorescent protein expressed under control of the same promoter, which evenly distributed throughout oligodendrocytes, α-synuclein was asymmetrically clustered in the cytosol of oligodendrocytes. The transgenic α-synuclein-positive profiles resembled the typical triangular and half moon-shaped GCIs of MSA patients (Lantos, 1998) at the light microscopic level. Moreover, the pathological phosphorylation at Ser129 was recapitulated in (PLP)-α-synuclein mice. Biochemical analysis confirmed the pathological insolubility of hyperphosphorylated, transgenic α-synuclein in this mouse model. However, in contrast to transgenic mice expressing high levels of α-synuclein in neurons (Neumann et al., 2002), oligodendroglial transgenic α-synuclein did not fibrillize upon aging. Possibly the employed oligodendrocyte-specific promoters do not lead to very high cytosolic α-synuclein concentrations, or oligodendrocytes have a greater capacity to suppress α-synuclein misfolding, either because of an abundance of small heat shock proteins (such as αB-crystallin) (Neri et al., 1997) or because oligodendrocytes have a lower vulnerability toward pro-aggregative risk

factors than aged neurons. One such risk factor may be exposure to environmental toxins. Transgenic (PLP)-αSYN mice exposed to the neurotoxin 3-nitropropionic acid displayed exacerbated neuronal loss, astrogliosis and microglial activation (Stefanova et al., 2005b). The MSA-like pathology in this combined α-synuclein / 3-nitropropionic acid mouse model caused severe motor impairment.

Another risk factor might be concomitant tauopathy. Tau protein, the microtubule-associated protein that is the major structural component of neurofibrillary tangles in Alzheimer's disease and progressive supranuclear palsy, is like α-synuclein a natively unfolded protein with little secondary structure that forms pathologic filaments with unusual solubility properties and protease resistance (Dickson et al., 1999). When a 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) promoter was employed to express human α-synuclein in transgenic mouse oligodendrocytes, no particular pathology and phenotype were initially noted unless these animals were cross-bred with mice that express [P301L]tau in oligodendrocytes (Giasson et al., 2003a). A subset of bigenic oligodendrocytes developed α-synuclein and tau fibrillar inclusions, as demonstrated by thioflavin S staining. Such amyloid formation coincided with motor impairments. It is tempting to speculate that in oligodendrocyte cytosol, α-synuclein and tau synergistically form individual fibrils, as was shown *in vitro* (Giasson et al., 2003a). Tau pathology has been noted in some MSA individuals (Giasson et al., 2003b; Jaros and Burn, 2000; Piao et al., 2001a) but more systematic studies are warranted to distinguish the potentially synergistic co-fibrillization of tau and α-synuclein from, e.g., co-morbid MSA and progressive supranuclear palsy.

Recently, Yazawa et al. (2005) followed the (CNP)-α-synuclein mice during aging and found a slowly progressive neurodegenerative condition associated with transgenic α-

synuclein accumulation in oligodendrocytes in an age-dependent manner, leading to a primary loss of the glial cells and a secondary neuronal degeneration (Yazawa et al., 2005). The insoluble fibrillar α-synuclein inclusions show striking resemblance to GCIs. The (CNP)-α-synuclein mouse has a normal life span; however, starting at three months of age, it begins to lose motor skills and paw strength. The decline is associated with brain atrophy especially severe at 2 years compared to littermates. In addition, neuronal and oligodendroglial cell loss is observed in the spinal cord. Electon microscopy analysis reveals degenerating glial cells and autophagocytosis of myelin. Neurons likewise show markers of injury and structural evidence of degeneration. Interestingly, neurons in older (CNP)-α-synuclein mouse express higher levels of endogenous mouse α-synuclein in their axons, presumably in response to oligodendrocyte degeneration.

Shults and colleagues tried to mimic MSA in a mouse by expressing human α-synuclein under the glial-specific MBP promoter (Shults et al., 2005). α-synuclein was highly expressed only in oligodendrocytes and formed detergent insoluble inclusions, which also contained phosphorylated α-synuclein. α-synuclein aggregation in oligodendrocytes was accompanied by mitochondrial alterations, and the white matter tracts of these animals displayed intense astrogliosis, myelin pallor, and decreased neurofilament immunostaining. Furthermore, widespread signs of neurodegeneration including decreased dendritic density and loss of dopaminergic were also observed in these mice. Starting at 2–4 months of age, the MBP-α-synuclein mice expressing high level of α-synuclein develop neurological alterations including tremors, ataxia, and seizure activity resulting in premature death at 6 months. Lower expressing mice show performance deficits at 6 month of age, presumably in response to oligodendrocyte degeneration. In fact, their decline was associated with the highest concentration of a-synuclein immunoreactive inclusions in oligodendrocytes

(Shults et al., 2005). This model further suggests that accumulation of insoluble filamentous α-synuclein in oligodendrocytes, leads to a primary loss of the glial cells and a secondary neuronal degeneration.

The major breakthrough demonstrated with the (CNP)-α-synuclein and (MBP)-α-synuclein mice is the identification of a secondary neurodegeneration following oligodendrocyte α-synucleinopathy. This provides evidence that neuronal degeneration can occur as a direct consequence of oligodendrocytic GCI-like pathologies and suggests that aberrant expression of α-synuclein in oligodendrocytes could be sufficient to cause MSA. However, the mechanisms leading to oligodendrocyte loss and subsequent neurodegeneration in this model remain unknown. Although it remains to be shown that oligodendrocytes induce the α-synuclein gene in human MSA patients, the (CNP)-α-synuclein and (MBP)-α-synuclein mice may allow the study how oligodendroglial α-synucleinopathy impairs myelination and axonal integrity. Moreover, as models of glial-driven neurodegeneration, they may be useful to test novel therapeutic approaches to treat MSA.

The different transgenic animal models for MSA provide insights into various aspects of the oligodendroglial phenotype and its role as a target in the disease, and are quite persuasive in implicating α-synuclein in the pathogenesis of MSA. These models indicate that a combination of genetic predisposition (ectopic α-synuclein expression in oligodendrocytes) and epigenetic factors (environmental toxins (Stefanova et al., 2005a), aging (Shults et al., 2005; Yazawa et al., 2005)) may contribute to MSA and represent useful tools to identify important pathways involved in α-synuclein toxicity which could be exploited for novel therapeutics in the treatment of MSA.

4 OLIGODENDROCYTES: POTENTIAL PRIMARY TARGETS IN MSA

Neurodegeneration in MSA affects selected gray matter "systems" whereas its hallmark inclusion-bearing cells are mostly found in white matter. Implicating the oligodendrocyte as the primary target, therefore, means providing a mechanism for region-specific oligodendrocyte dysfunction or a mechanism whereby oligodendrocyte dysfunction leads to pronounced destruction of selected vulnerable neuronal populations. To date there is little evidence that oligodendrocytes may be subtyped according to the neuronal populations they subserve. Older classifications of Mori and LeBlond (Mori and Leblond, 1970) and Stensaas and Stensaas (Stensaas and Stensaas, 1968) subtyped oligodendrocytes based on fine structural features of the oligodendrocyte nucleus and processes and myelin sheaths, respectively, providing indirect evidence for functional specialization, but no conclusive evidence for tract specificity. Additionally, single oligodendrocytes have been shown to myelinate axons of different anatomical tracts, casting some doubt on the possibility that oligodendrocytes may be classified based on the functional properties of corresponding axons (Sternberger et al., 1978). In MSA, GCIs are reported to involve so-called perivascular, perifascicular, and perineuronal oligodendrocytes without further qualification as to subtype. In essence, then, GCIs involve all morphological types of oligodendrocytes with varying frequency in specific anatomical regions (Lantos and Papp, 1994; Papp and Lantos, 1994a). It therefore appears that oligodendroglial disturbance in MSA, whether or not it occurs primarily, tends to be more generalized and would likely have to occur in conjunction with intrinsic vulnerability of selected neuronal populations, the nature of which remains to be determined. It is noteworthy in this regard that axonal damage is known to occur in otherwise classical demyelinating conditions (Griffin et al.,

1996; Trapp et al., 1998). Furthermore, the critical trophic influences that are described between oligodendrocytes and axons (Kaplan et al., 1997) indicate that oligodendroglial pathology would likely affect neuronal function.

4.1 Oligodendrocyte functions in the CNS

Glial cells represent the vast majority of cells in the CNS and outnumber neurons by 10 to 1 and occupy half of the CNS space (Kettenmann 1995; Zhang 2001). These cells help to construct the nervous system during embryonic development and maintain its functions. Rio Hortega introduced the term *oligodendroglia* to describe neuroglial cells with few processes in material stained by metallic impregnation techniques (Baumann and Pham-Dinh, 2001). An oligodendrocyte extends many processes, each of which contacts and repeatedly envelopes a stretch of axon with subsequent condensation of this multispiral membrane-forming myelin (Bunge et al., 1962; Bunge, 1968) (Figure 6). The main and evident function of oligodendrocytes is the formation of a myelin sheath around most axons in the CNS (Figure 6). Myelin functions as an insulator of the axons, and its structure facilitates rapid transmission of impulses. Oligodendrocytes are able to myelinate up to 50 axonal segments, depending on the region of the CNS. On the same axon, adjacent myelin segments belong to different oligodendrocytes.

In addition of being an insulating sheath that allows neurons to conduct electrical signals at high speed, myelin is involved in clustering of sodium channels at the node of Ranvier (figure 6) during axogenesis, in axonal development and maintenance. Finally, myelin regulates axonal growth and regeneration (Baumann and Pham-Dinh, 2001). Myelin contains proteins such as Nogo (Schwab, 2004) have been demonstrated to contribute to regenerative failure after CNS injury and to the limitation of lesion-induced plasticity.

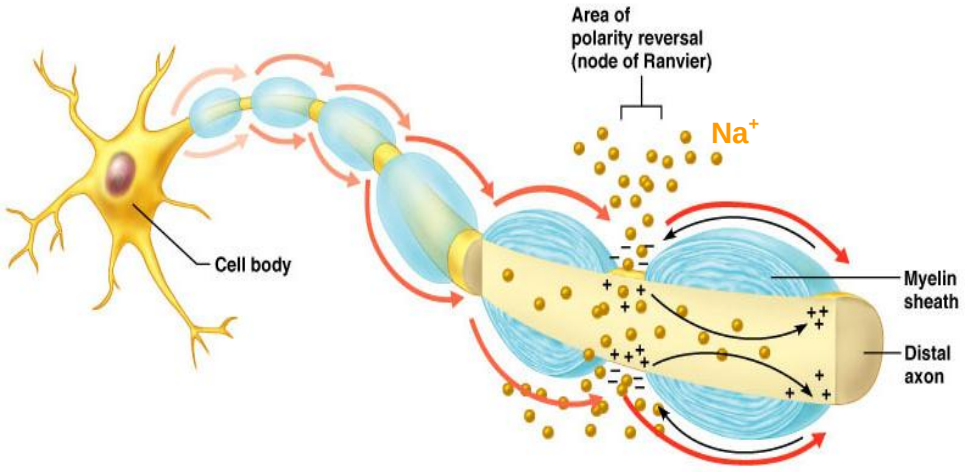


Figure 6: The oligodendrocyte and the mature myelin sheath. An oligodendrocyte sends a glial process forming compact myelin spiraling around an axon. Cytoplasm is trapped occasionally in the compact myelin. In transverse sections, this cytoplasm is confined to a loop of plasma membrane, but along the internode length, it forms a ridge that is continuous with the glia cell body. In the longitudinal plan, every myelin unit terminates in a separate loop near a node. Within these loops, glial cytoplasm is also retained. The tight wrapping of myelin prevents any ionic exchange or spread of electric current, therefore the action potential can only occur at the nodes of Ranvier which are devoid of myelin. As shown above, when the action potential is present at one node, the influx of Na^+ ions causes the displacement of K^+ ions down the axon. Thus the action potential jumps from node to node. Saltatory conduction increases conduction velocity and is very energy efficient, as only a small part of the axon is involved in the exchange of ions, much fewer ions need to be pumped back after the action potential has passed. [Adapted from Bunge et al., 1968]

Although the oligodendrocyte is mainly a myelin-forming cell, there are also satellite oligodendrocytes that may not be directly connected to the myelin sheath. Satellite oligodendrocytes are located in the grey matter, perineuronal and regulate the microenvironment around neurons (Ludwin, 1997).

4.2 Origin of oligodendrocytes

a Neuron-glia decision

Both intracellular factors as well as extracellular factors may exert influence on whether a neural cell becomes neuron or glia. Accumulating evidence suggests that the Notch receptor promotes glial development at least partially via repressing the neurogenic basic helix-loop-helix protein (bHLH) transcription factors, promoters of neuronal fate (reviewed in (Kageyama and Nakanishi, 1997). Notch signaling instructively commit CNS stem cells into the astroglial lineage, thus fulfilling the criterion for a molecular switch between neuronal and glial fate (Morrison et al., 2000; Tanigaki et al., 2001). Neurons are generated before glial cells (Lu et al., 2000; Morrison, 2000; Yu et al., 1994; Zhou et al., 2000). Environmental factors present during early embryonic development favour neuronal development whereas those present at later stages favour glial development. Once a cell fate is chosen, other mechanisms presumably will step in to permanently lock the cell in this fate. In addition, neural stem cells may contain an internal timer that counts time or cell division and that determines the sequential production of neuron and glia (Morrison et al., 2000).

b Oligodendrocyte specification

Oligodendrocyte precursors originate from neuroepithelial cells of the ventricular zones, at very early stages during embryonic life. As mentioned before, neurogenic bHLH factors control the neuron-glia decision. The bHLH transcription factors Olig2 and Olig1 are absolutely required for oligodendrocyte fate determination (Balasubramaniyan et al., 2004; Lu and Sloan, 2002; Lu et al., 2000; Zhou and Anderson, 2002). Olig1,2 double null mutants mice or neurosphere cultures fail to give rise to any oligodendrocytes (Lu et al., 2002; Zhou and Anderson, 2002).

Sonic Hedgehog (Shh), a morphogen that regulates the transcription of different target genes at different concentrations, is a potent inducer and obligatory for oligodendrocyte

generation in both rostral and caudal neural tube (Alberta et al., 2001; Nery et al., 2001; Orentas et al., 1999; Park et al., 2002; Tekki-Kessaris et al., 2001b). Shh exerts its pro-oligodendrocyte functions by inducing Olig genes (Lu et al., 2000; Nery et al., 2001). However Olig genes cannot substitute for Shh, as shown in zebrafish where forced Olig expression largely failed to rescue the oligodendrocyte failure in the absence of Shh signalling (Park et al., 2002). Shh therefore must induce additional genes that cooperate with Olig to promote oligodendroglia fate.

Members of the bone morphogenetic proteins, on the contrary, repress Olig gene transcription even in the presence of Shh and thereby act as potent inhibitors of oligodendrocyte fate (Gross et al., 1996; Mabie et al., 1999; Mehler et al., 2000; Mekki-Dauriac et al., 2002).

In summary, oligodendroglia fate specification requires the absence of general glial repressors such as neurogenic bHLH factors and the induction of Olig genes and their partners.

c Spatial origin of oligodendrocytes

Despite the wide distribution of mature oligodendrocytes throughout the CNS in adult vertebrate, it is now well established that oligodendrocyte precursors are generated in restricted regions of the CNS. Mapping the birth place of glial cells has always been problematic due to the extensive migratory behavior of these cells. Oligodendrocyte precursors found in the mid- and forebrain originate from the ventral plate of the neural tube (Spassky et al., 1998). Converging studies suggest that oligodendrocytes predominantly come from the ganglionic eminence area and later migrate throughout the brain (Olivier et al., 2001a; Tekki-Kessaris et al., 2001a; Woodruff et al., 2001). Oligodendrocytes may also derive from other brain loci, sites of oligodendrogliogenesis

(Olivier et al., 2001b; Spassky et al., 1998). The initial restricted localization of oligodendrocyte precursors in the ventral plate of the neural tube appears not to be limited to the mid- and forebrain (Spassky et al., 1998). Earlier this year Cai et al., and Vallstedt et al., provided compelling evidence for a second dorsal origin of oligodendrocyte precursors in the hindbrain and spinal cord while oligodendrocyte precursors have previously been localized to the ventral midline (Cai et al., 2005; Vallstedt et al., 2005).

The subventricular zone is a germinal matrix of the forebrain that first appears during the later third of murine embryonic development (Doetsch et al., 1997). It enlarges during the peak of gliogenesis, between 5 and 20 days in postnatal life, and then shrinks but persists into adulthood. The majority of progenitors within this germinal matrix are glial precursors that generate astrocytes or oligodendrocytes and a rare cell will develop into both neurons and glia (Levison et al., 1993; Levison and Goldman, 1993; Luskin et al., 1988; Price et al., 1988). In cerebrum and cerebellum, oligodendrocytes arise postnatally from the SVZ of the lateral ventricles (Reynolds and Wilkin, 1988) (Levison et al., 1993; Zerlin et al., 1995). Oligodendrocyte progenitors then migrate long distances away from these zones and populate the developing brain to form white matter throughout the brain.

4.3 Oligodendrocyte precursor cell (OPC) proliferation and differentiation

a Oligodendrocyte precursor proliferation

OPCs undergo a series of differentiation steps to give rise to mature, multipolar oligodendrocytes with myelin sheets. Recently, several oligodendrocytes precursor markers have been discovered, including PDGF receptor-α (PDGFRα), Sox10, Nkx2.2, $Olig_{1/2}$ and PLP/DM-20 mRNA (Lu et al., 2000; Ono et al., 1995; Piao et al., 2001b; Timsit

et al., 1995; Timsit et al., 1992; Yu et al., 1994; Zhou et al., 2000). OPCs are actively proliferating and possess migratory properties.

A number of extracellular factors have been found to influence the proliferation of OPC. While OPC proliferates extensively in culture in the presence of PDGF, the absence or withdrawal of PDGF induces immediate terminal differentiation (Noble et al., 1988; Raff et al., 1988). Overexpression of PDGF-A in transgenic mice resulted in increased proliferation of OPC (Calver et al., 1998) whereas both the ligand (PDGF-A) and receptor (PDGFRα) knockout showed a severe loss of oligodendrocytes (Fruttiger et al., 1999; Klinghoffer et al., 2002). These experiments firmly established PDGF-PDGFRα ligand-receptor pair as one major regulator of OPC proliferation. The basic fibroblast growth factor (bFGF) is also a major inducer of OPCs proliferation (Villa et al., 2000) and the combined action of PDGF and bFGF can keep OPC in a proliferative state almost indefinitely *in vitro* (Bogler et al., 1990).

b Oligodendrocyte precursor differentiation and maturation

Oligodendrocytes differentiation and maturation takes place in the mammalian CNS largely in early postnatal life and involves morphological changes as well as expression of various antigenic markers. Oligodendrocyte progenitors are characterized by their bipolar morphology and by the presence of specific markers such as glycolipids recognized by the A2B5 antibody and a chondroitin sulfate proteoglycan called NG2 (Nishiyama et al., 1999). After their migration in the mammalian CNS, oligodendrocyte progenitors settle along fiber tracts of the future white matter and then transform into preoligodendrocytes (Figure 7), multiprocessed cells which keep the property of cell division and express the sulfatide O4 on their cell surface (Sommer and Schachner, 1981). At this stage, they are less motile (Orentas and Miller, 1996), or even postmigratory (Pfeiffer et al., 1993a), and

they lose their mitogenic response to PDGF as a consequence of PDGFR downregulation (Hart et al., 1989; Pringle and Richardson, 1993). The preoligodendrocytes become immature oligodendrocytes, characterized in the rat by a multiramified morphology, the appearance of the marker galactocerebroside (GalC) and the loss of expression of A2B5 antigens on the cell surface. CNP, the earliest known myelin-specific protein to be synthesized by developing oligodendrocytes, is expressed at the same time as GalC (Pfeiffer et al., 1993b; Reynolds and Wilkin, 1988; Sprinkle, 1989). Myelin-specific protein expression such as such as myelin basic protein (MBP), PLP and myelin associated glycoprotein (MAG) occurs 2-3 days after CNP expression, before myelin formation (Butt et al., 1995; Dubois-Dalcq et al., 1986; Hardy and Reynolds, 1993; Monge et al., 1986; Pfeiffer et al., 1993a). Then the number of processes is reduced in the mature oligodendrocytes together with the expression of myelin oligodendrocyte glycoprotein (MOG), the latest marker of oligodendrocyte maturation (Hardy et al., 1996). The mature oligodendrocytes will myelinate if the processes contact an axon. The capacity of oligodendrocyte progenitors to differentiate into oligodendrocyte, is a carefully orchestrated event *in vivo* to ensure timely myelination of the axons, and is intrinsic to the lineage (Temple and Raff, 1986). In fact, in the absence of neurons, MOG-positive oligodendrocytes can clearly make a myelin-like membrane (Sarlieve et al., 1983). Nevertheless, coculture with neurons increases myelin gene expression, such as MBP, PLP, MAG (Matsuda et al., 1997). A similar myelin-gene induction by neuronal contact with oligodendrocytes is also observed *in vivo* (Kidd et al., 1990); in premyelinating oligodendrocytes, axonal contact induces the switch from DM-20 to PLP isoform expression.

Because mature oligodendrocytes cannot migrate, preventing premature differentiation of progenitors is crucial for ensuring that they successfully make it to their final destination. Premature oligodendrocyte differentiation is effectively prevented by an inhibition mechanism recently shown to occur in gliogenesis, the Notch pathway (Wang et al., 1998). Notch activation generally leads to the expression of Hes family inhibitory basic bHLH transcription factors which inhibit terminal differentiation of optic nerve OPC (reviewed by Kageyama and Nakanishi 1997). Thus, Notch acts as a negative signal for OPC differentiation *in vivo.* The expression of Jagged, a neuronal Notch ligand, is downregulated while embryonic development progresses, allowing myelination to occur in a controlled manner (Wang et al., 1998).

Two transcription factors necessary for oligodendrocyte terminal differentiation, Sox10 and Nkx2.2, have recently been discovered. (Stolt et al., 2002; Xu et al., 2000; Zhou et al., 2000). Studies in knockout mice revealed that loss of either Sox10 or Nkx2.2 results in hypomyelination and loss of mature oligodendrocyte markers such as PLP and MBP without affecting the development of oligodendrocyte precursors (Qi et al., 2001; Stolt et al., 2002).

Recently, it was reported that the neurotransmitter adenosine released during action potential firings could potently inhibit OPC proliferation while promoting their terminal differentiation and myelination (Stevens et al., 2002).

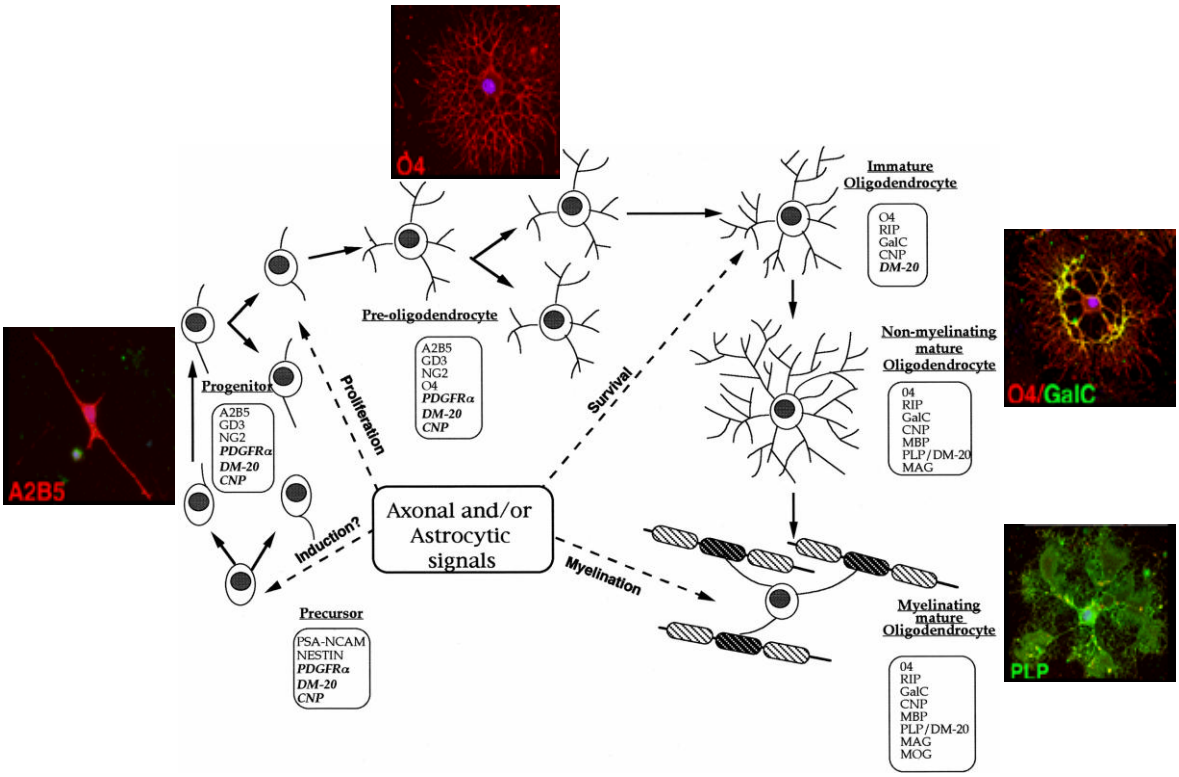


Figure 7: **Developmental stages of cells of the oligodendrocyte lineage.** Schematic drawing of the morphological and antigenic progression from precursor cells to myelinating mature oligodendrocytes, through progenitors, preoligodendrocytes, and immature non myelinating oligodendrocytes. Timing of neuronal and astrocytic signaling is indicated. Stage-specific markers are boxed. RNAs are in italics. [Modified from (Lubetzki, 1997)]

Chapter 2: SPECIFIC AIMS

In contrast to the well-studied neuronal α-synuclein pathology, both causes and effects of oligodendroglial α-synucleinopathy in MSA are more enigmatic. While the presence of α-synuclein in GCIs was identified almost one decade ago, we still do not know with certainty if and how α-synuclein provokes the disease. In mature brain, oligodendrocytes express undetectable levels of α-synuclein (Miller et al., 2005; Solano et al., 2000a). Thus, ectopic expression of α-synuclein could be causally linked to MSA pathology. To test the hypothesis that α-synuclein expression in oligodendrocytes leads to the formation of GCIs, transgenic mouse models were developed. Although key features of MSA pathology were recapitulated in transgenic animal models, neither model as yet yields any specifics on how α-synuclein aggregates in MSA and selectively injures or kills oligodendrocytes. In particular, questions remain regarding the role of α-synuclein fibrils, protofibrils, oligomers, GCIs, ubiquitination, and α-synuclein-interacting proteins in oligodendrocyte dysfunction and cell death.

The aim of my PhD thesis was to further clarify these complex issues and to investigate the molecular mechanisms leading to oligodendroglial α-synucleinopathy and its cellular consequences. Therefore, I developed an experimental system based on overexpression of human α-synuclein in primary mouse oligodendrocytes. In this context, the following questions were addressed:

SPECIFIC AIMS

1. When during oligodendrocyte differentiation is transgenic α-synuclein expressed in primary mouse oligodendrocytes derived from (PLP)-α-synuclein transgenic mice?
2. What are the cellular consequences of α-synuclein expression on transgenic oligodendrocytes?
3. What is the contribution of an impairment of the oligodendroglial ubiquitin-proteasome system to the pathogenesis of MSA?
4. Do high α-synuclein levels reached by lentiviral delivery lead to the aggregation of ectopic α-synuclein?
5. To what extent do α-synuclein aggregates in culture resemble GCIs found in MSA patients?
6. What are the cellular consequences of α-synuclein aggregation for affected oligodendrocytes?
7. Can anti-aggregative gene therapy reduce α-synuclein mediated cytotoxicity?
8. Which molecular mechanisms induce death of affected oligodendrocytes in culture? Could these mechanisms also be responsible for glial cell loss in MSA?

The establishment of a cell culture model of MSA is easier to manipulate than transgenic animals and should provide us with an experimental system to investigate the risk factors which may enhance and/or accelerate α-synuclein aggregation and the molecular mechanisms underlying oligodendrocyte cell death in MSA. Such a model provide avenues to evaluate many therapeutic compounds that could inhibit the formation of α-synuclein fibrils or rescue dysfunctional oligodendrocytes in culture, with the long term goal of identifying drug targets for MSA treatment.

Chapter 3: EXPERIMENTAL PROCEDURES

Animals

Homozygous transgenic (PLP)-α-synuclein mice were generated in which human wild-type α-synuclein was driven by a PLP promoter (Kahle et al., 2002b). Homozygous transgenic mice overexpressing the enhanced green fluorescent protein under the PLP promotor (PLP-eGFP) mice and wild-type mice with the same genetic background were used as controls.

Antibodies

The following primary antibodies were used: mouse monoclonal A2B5 (IgM, American Tissue Culture Collection, 1:5), rabbit polyclonal anti-NG2 antibody (Chemicon, 1:100), mouse monoclonal O4 (IgM, Sommer and Schachner, 1:5), mouse monoclonal anti-GalC (IgG3, 1:10, (Ranscht et al., 1982)), mouse monoclonal anti-myelin basic protein (MBP) (SMI 94 Stenberger monoclonals incorporated, 1:1000), mouse monoclonal Mab42 anti-α-synuclein antibody (1:1000, BD Transduction Laboratories), human-specific rat monoclonal α-synuclein antibody (15G7, hybridoma supernatant 1:10 (Kahle et al., 2000)), polyclonal anti-ubiquitin (Dako, 1:100), mouse monoclonal 1510 anti-ubiquitin (Chemicon, 1:1000), mouse monoclonal against the α-subunits of 20S proteasome (Affiniti, 1:100), rabbit polyclonal antibody raised against phosphorylated α-synuclein at Ser129 ((Fujiwara et al., 2002), 1:100 for cytochemistry and 1:1000 for western blot), mouse monoclonal antibody Syn303 raised against oxidized α-synuclein ((Giasson et al.,

2000), 1:200 for cytochemistry and 1:1000 for western blot), rabbit polyclonal anti-4-Hydroxy-2-Nonenal (HNE) Michael adducts (Calbiochem, 1:200), polyclonal anti-hsp 70 (Stressgen, 1:200), rabbit polyclonal anti-hsp 40 (Stressgen, 1:200), mouse monoclonal anti αB-crystallin (MBL, 1:100), rabbit polyclonal anti 14-3-3 antibody (Cell signaling, 1:100), rabbit polyclonal p25 antibody (gift from PH. Jensen, 1:100), rabbit polyclonal activated caspase 3 (PharMingen, 1:300), monoclonal anti-GFP antibody (Clontech, 1:1000), rabbit polyclonal anti-Fas antibody (C20, Santa Cruz, 1:50 for immunohistochemistry), mouse monoclonal anti-Fas antibody (1:1000 for western blot, BD Transduction Laboratories).

Secondary fluorescent antibodies were Alexa Fluor 488- and 594-conjugated goat anti-mouse, rabbit or rat antibodies (Molecular Probes, 1:1000). Peroxidase-conjugated anti-mouse, -rat and -rabbit IgGs were purchased from Sigma (1:5000).

Purification and Culturing of Oligodendrocyte Precursor Cells

OPCs were purified from newborn (PLP)-α-synuclein, (PLP)-eGFP and wild-type C57Bl 6 mouse brain by Percoll gradient as previously described (Lubetzki et al., 1992). Briefly, the primary mixed glial cultures were obtained from forebrains of 2-day-old mice. Mice were killed by decapitation, and the forebrains were dissected before being dissociated, first mechanically and then by digestion with 0.1% trypsin (Sigma, 15min at 37°C). Cells were washed in Hank's balanced solution (Flow Laboratories), passed through 150 and 63mm nylon meshes and layered on a Percoll (Pharmacia) density gradient. The oligodendrocyte progenitor -rich fraction was then washed twice by centrifugation and

resuspended in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 10% fetal calf serum (FCS) (Eurobio). The cells were plated out onto 14-mm-poly-L-lysine (Sigma) coated glass coverslips (OSI) or plastic 24-well plates (Costar) at a density of $50x10^3$ cells/well and cultured in a 37°C 5% CO_2 incubator. For biochemical analysis of α-synuclein aggregates, oligodendrocytes were plated in 35mm diameter wells, at a density of $800x10^3$ cells/well.

The purified oligodendrocyte progenitors were cultured in Bottenstein and Sato medium (BS; (Bottenstein and Sato, 1979), supplemented with 1% FCS, 1% penicillin-streptomycin (ATGC Biotechnologie), 10ng/ml recombinant PDGF-AA (Peprotech). After 7 days in culture, the medium was switched to a differentiating medium (proliferating medium without PDGF).

The purity of oligodendroglial cultures assessed by immunostaining with O4 and GalC antibodies ascertained that 2 days after plating, 85% of the cells were O4 positive immature oligodendrocytes.

Lentiviral vector and primary oligodendrocyte transduction

Lentiviral vectors were produced using a triple transfection system in 293T cells with the following plasmids:

1. A packaging plasmid encoding structural viral proteins and enzymes. The packaging signal has been deleted.
2. An envelope plasmid encoding the envelope of vesicular stomatitis virus

3. A transfer plasmid encoding the transgene under control of a promoter. It contains cis-acting signals for encapsidation (ψ), reverse transcription and integration flanked by two long terminal repeats (LTR)

The vector particles are released in the supernatants and were isolated by ultracentrifugation. The vectors are pseudotyped with the envelope of VSV to increase tropism and stability of the vectors. Lentiviral vectors produced by this method are able to transduce non-dividing cells such as neurons and oligodendrocytes. The backbone of recombinant lentiviral vectors expressing eGFP (LV-eGFP), α-synuclein (LV-αSYN) and β-synuclein (LV-βSYN) under the mouse phosphoglycerate kinase promoter (PGK) was as follow:

Viral stocks were concentrated 10-fold and the biologic titers of concentrated vector preparations ranged between 1-3x10^8 Infecting Unit/mL. After 7 days in culture, when about 80% of the cells were O4-positive, oligodendrocyte progenitors were exposed to lentiviral vectors at the doses of 50ng/μL (p24 protein concentration). The transduction efficiency of oligodendrocytes was about 70%. 2 days after transduction, 10μM of proteasome inhibitor was added to the oligodendrocyte cultures for 24h.

Immunostaining

For double immunofluorescence staining of immature oligodendrocytes cultures, involving surface and cytoplasmic antigens, oligodendrocytes grown on glass coverslips were washed once with phosphate-buffered saline (PBS), and fixed with 4% paraformaldehyde (Sigma) in PBS for 5min at RT. After washing with PBS, fixed cells were blocked with 10% FCS in PBS for 30min and incubated overnight at 4°C with the primary antibody (A2B5, O4 or GalC) diluted in the blocking solution. The following day, the cells were washed with PBS and then incubated 1h with the secondary antibody at RT. After washing, cultures were fixed 10min with 4% paraformaldehyde, washed in PBS, and incubated with primary antibody 1h at RT with the primary antibody diluted in blocking solution containing 0.02% Triton-X-100 (Sigma) for cytosolic staining. Cells were washed, incubated for 1h at RT with the secondary antibody and counterstained with the nuclear dye Hoechst 33342 (Sigma, 1μg/ml). After washing in PBS, coverslips were mounted in Fluoromount (Southern Biotechnology Associates).

For double immunostaining of mature oligodendrocytes involving only cytoplasmic antigens, cells were fixed with 4% paraformaldehyde in PBS for 15min at RT. After washing with PBS, fixed cells were blocked with 10% FCS in PBS for 30min and incubated 1h at RT with the primary antibody diluted in blocking solution containing 0.02% Triton-X-100. Cells were washed with PBS, incubated with the secondary antibody 1h at RT and counterstained with Hoechst dye before mounting.

For quantification analysis, cells were visualized with Eclipse TE300 Microscope fluorescence microscope (Nikon) using 20X or 40X objectives, and phase contrast microscopy with 40X objective, as indicated. Images were acquired with Simple PCI software (Compic Inc. Imaging Systems).

To demonstrate simultaneous immunocytochemical localization, cells were visualized using 63X or 100X oil-immersion objectives with axioplan 2 imaging Zeiss Microscope or with LSM510 Zeiss confocal microscope. Images were acquired with FluoUp Mercator software (Explora Nova) and LSM 10 Meta (Zeiss), respectively.

Thioflavin S Staining

Cells were washed once with PBS, fixed 30min with 4% paraformaldehyde and subjected to immunofluorescence staining as described above. After three PBS washes, cells were incubated with 0.005% thioflavin S (diluted in 70% ethanol, Sigma) for 8min, washed three times in ethanol 70%, once in H_2O, and then mounted.

Histochemistry on human brains

Sections from either formalin or ethanol-fixed paraffin-embedded brain tissue, as well as frozen tissue from MSA cases and controls, were obtained from the German Brain Bank "Brain-Net" and the Center for Neurodegenerative Disease Research, University of Pennsylvania. For immunohistochemistry, the avidin-biotin-peroxidase method with 3,3'-diaminobenzidine for color development or the alkaline phosphatase/anti-alkaline phosphatase system (DAKO, Germany) with neufuchsin as the chromogen were used. To enhance immunoreactivity, tissue sections were boiled in 10 mM citrate buffer, pH 6.0, in a microwave oven (5 times for 3min each). To block non-specific antibody binding sites, tissue sections were incubated in 2% BSA / 0.01% (v/v) Triton X-100 for 30min at room temperature. An improved thioflavin S staining method (Guntern et al., 1992) was performed. Double-labeling immunfluorescence of Fas and a-synuclein was performed with Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 594-conjugated goat

anti-mouse (Molecular Probes, 1:1000) as secondary antibodies. Autofluorescence was blocked by incubating the sections with 0.3 % Sudan Black in 70% ethanol (Romijn et al., 1999). Sections were mounted in Vectorshield with DAPI (Vector Laboratories).

Immunoelectron Microscopy

Immunogold labeling for electron microscopy was performed on oligodendrocytes overexpressing either eGFP or wild-type α-synuclein. After 8-10 days in culture, some of the oligodendrocytes were treated with proteasome inhibitor for 24h. Cells were then rinsed with PBS, fixed with 4% paraformaldehyde for 45min, washed three times in PBS then incubated for 1h in PBS, 0.2% BSA, 0.02% gelatine blocking buffer. Cells were rinsed three times with 0.2M PBS and incubated overnight at 4°C with 15G7 (1:10) in PBS, 0.2% BSA. The day after, the cells were extensively washed with filtered PBS and incubated 3h at RT with goat anti-rat immunoglobulins conjugated to 10nm gold particles (British Biocell, 1:100). To determine background staining, 15G7 antibody was omitted. Cells were then washed in PBS, fixed 30min with 2% glutaraldehyde, and then postfixed in 1% osmium tetroxide for 30min on ice in the dark. Cells were washed in distilled water, dehydrated in a graded series of ethanol solutions in the specific order: ethanol 50% (5min) and ethanol 70% (5min). Cells were treated with uranyl acetate 1% in 70% methanol 30min (in the dark) and bathed 5min in ethanol 95% (5min). Oligodendrocytes were pre-embedded in a mix ethanol/epon (ratio 2/1 for 5min; and then switched to 1/1 for 5min, then to a ratio of 1/2 for 5min). Cells were then embedded in Epon (2 baths in pure epon for 5min each followed by a 24h polymerization step at 60°C). Semi-thin sections (0.10μm) were cut with an ultramicrotome, counterstained with toluidine blue. When the

cell layer was reached, ultra-thin sections (50-60nm) were laid on a copper grid and counterstained with conventional techniques (uranyl acetate and lead citrate).

Sections were examined with a JEOL 1200EX II transmission electron microscope. Images were analyzed with Analysis Docu Soft Imaging System.

Proteasome Inhibition

Differentiated oligodendrocytes were exposed to 0.01-10μM epoxomicin or MG-132 (Calbiochem) for 2-24h. After the indicated time, cells were fixed and stained with 15G7 for visualization and quantification of α-synuclein aggregates. The composition of α-synuclein aggregates was characterized by double staining for α-synuclein (normal, nitrated or phosphorylated α-synuclein), HNE, ubiquitin, 20S proteasome subunits, hsp70, hsp40, αB crystallin, 14-3-3 chaperone protein, tubulin polymerization promoting protein TPPP/p25α protein. Cells were counterstained with Hoechst nuclear dye as described above.

Quantification of α-synuclein Inclusions

Punctate aggregates were detected as many small sized-α-synuclein positive inclusions throughout the cytosol, whereas the GCI-like inclusions were single, compact and perinuclear. For each condition, 500 oligodendrocytes from randomly selected fields were analyzed in triplicates and each experiment was repeated at least three times.

Quantification of Ubiquitinated Cytoplasmic Inclusions

To determine the number of oligodendrocytes containing ubiquitinated cytoplasmic inclusions, cells were stained with polyclonal anti-ubiquitin antibody and visualized under standard epifluoresence using 40X objective. Inclusions were scored as positive if the ubiquitin immunoreactivity was localized to a discrete area, distinct from the nucleus, not encompassing the entire cytoplasmic volume. For each condition, 500 oligodendrocytes from randomly selected fields were analyzed in triplicates and each experiment was repeated three times.

Treatment with Caspase Inhibitors

After 2 weeks in culture, the medium was replaced with BS medium containing the broad spectrum caspase inhibitor z-VAD-fmk or specific inhibitors z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk against caspases-3,-8 and -9, respectively (Calbiochem; final concentration 300μM). Each compound was added to 3 separate wells. Control cultures were returned to BS medium in the absence of caspase inhibitors.

Cultures were incubated for 20min and treated with 10μM epoxomicin (or BS medium for control cultures) at 37°C 5% CO_2 for a further 24h, fixed using 4% paraformaldehyde in PBS and immunostained for α-synuclein, activated caspase-3 and counterstained with Hoechst nuclear dye.

Treatment with Death Ligands

Oligodendrocytes were cultured as described above and then treated for 18h with soluble Fas ligand (sFasL; 5ng/mL, Upstate), membrane bound Fas ligand (mFasL; 5ng/mL,

Upstate), mouse NGF (100ng/mL, Alomone Labs), TNFα (100ng/mL, Chemicon), TRAIL (300ng/mL, Chemicon) and sometimes preincubated with neutralizing Fas antibody (1μg/mL Alexis). Cells were stained with propidium-iodide and calcein (Live dead kit, Molecular Probes). Cells were scored as alive if the cytosol was intensely stained with calcein and as apototic if the nucleus was red. For each condition, 5 randomly selected fields of 100 oligodendrocytes were analyzed in triplicates and the experiment was repeated three times.

Caspase-3 Activation

Cultured oligodendrocytes were treated as described above and then immunocychemically stained with anti-activated caspase-3 antibody to detect caspase-3 activation. Cells were scored as positive if the cytosol was intensely activated-caspase-3 immunoreactive. For each condition, 5 randomly selected fields of 100 oligodendrocytes were analyzed in triplicates and each experiment was repeated at least three times.

Visualization of Apoptotic Nuclei

Cells were scored as apoptotic based on Hoechst staining showing condensed or fragmented chromatin and nuclear morphology. To quantify apoptotic cell death, the percentage of cells with condensed and fragmented chromatin nuclei in 5 fields of 100 cells was determined. Each experiment was conducted at least three times.

Fractionation of α-synuclein Aggregates

Sequential detergent soluble extraction (Baba et al., 1998; Dickson et al., 1999; Kahle et al., 2001) was used to detect insoluble α-synuclein molecules after treating transgenic oligodendrocytes with epoxomicin. Briefly, mature (PLP)-α-synuclein and (PLP)-eGFP oligodendrocytes were exposed to epoxomicin (10μM) for 24h , washed twiced with cold PBS and harvested on ice in Tris-buffered saline (TBS) + β-glycerophosphate (50mM) and sodium orthovanadate (1mM), complete protease inhibitors (Roche). The cells were sonicated and the cytosolic fraction was isolated after centrifugation. Equal amount of protein from both control and epoxomicin treated cultures was determined by the Bradford Protein Assay (Bio-Rad) and the 100,000 xg pellet was extracted in 5% sodium dodecylsulfate (SDS). The remaining detergent insoluble pellet was solubilized with 8M urea / 5% SDS.

The fractions were loaded onto a 15% SDS polyacrylamide gel (15% SDS-PAGE), separated electrophoretically, and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The PVDF membranes were briefly washed in methanol, blocked for 1h at RT with TBS containing 1.0% Tween-20 and 5% dehydrated skim milk to block nonspecific protein binding, and probed overnight at 4°C with anti-α-synuclein monoclonal antibodies 15G7, Syn303, phosphorylated α-synuclein polyclonal antibodies and monoclonal anti-GFP.

Changes in ubiquitination were visualized by probing the membranes with anti-ubiquitin 1510 monclonal antibody.

After several washes, the membranes were incubated with horseradish peroxydase conjugated secondary antibodies, and after additional washes, the immunoreactive bands

were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Membranes were exposed to Kodak X-ray films.

Biochemistry for Fas expression

Cells were washed 2 times with PBS before being lysed 20min on ice in STEN lysis buffer containing 50mM Tris pH 7.6, 2mM EDTA, 0.2% (v/v) NP-40 und 150mM NaCl. Cells were centrifuged 20min at 16 000 x*g* at 4°C. Protein concentration was determined with Bradford test and 30µg of total protein was loaded on a 15% SDS gel. MSA and control brains were homogenized on ice with a glass potter in STEN lysis buffer and homogenates were briefly sonicated and centrifuged 20min at 16 000 x*g* at 4°C. Protein concentration was determined with Bradford test and 80µg of total protein was loaded on 15%SDS gel. Western blot was performed as described above and changes in Fas expression levels were visualized by probing the membranes with anti-Fas monclonal antibody (Transduction Laboratories), and equal loading was confirmed by reprobing with mAb B-5-1-2 against α-tubulin (Sigma).

Frozen tissue from MSA cases and controls were obtained from the German Brain Bank "Brain-Net". Brain samples were homogenized on ice with a glass potter in STEN lysis buffer and homogenates were briefly sonicated and centrifuged for 20min with 16,000 x *g* at 4°C. Protein concentration was determined with Bradford test and 80µg of total protein was subjected to 15% SDS-PAGE. Western blot analysis was performed as described above.

Proteasome activity

The proteasome activity was determined as described (Lightcap et al., 2000). Proteasome activity was measured 8 days after differentiation. Oligodendrocytes cultured with proteasome inhibitors or left untreated were washed twice wit PBS and harvested on ice in 25mM Tris buffer. Cells were centrifuged and lysed by brief sonication and the fluorogenic substrates Suc-LLVY-AMC, Z-LLE-AAMC and Boc-LRR-AMC, were added to measure the chymotryptic-like, tryptic-like and post-acidic activities, respectively. The proteasome activities were monitored by the fluorescence activity (λex: 380 nm; λem: 460 nm) (Keller et al., 2000). The relative activity was standardized by protein concentration which was determined using the Bio-Rad assay kit (Bio-Rad laboratories, CA).

Statistical Analysis

Student's *t* tests and one-way ANOVA followed by Dunnett's test were used to determine whether differences between groups were significant. Data are expressed as means +/- SEM of at least three independent experiments performed in triplicates. Statistical significances were defined as *P< 0.05; **P< 0.001; ***P< 0.0001.

Chapter 4: RESULTS

1 Transgenic α-Synuclein Expression in Primary Oligodendrocytes Derived From (PLP)-α-synuclein Mice

To determine when during oligodendrocyte differentiation is transgenic α-synuclein expressed in culture, I isolated neuroglial progenitor cells from (PLP)-α-synuclein mice, and induced the oligodendroglial lineage by mitogen deprivation (Lubetzki et al., 1992). Oligodendrocyte differentiation in these cultures was monitored by immunostaining with stage-specific markers, including the cell surface protein A2B5, the chondroitin sulphate proteoglycan NG2, the ganglioside O4 and GalC, and the integral MBP. The primary cultures were highly enriched in oligodendrocyes, with 80-85% of the cell population being O4-positive.

Transgenic α-synuclein expressed under control of the PLP promoter was detectable already in A2B5- and NG2-positive oligodendrocyte precursors (Figure 8), consistent with the very early induction of the *DM-20*/*PLP* gene *in vivo* (LeVine et al., 1990). Expression of the transgenic α-synuclein persisted throughout oligodendrocyte differentiation in culture (Figure 8), as evidenced by human-specific α-synuclein immunoreactivity in the O4-positive immature stage, the GalC-positive mature stage, and the myelin basic protein positive pre-myelinating stage. Specificity of the human α-synuclein 15G7 antibody was demonstrated by the absence of immunostaining in primary oligodendrocytes derived from (PLP)-eGFP transgenic mice.

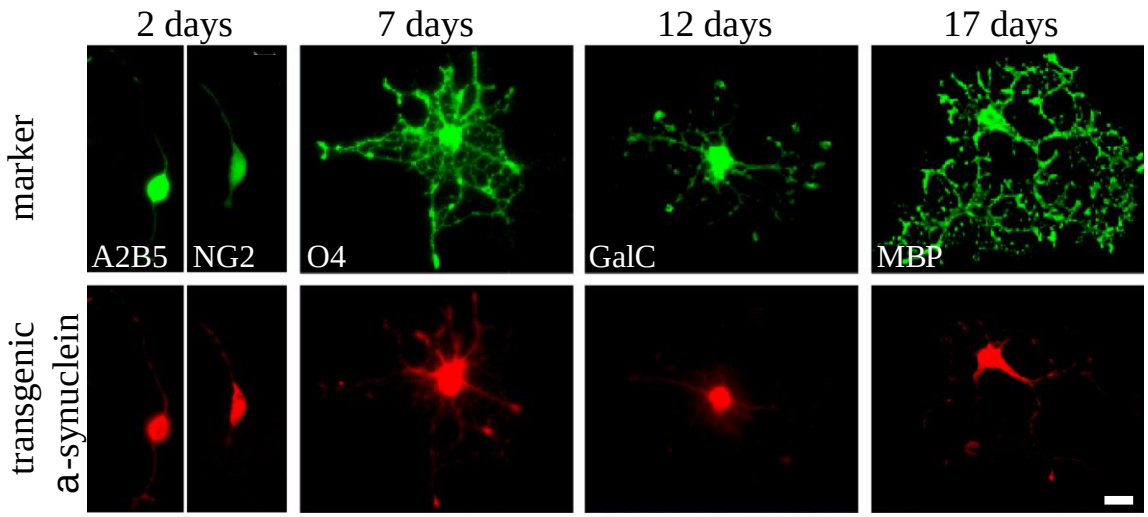


Figure 8: Oligodendrocyte progenitor cells were isolated from transgenic mouse forebrains. After being cultured for 2, 7, 11 and 17 days, primary α-synuclein transgenic oligodendrocytes were fixed for double-labeled immunostaining analysis with the indicated oligodendrocyte markers (green; upper panels) and 15G7 anti-α-synuclein (red; lower panels). Diffuse α-synuclein staining was detected throughout the cytoplasm and the processes from the oligodendrocyte progenitor stage throughout the maturation process. *Scale bars, 10μm.*

2 Cellular Consequences of α-synuclein Expression on Transgenic Oligodendrocytes

Compared to wild-type and control eGFP transgenic oligodendrocytes, the number of A2B5-positive precursor cells decreased about 2 days earlier in α-synuclein transgenic cultures, concomitant with an equally accelerated appearance of O4-positive immature oligodendrocytes (Figure 9). The effect of transgenic α-synuclein on oligodendrocyte maturation was less marked. The development of α-synuclein transgenic GalC-positive mature oligodendrocytes was accelerated by approximately 1 day, and no effect of α-

synuclein was observed when measuring the appearance of MBP-positive pre-myelinating oligodendrocytes after 2 weeks in culture.

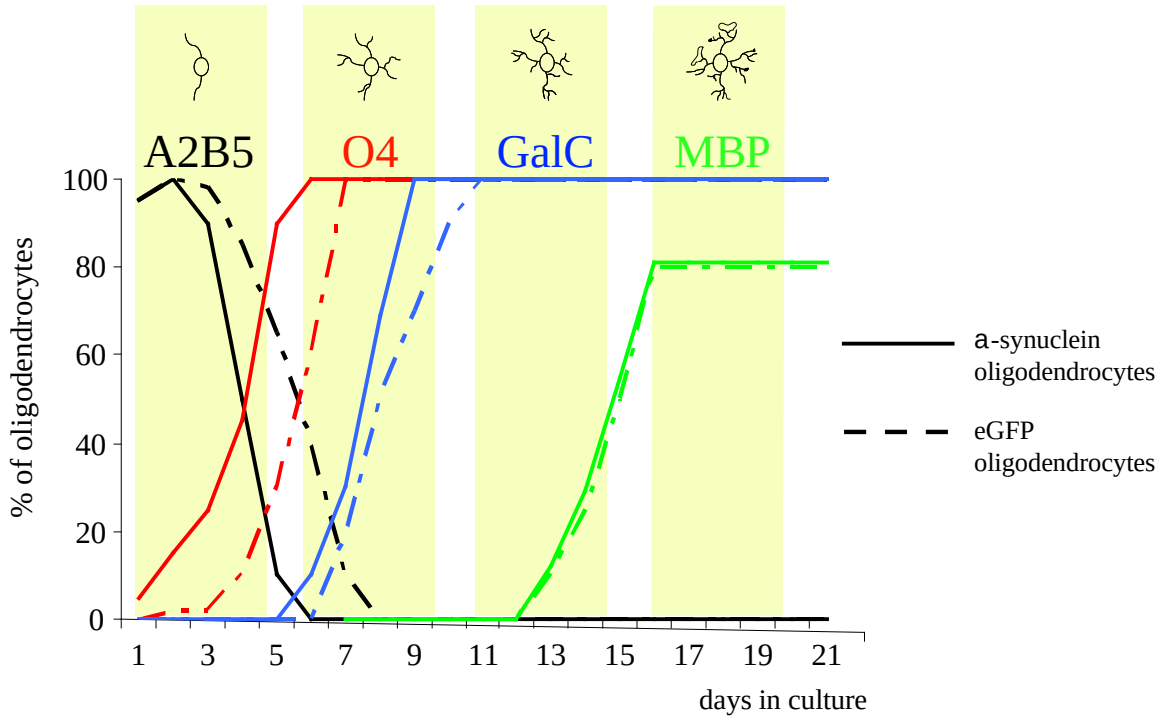


Figure 9: Oligodendrocyte progenitor cells isolated from α-synuclein (solid lines) and eGFP (broken lines) transgenic mice were kept in culture for up to 21 days and labeled with oligodendrocyte markers (black=A2B5; blue=O4; green=GalC; red=MBP). An accelerated differentiation could be observed in α-synuclein transgenic oligodendrocytes.

Rat oligodendrocyte cultures have been reported to withdraw from the cell cycle and differentiate faster from the A2B5-positive progenitor stage along the oligodendroglial lineage upon partial proteasome inhibition with low doses of lactacystin and MG-132 (Pasquini et al., 2003). α-synuclein expression decreased proteasome activity in cell lines (Tanaka et al., 2001), possibly because α-synuclein fibrils directly inhibit the 20S proteasome (Lindersson et al., 2004; Snyder et al., 2003). Therefore the effects of ectopic

α-synuclein expression on oligodendrocyte proteasomal activities were addressed by measuring the hydrolysis rates of fluorogenic proteasome substrates in non-transgenic as well as eGFP and α-synuclein transgenic oligodendrocytes. No significant difference in any proteasomal activity was observed in eGFP transgenic oligodendrocytes compared to non-transgenic oligodendrocytes. Interestingly, primary α-synuclein oligodendrocytes showed a significant decrease in the chymotryptic proteasomal activity (Figure 10). The tryptic and postacidic proteasomal activities were reduced to a lesser extent in α-synuclein transgenic oligodendrocytes. Consistently, aggregated preparations of recombinant α-synuclein selectively inhibited the chymotryptic activity of 20S proteasomes *in vitro* (Lindersson et al., 2004), but the mechanism of such an unusual inhibition of the chymotryptic proteasome activity by α-synuclein remains to be rigorously established.

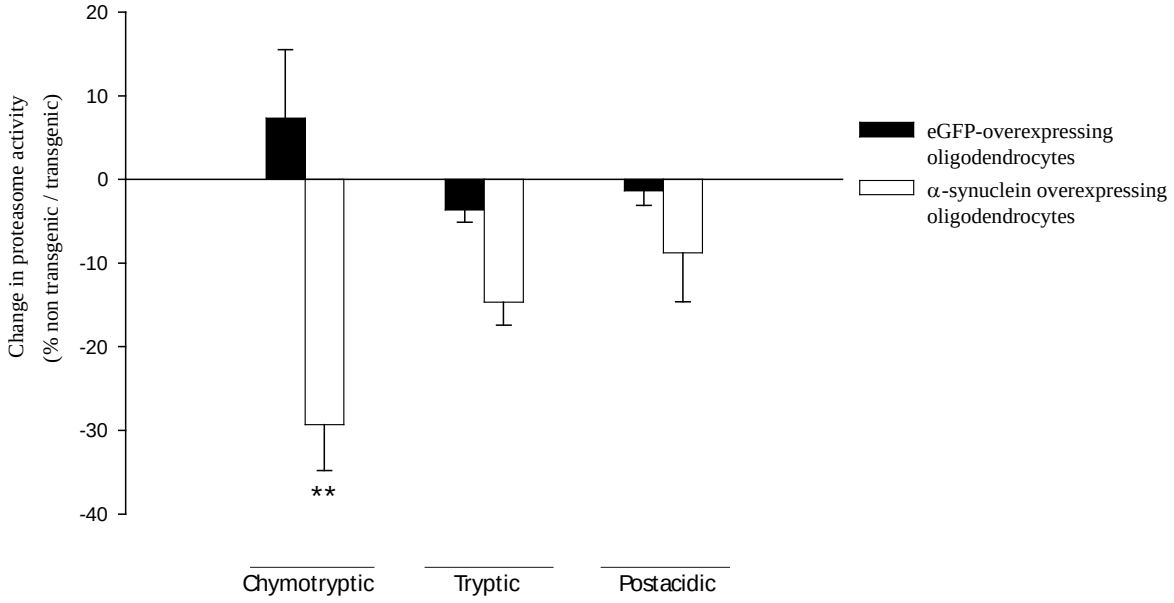


Figure 10: Primary wild-type, eGFP and α-synuclein transgenic oligodendrocytes were cultured for 2 weeks and lysed. The fluorogenic substrates Suc-LLVY-AMC, Z-LLE-AAMC and Boc-LRR-AMC, were added to measure the chymotryptic-like, tryptic-like and post-acidic activities, respectively and changes in proteasome activities were determined as percentage of proteasome activities in wild-type oligodendrocytes. Data are expressed as means +/- SEM of at three independent experiments performed in triplicates. Student *t* test; **P<0.001.

3 Influence of UPS Impairment on Oligodendroglial α-Synucleinopathy

3.1 Proteasome inhibition causes time- and dose-dependent formation of α-synuclein inclusions in transgenic oligodendrocytes

To exacerbate the inhibition of the chymotryptic proteasome activity in α-synuclein transgenic oligodendrocytes and to determine the contribution of an impairment of the oligodendroglial UPS to the pathogenesis of MSA, I challenged primary oligodendrocytes with the epoxyketone epoxomicin. In the absence of inhibitor, the transgenic α-synuclein in primary oligodendrocytes was diffusely distributed throughout the cytosol and the processes. Under basal conditions, α-synuclein aggregates were rarely seen under the microscope (5% of total α-synuclein transgenic oligodendrocytes), indicating that α-synuclein expression in primary oligodendrocytes derived from (PLP)-α-synuclein mice is not enough to induce MSA neuropathology. However, after a 2h treatment with a saturating concentration of epoxomicin (10μM), small α-synuclein aggregates developed throughout the cytosol of the oligodendrocytes (Figure 11). Between 6-12h of proteasome inhibition, the small aggregates clustered into larger aggregates, which later appeared to converge into a single round or triangular α-synuclein aggregate near the nucleus (Figure

11). I confirmed specificity of the observed effects with a different proteasome inhibitor, the peptide aldehyde MG-132 (Kisselev and Goldberg, 2001).

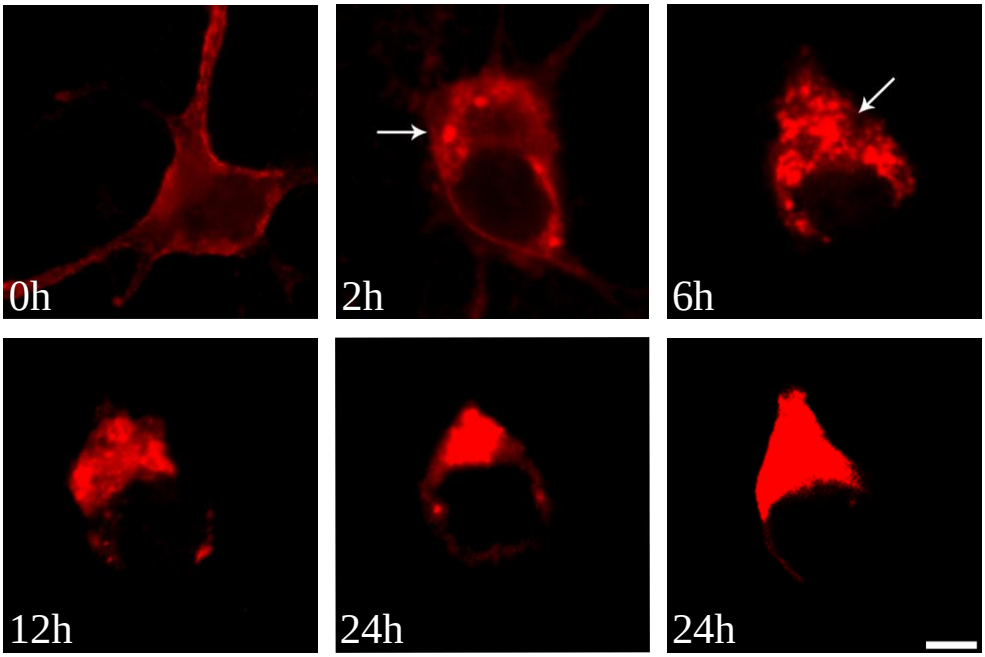


Figure 11: Cultures were treated with 10μM of epoxomicin for the indicated times. Transgenic human α-synuclein aggregates was detected by immunostaining with 15G7 antibody and visualised by confocal microscopy. Note the time-dependent changes in α-synuclein localization from diffuse in the cell body and processes (0h) to small aggregates scattered throughout the cytosol after 2h (arrows) to large perinuclear aggregates after 24h (arrowheads). *Scale bar, 10μM.*

Quantitative dose-response analysis showed that the number of oligodendrocytes with several small α-synuclein aggregates increased from 5.5% ± 1.1 in control cultures to 19.8% ± 3 in the presence of 0.01μM epoxomicin. Exposure to higher doses of proteasome inhibitor (0.1-10μM) was accompanied with a gradual decrease of oligodendrocytes with small α-synuclein aggregates concomitant with a dramatically increased formation of large perinuclear aggregates in up to 42.5% ± 2.5 of the oligodendrocytes (Figure 12).

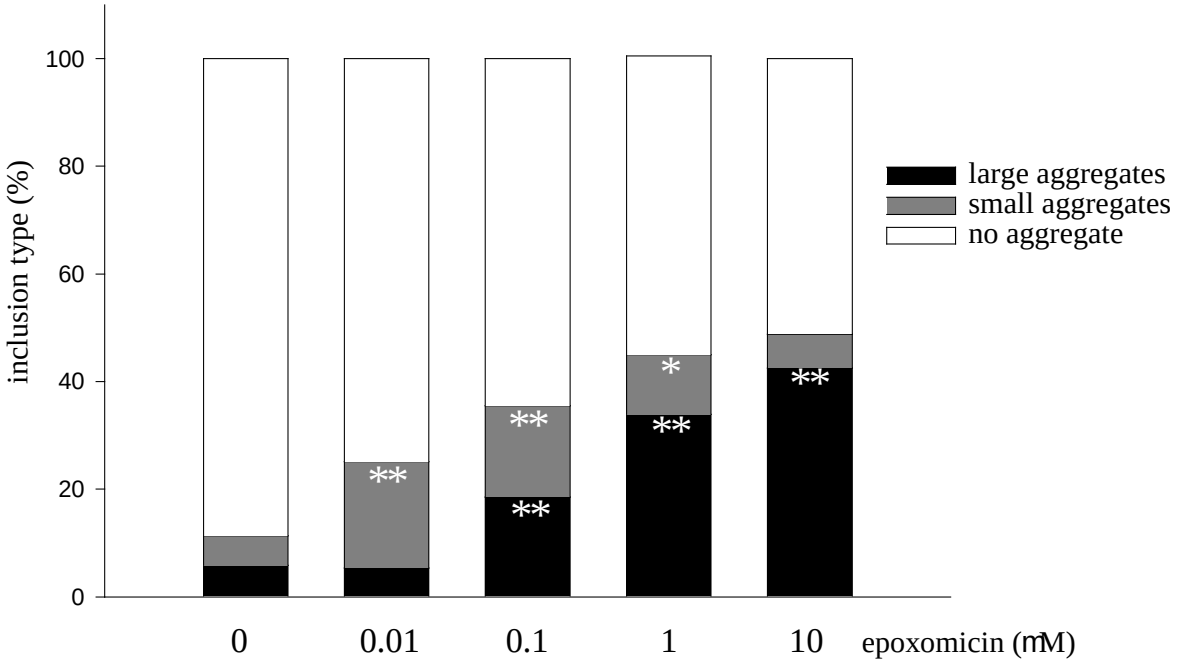


Figure 12: Primary α-synuclein transgenic oligodendrocytes were treated with 0-10μM epoxomicin for 24h. Cells were then stained with 15G7 anti-α-synuclein antibody and the number of oligodendrocytes containing small or large α-synuclein aggregates were counted under standard epifluoresence. Small aggregates were scored when detected as several small sized-α-synuclein immunopositive inclusions throughout the cytosol, whereas the large inclusions were single, compact and perinuclear. For each condition, 500 oligodendrocytes from 3 randomly selected fields were analyzed. Data are expressed as means from 5 independent experiments conducted in triplicate. The *asterisks* indicate statistical differences between the percentages of primary α-synuclein transgenic oligodendrocytes bearing aggregates when treated with epoxomicin compared with control (0μM) conditions. Student *t* test; *p< 0.05; **P<0.001.

3.2 Proteasome inhibition elevates α-synuclein levels and induces the formation of detergent insoluble α-synuclein inclusions

Although it is not clear if α-synuclein is directly degraded by the proteasome in a ubiquitin-dependent manner (Bennett et al., 1999; Tofaris et al., 2001), it was shown previously that proteasome inhibition in neuronal cells causes the formation of insoluble,

ubiquitinated α-synuclein aggregates (Rideout et al., 2004; Rideout et al., 2001). To assess the influence of proteasome inhibition on α-synuclein turnover and solubility in oligodendrocytes, differentiated primary oligodendrocyte cultures treated with epoxomicin were lysed. The lysates were fractionated into buffer-soluble, SDS-soluble, and SDS-insoluble material (urea extract) (Kahle et al., 2002b), and Western blot analysis was performed with antibodies against α-synuclein.

In control cultures derived from eGFP transgenic mouse oligodendrocytes, only small amounts of endogenous α-synuclein were detected, consistent with a previous report on wild-type rat oligodendrocytes (Richter-Landsberg et al., 2000). Endogenous α-synuclein migrated as a single band with an apparent molecular mass of 16kDa, corresponding to monomeric α-synuclein, and was found in the buffer-soluble (cytosolic) fraction (Figure 13A). Treatment with 10μM epoxomicin raised the steady-state level of soluble, monomeric α-synuclein (Figure 13A), demonstrating that α-synuclein is turned over by the proteasome in primary oligodendrocytes. There was no formation of insoluble α-synuclein aggregates in eGFP-transgenic oligodendrocyte cultures even in the presence of saturating concentrations of epoxomicin. In contrast, α-synuclein transgenic oligodendrocytes contained considerable amounts of buffer-insoluble α-synuclein in the SDS fraction. However, α-synuclein transgenic oligodendrocytes did not contain detectable amounts of detergent-insoluble α-synuclein in the absence of proteasome inhibitor. Steady-state levels of transgenic human α-synuclein in these cultures were greatly increased upon proteasome inhibition, and a rather large proportion of α-synuclein could only be recovered under harsh denaturing detergent conditions (extraction with 8M urea / 5% SDS) (Figure 13B). Much of the SDS-insoluble, α-synuclein-immunoreactive material migrated as >40kDa

high molecular weight smear, and was previously shown to be characteristic for human MSA patients (Campbell et al., 2001; Kahle et al., 2002b). Taken together, proteasome inhibition in primary α-synuclein transgenic oligodendrocytes causes the formation of inclusions containing insoluble α-synuclein aggregates.

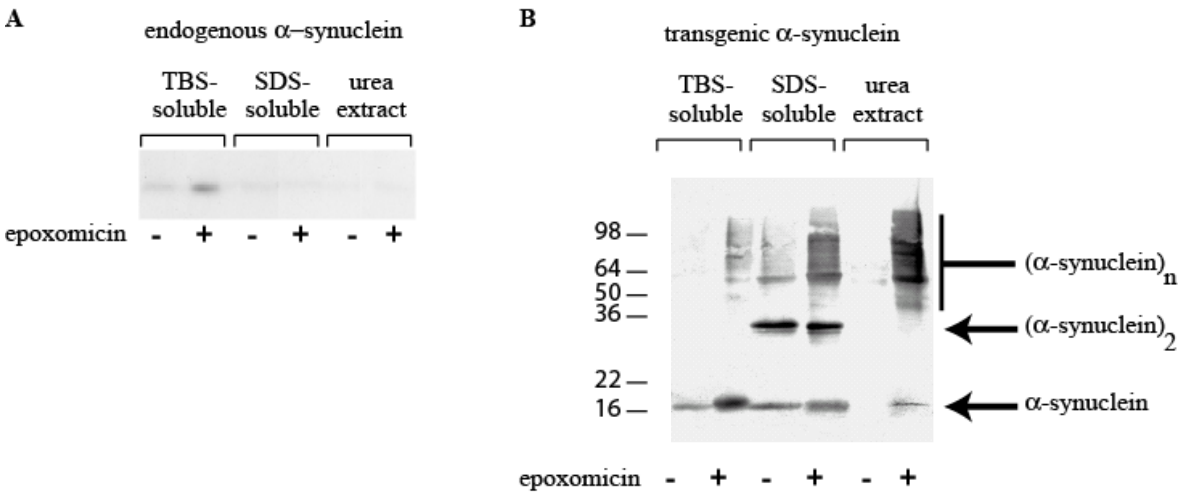


Figure 13: (A, B) Transgenic α-synuclein primary oligodendrocytes were challenged without (-) or with 10μM epoxomicin for 24h and washed twice with cold PBS. Cells were then fractionated into TBS-soluble, SDS-soluble and urea extracts. These fractions were electrophoresed and Western blotted, and probed with Mab42 anti-α-synuclein (A), and human-specific 15G7 anti-α-synuclein (B)

4 High Levels of α-Synuclein are Sufficient to Induce the Formation of Inclusions in Primary Oligodendrocytes

Most likely epoxomicin treatment elevates the α-synuclein levels beyond a threshold above which spontaneous aggregation of α-synuclein occurs. To corroborate this point, primary wild-type oligodendrocytes were transduced with a lentiviral vector encoding α-

synuclein, which has been previously demonstrated to cause degeneration in dopaminergic nigral neurons in the rat brain (Lo Bianco et al., 2002). As controls lenti-eGFP, and more specifically, a novel lentiviral construct encoding the non-amyloidogenic β-synuclein were used.

Comparative Western blotting revealed that human α-synuclein expression levels in primary mouse oligodendrocytes 2 days after transduction with a lentiviral vector were much higher than the basal levels detected in primary oligodendrocytes derived from PLP-α-synuclein transgenic mice (Figure 14A). Thus, acute lentiviral expression of α-synuclein driven by a PGK promoter is stronger compared to transgenic expression in the (PLP)-α-synuclein mouse derived oligodendrocytes. Importantly, a considerable fraction of the α-synuclein transduced into wild-type oligodendrocytes was recovered from the SDS-insoluble fraction even in the absence of proteasome inhibitor (Figure 14B). Thus, lentiviral vectors allow high-level overexpression of α-synuclein without potential side effects due to proteasome inhibition.

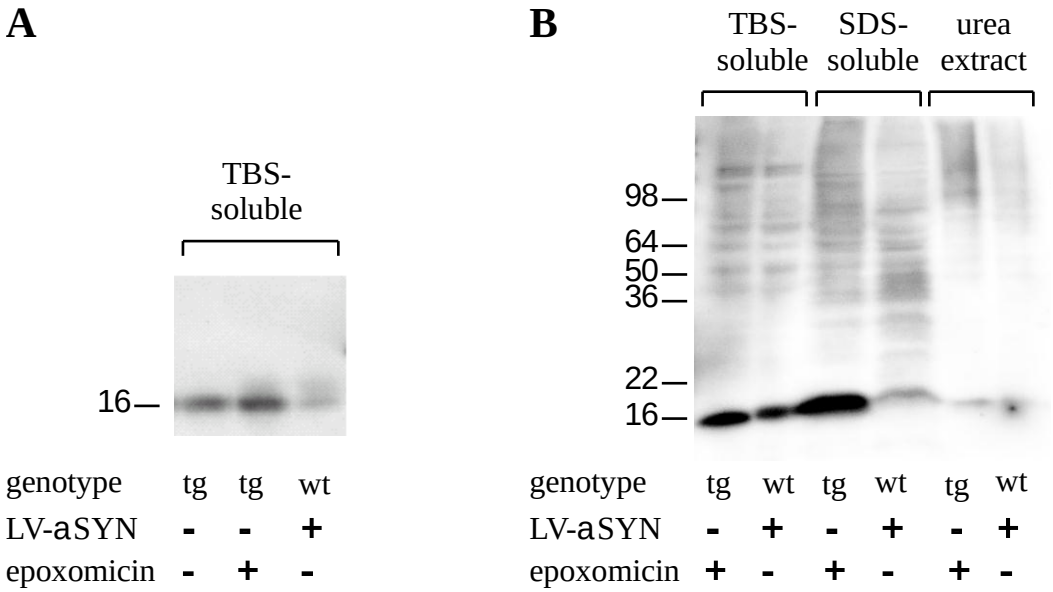


Figure 14: (A) Primary oligodendrocyte cultures derived from (PLP)-α-synuclein transgenic (tg) mice were challenged without (-) or with 10μM epoxomicin for 24h and wild-type derived oligodendrocytes were exposed for 2 days to LV-αSYN, as indicated. Cells were then fractionated into TBS-soluble. These fractions were electrophoresed and Western blotted, and probed with human-specific 15G7 anti-α-synuclein. (B) Similarly high levels of α-synuclein were expressed after 1 day

epoxomicin of transgenic (PLP)-α-synuclein oligodendrocytes and 2 days after transduction of wild-type mouse oligodendrocytes with LV-αSYN. Cultures were lysed and Western probed with human-specific 15G7 antibody.

Two days after transduction, lentivirally delivered eGFP and β-synuclein was evenly distributed throughout the cytosol of O4-positive primary wild-type mouse oligodendrocytes (Figure 15A). In contrast, a significant fraction of the O4-positive oligodendrocytes transduced with LV-αSYN showed perinuclear inclusions similar to those found in epoxomicin-treated α-synuclein transgenic oligodendrocytes (Figure 15A, B). The GCI-like fibrillar nature of these inclusions was confirmed by staining with thioflavin S (Figure 15B). While 32.3 ± 5.3% of oligodendrocytes transduced with α-synuclein lentiviral vectors developed cytoplasmic inclusions, less than 10% contained cytosolic aggregates in control cultures exposed to LV-eGFP and LV-βSYN (Figure 15B).

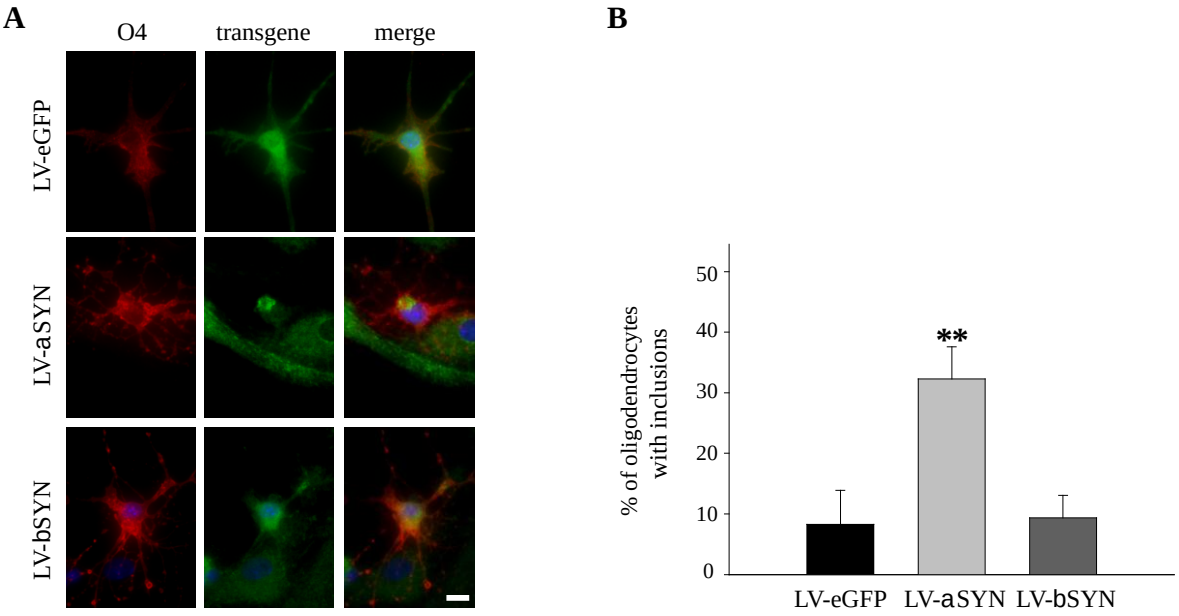


Figure 15: (A) Wild-type mouse oligodendrocytes were transduced with recombinant lentiviral vectors harboring eGFP, α-synuclein, and α-synuclein, as indicated. Two days after transduction, oligodendrocytes were identified by O4 staining (left panels) and eGFP, β-synuclein or α-synuclein immunostained (middle panels). Note that the few (O4-negative) astrocytes present in the cultures were also transduced by the lentiviral α-synuclein vector, but in these cells α-synuclein did not form GCI-like inclusions. Nuclei were counterstained with Hoechst 33258 (blue; right panels). *Scale bar, 10μm.* (B)

Lentiviral mediated α-synuclein expression was sufficient to induce the formation of cytoplasmic inclusions while eGFP and β-synuclein transduced oligodendrocytes hardly developed any inclusions.

5 Inclusions Formed in Culture Resemble Human Pathological GCIs

5.1 Characterization of the inclusions formed *in vitro*

The resemblance in morphology between α-synuclein inclusions in primary oligodendrocytes and GCIs in MSA is striking, as evidenced by the picture below:

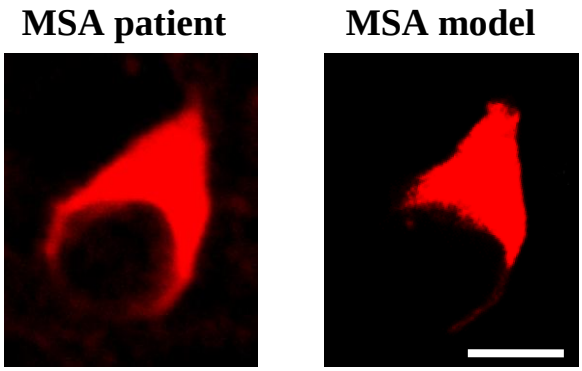


Figure 16: Treatment of α-synuclein transgenic primary oligodendrocytes with proteasome inhibitors caused the formation of α-synuclein inclusions that resembled human pathological GCIs. *Scale bar, 10μm.*

To characterize the nature of these α-synuclein aggregates and to determine their degrees of homology with human GCIs, I decided to perform a thoroughful and lengthy analysis of these inclusions using established markers of GCI pathology.

Long before α-synuclein was found to be the main component of GCIs, ubiquitin was the reference marker of the glial inclusions found in MSA (Papp et al., 1989b). Biochemical

analysis of the inclusions found *in vitro* revealed that they also contain ubiquitin. In fact, high molecular mass protein smears reacted with anti-ubiquitin on Western blots of the detergent-insoluble fractions prepared from epoxomicin-treated (PLP)-α-synuclein oligodendrocytes (Figure 17A). Moreover, double-label confocal microscopy demonstrated co-localization of α-synuclein and ubiquitin within the inclusions that formed in proteasome-inhibited transgenic oligodendrocytes (Figure 17B, C), while oligodendrocytes not challenged with epoxomicin exhibited low-level and diffusely distributed ubiquitin immunoreactivity throughout the cytosol. Ubiquitin immunoreactivity was intense in the inner area of the small aggregates (Figure 17B). Interestingly, in the large perinuclear aggregates, ubiquitin immunoreactivity displayed spatial variations in intensity so that some regions of an individual aggregate were intensely stained, while other regions remained unstained (Figure 17C). Quantitative analysis revealed that 43.7% ± 3.7 of the large perinuclear inclusions were α-synuclein-immunopositive and ubiquitin-negative, and 52.4% ± 2 were immunopositive for both ubiquitin and α-synuclein. The remaining 3.9% of the inclusions were detected only with anti-ubiquitin. A similarly small percentage of ubiquitin-immunopositive inclusions were detected in proteasome-inhibited control eGFP-transgenic oligodendrocytes. Although ubiquitin is often used as a marker of GCIs (Kato et al., 1991; Papp et al., 1989a), only a subset of α-synuclein-positive GCIs is ubiquitin-immunoreactive (Gai et al., 1998; Sampathu et al., 2003). Taken together, ubiquitination of α-synuclein is not the cause of aggregate formation but may rather be a secondary consequence of α-synuclein aggregation. Consistent with mobilization of the UPS, some of the small and large α-

synuclein aggregates in epoxomicin-treated transgenic oligodendrocytes contained 20S proteasome α-subunits co-localizing with α-synuclein (Figure 17B, C).

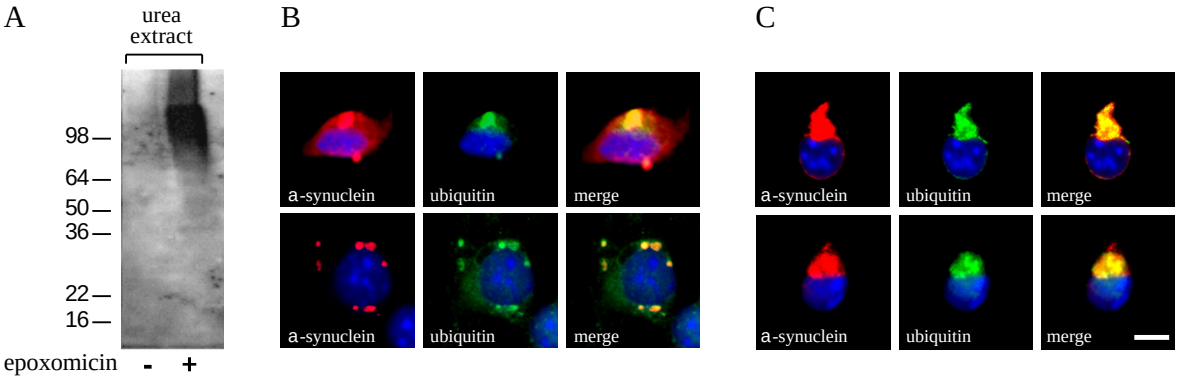


Figure 17: (A) Urea extracts were prepared from primary (PLP)- α-synuclein oligodendrocyte cultures that were treated without (-) or with (+) 10μM epoxomicin for 1 day. The material was Western blotted and probed with anti-ubiquitin, revealing the presence of high-molecular mass protein smears in the insoluble fraction of proteasome-inhibited oligodendrocytes. (B-C) Colocalization of α-synuclein (red) with ubiquitin (green) and 20S proteasome α-subunits (green) in the small (B) and large (C) aggregates was demonstrated by dual-labeling confocal microscopy. Nuclei were counterstained with Hoechst dye (blue). *Scale bar, 10μm.*

Heat shock proteins (hsps) are molecular chaperones, which directly prevent the misfolding of aggregation-prone proteins and thereby modulate neurodegeneration and oligodendrocyte damage (Muchowski and Wacker, 2005; Richter-Landsberg and Bauer, 2004). The major cytosolic chaperone hsp70 and its cofactor hsp40 as well as the small hsp αB-crystallin co-localized with α-synuclein aggregates in proteasome-inhibited transgenic oligodendrocytes, closely reflecting the accumulation of these hsps in GCIs of human MSA patients (Figure 18A-I). The apparent recruitment of the chaperones hsp70 / hsp40 and αB-cystallin may reflect an adaptive cellular response to the accumulation of misfolded proteins.

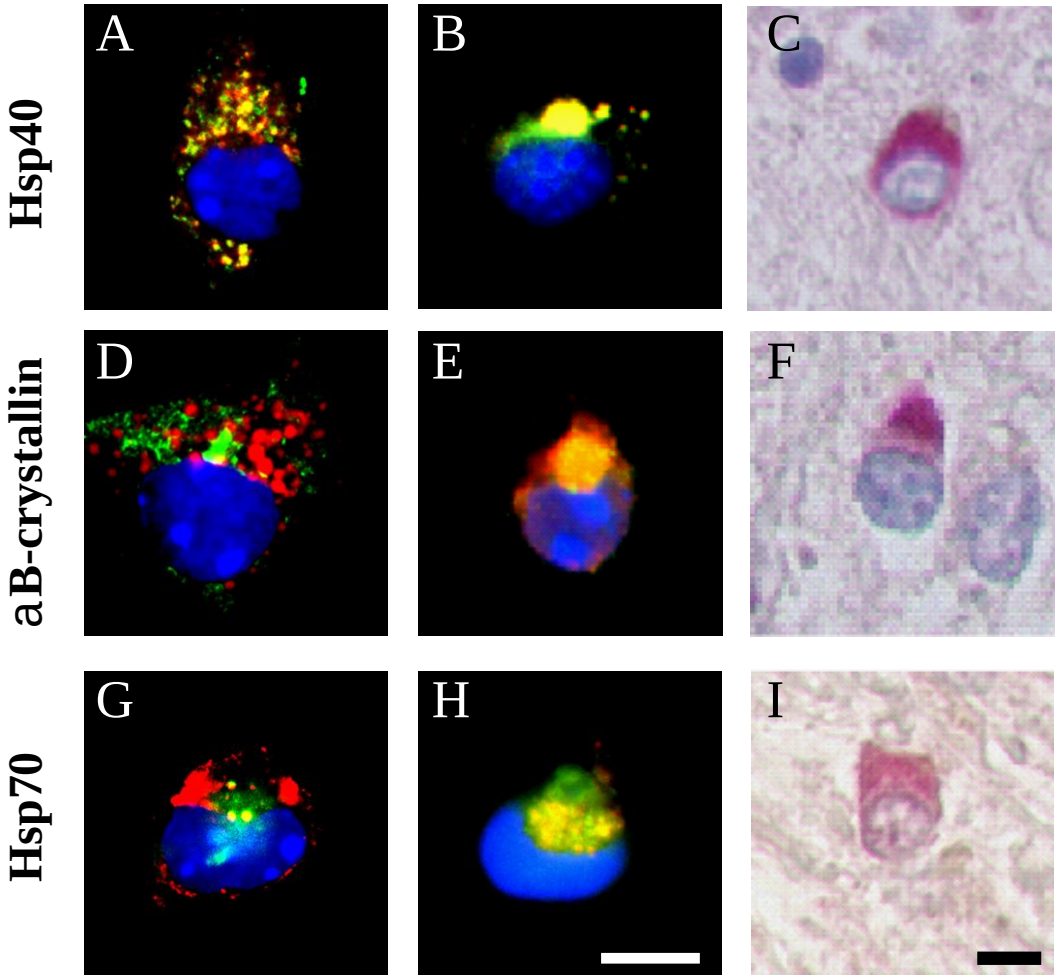


Figure 18: (A-I) Primary α-synuclein transgenic oligodendrocytes challenged with epoxomicin were double labeled with antibodies against α-synuclein (red) and hsp40, hsp70 and αB-crystallin (green). Yellow signal depicts the presence of these stress proteins in small (A, D, G) and large (B, E, H) α-synuclein aggregates. Nuclei were counterstained with Hoechst dye (blue). Hsp40, hsp70, and αB-crystallin are also present in GCIs of MSA patients. *Scale bars, 10μm*.

It has been suggested that the induction of αB-crystallin serves to maintain the integrity of the microtubular network, but sustained proteasome inhibition would overwhelm the chaperone capacity of oligodendrocytes, leading to a disruption of the microtubular network that is most important for oligodendrocyte viability (Goldbaum and Richter-

Landsberg, 2004). In my experimental system, I did not note any obvious aberrations of the microtubule-associated protein tau. However, inclusions formed in epoxomicin-treated α-synuclein transgenic oligodendrocytes were immunopositive for the tubulin polymerization promoting protein p25α (Figure 19A), which has been described to induce aberrant microtubule assemblies (Hlavanda et al., 2002) and stimulate α-synuclein fibrillization (Lindersson et al., 2005). Indeed, p25α immunoreactivity was detected in GCIs of MSA patients (Lindersson et al., 2005).

Furthermore, the microtubular network of oligodendrocytes might be influenced by 14-3-3 proteins. For example, 14-3-3ζ was reported to bind to tau and regulate its phosphorylation (Hashiguchi et al., 2000). Although there is generally no tau pathology in MSA patients and therefore very little overlap between tau and 14-3-3 immunoreactivity in GCIs (Giasson et al., 2003b), these pathological lesions do contain considerable 14-3-3 (Kawamoto et al., 2002). Similar to GCIs in MSA patient brains, inclusions formed in proteasome inhibited primary α-synuclein oligodendrocytes contain 14-3-3 (Figure 19B). The localization of 14-3-3 in these α-synuclein inclusions could simply reflect physical interaction between these two molecules (Ostrerova et al., 1999). Alternatively, the apparent recruitment of 14-3-3 into GCIs might indicate a specific interaction with α-synuclein whose aggregation is promoted by phosphorylation.

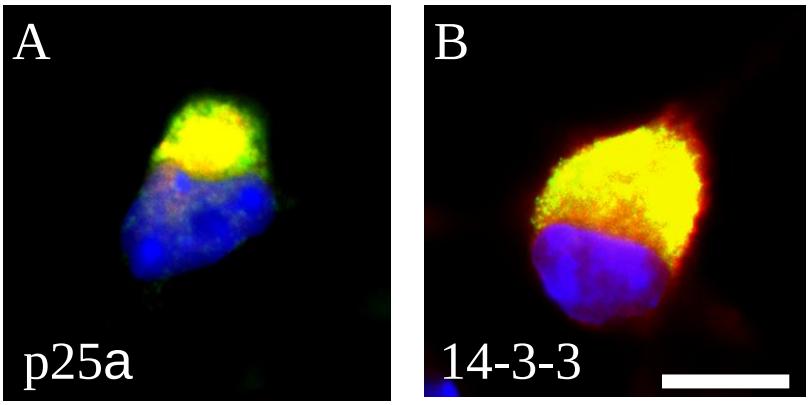


Figure 19: (A, B) Co-localization of transgenic α-synuclein in inclusions formed in epoxomicin-treated primary oligodendrocytes with p25α (A) and 14-3-3 (B) is evidenced by the yellow overlay of red α-synuclein signal and green marker signal. *Scale bar, 10μm*.

5.2 Pathological modifications of α-synuclein in proteasome-inhibited transgenic oligodendrocytes

The aggregation of α-synuclein into neuropathological lesions is accompanied by characteristic post-translational modifications, including phosphorylation at Ser129 (Fujiwara et al., 2002) and oxidative nitration (Giasson et al., 2000). Such pathological α-synuclein modifications were also prominently visualized in GCIs of MSA patients (Figure 20) using antibodies against Ser129-phosphorylated α-synuclein (Fujiwara et al., 2002) and oxidized α-synuclein (Duda et al., 2002), respectively. Likewise, some of the inclusions that developed in primary α-synuclein transgenic oligodendrocytes exposed to proteasome inhibitors were also decorated with these diagnostic antibodies (Figure 20). Phosphorylated α-synuclein and oxidized α-synuclein were more restricted to inclusions compared to overall α-synuclein staining with the 15G7 antibody, confirming the pathological nature of such modified α-synuclein.

The apparent oxidation of α-synuclein protein that specifically became detectable after proteasome inhibition in α-synuclein transgenic oligodendrocytes indicates that the

environment within these cells is oxidizing, consistent with the notion that UPS failure causes oxidative stress (Hyun et al., 2004; Lee et al., 2001c). Oxidative nitration of proteins is evidenced by strong immunolabeling of cytosolic α-synuclein inclusions with an antibody that recognizes 3-nitrotyrosine (3-NT) (Figure 20; insert).

Oxygen radicals can damage not only proteins, but also lipids. For example, polyunsaturated fatty acids are peroxidized by reactive oxygen species, and further oxidation leads to breaking of the acyl chain into toxic aldehyde metabolites. A marker of such lipid peroxidation is 4-hydroxy-2-nonenal (HNE), which can be detected by specific antisera (Uchida, 2003). Weak but significant immunostaining of HNE was observed in GCIs of human MSA patients as well as in the α-synuclein inclusions of proteasome-inhibited transgenic oligodendrocytes (Figure 20). Thus, proteasome inhibition in α-synuclein transgenic oligodendrocytes appears to generate free radicals, which oxidize proteins and lipids like in human patients.

α-synuclein within aggregates has an "amyloid" structure that is rich in β-pleated sheets (Conway et al., 2000a), which can be selectively stained with thiazole dyes such as thioflavin S. Indeed, GCIs in postmortem tissue from MSA patients could be stained with thioflavin S (Figure 20) following the procedure described by (Schmidt et al., 2001). In primary α-synuclein transgenic oligodendrocytes challenged with proteasome inhibitors, only 1.7% ± 0.5% of small α-synuclein aggregates, but 54% ± 3.2% of large α-synuclein aggregates were positively stained with thioflavin S (Figure 20). Thus, the peripheral small α-synuclein aggregates were not rich in "amyloid" fibrils, a property that appears to be acquired as the aggreates converge into large perinuclear GCI-like structures.

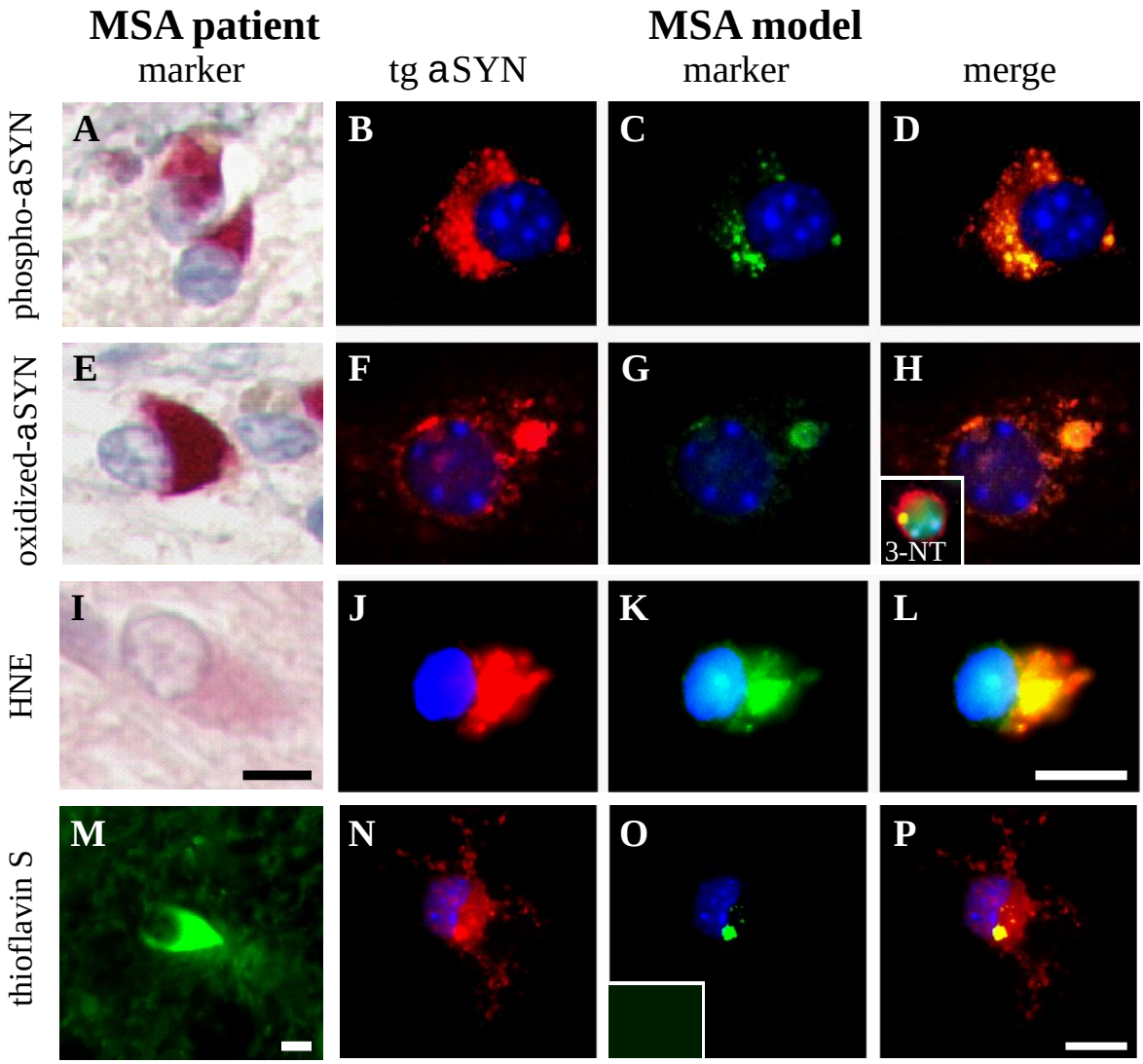
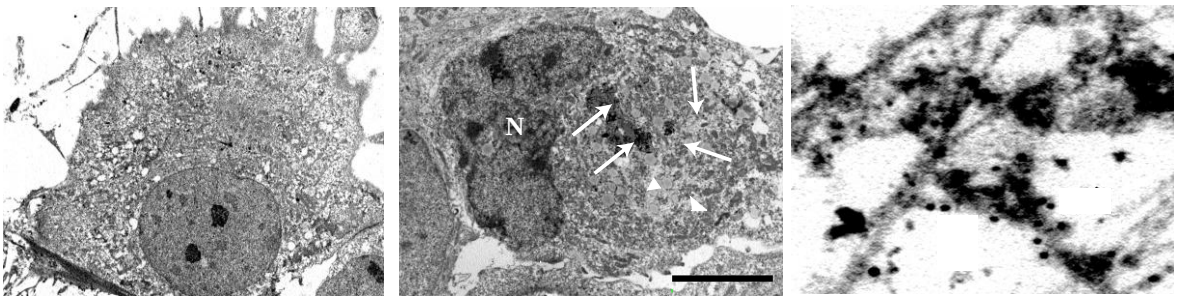


Figure 20: Sections from MSA brains and α-synuclein transgenic oligodendrocytes challenged with epoxomicin were stained with antibodies against markers of pathological modifications and oxidative stress. Both GCIs and inclusions in epoxomicin-treated oligodendrocytes were immunoreactive with antibodies against α-synuclein phosphorylated at Ser129 and oxidized α-synuclein. Generalized protein oxidation in GCI-like inclusions was detected with antiserum against 3-nitrotyrosine (insert). The presence of oxidized lipid in GCIs and α-synuclein aggregates was revealed by 4-hydroxynonenal staining. The amyloid nature of aggregated α-synuclein was revealed by thioflavin S staining (N-P), with the large perinuclear inclusions (loer left insert O) showing great similarities to GCIs (M). Note that all markers tended to be confined to the perinuclear inclusions in the cell culture model. Nuclei were counterstained with Hoechst dye (blue). *Scale bars, 20µm (A-L); 10µm (M-P).*

To further establish the fibrillar nature of the α-synuclein aggregates immunogold electron microscopy was performed on proteasome inhibitor treated transgenic oligodendrocytes. Immunogold-labeled α-synuclein fibrils were found neither in primary eGFP-transgenic nor in α-synuclein transgenic oligodendrocytes in the absence of proteasome inhibitor, or in epoxomicin-treated primary eGFP transgenic oligodendrocytes (not shown). In contrast, electron-dense aggregates were detected in primary α-synuclein transgenic oligodendrocytes challenged with epoxomicin (Figure 21). Immunogold labeling depicted large granulo-filamentous structures. These aggregates contained α-synuclein-positive fibrils, which appeared as straight filaments with a diameter of 15-25nm (Figure 21), consistent with the reported diameters of α-synuclein fibrils in GCIs (Gai et al., 2003; Tu et al., 1998).



control epoxomicin treated

Figure 21: Pre-embedding immunogold labeling electron microscopy was performed on primary α-synuclein transgenic oligodendrocytes. In the absence of proteasome inhibitor no immunogold-labeled α-synuclein fibrils were found (left panel). After treatment with epoxomicin, large aggregates formed in juxtaposed to indented nuclei (N). The spherical inclusions contained autophagosomes (arrows) and lipid droplets (arrowheads). Randomly arranged filaments associated with granular material were resolved at higher magnification. The fibrils were decorated with anti-α-synuclein immunogold particles (right panel). *Scale bar, 10µm* (mid panel).

6 Cellular Consequences of α-synuclein Aggregation for Affected Oligodendrocytes

6.1 Sensitization of α-synuclein transgenic oligodendrocytes to proteasome inhibitor mediated apoptosis

Up-regulation of the apoptosis-related protein Bcl-2 and terminal deoxynucleotidyl transferase-mediated nick end-labeling evidenced apoptosis in MSA patient oligodendrocytes (Probst-Cousin et al., 1998). Therefore I investigated if the formation of α-synuclein inclusions in transgenic oligodendrocytes was accompanied by apoptosis. Light microscopical examination showed that proteasome-inhibited transgenic oligodendrocytes displayed altered morphologies with shortened processes, shrunken cell bodies, and condensed chromatin. Electron microscopy of α-synuclein oligodendrocytes treated for 24h with 10μM epoxomicin demonstrated apoptotic changes, including invagination and disruption of the nuclear membrane and chromatin condensation. Vacuolizations within the cytosol were frequently observed, most notably autophagic vacuoles with double or single membrane containing electron-dense granular material. Mitochondria accumulated around the α-synuclein aggregates, as evidenced by cytochrome C immunofluorescence staining (Figure 22A) and electron microscopy (Figure 22B; white arrows). Most of the mitochondria appeared swollen, and α-synuclein was often found to be associated with the outer mitochondrial membrane (Figure 22C), suggesting a direct involvement of α-synuclein in oligodendrocyte apoptosis.

It has been suggested that some toxic α-synuclein folding intermediate might directly damage the mitochondria, potentially initiating an apoptotic cell death cascade (Hashimoto et al., 2003; Volles and Lansbury, 2003). Swollen mitochondria will eventually rupture and release cytochrome C, which in turn activates an apoptotic cascade that activates caspase-3 (Jiang and Wang, 2004; Polster and Fiskum, 2004). Indeed, immunostaining of activated caspase-3 was observed in epoxomicin-treated transgenic oligodendrocytes, particularly those with high levels of α-synuclein accumulation (Figure

22D-F). Consistent with the sequence of events during apoptosis, activation of caspase-3 preceded chromatin condensation and the final breakdown of the cell.

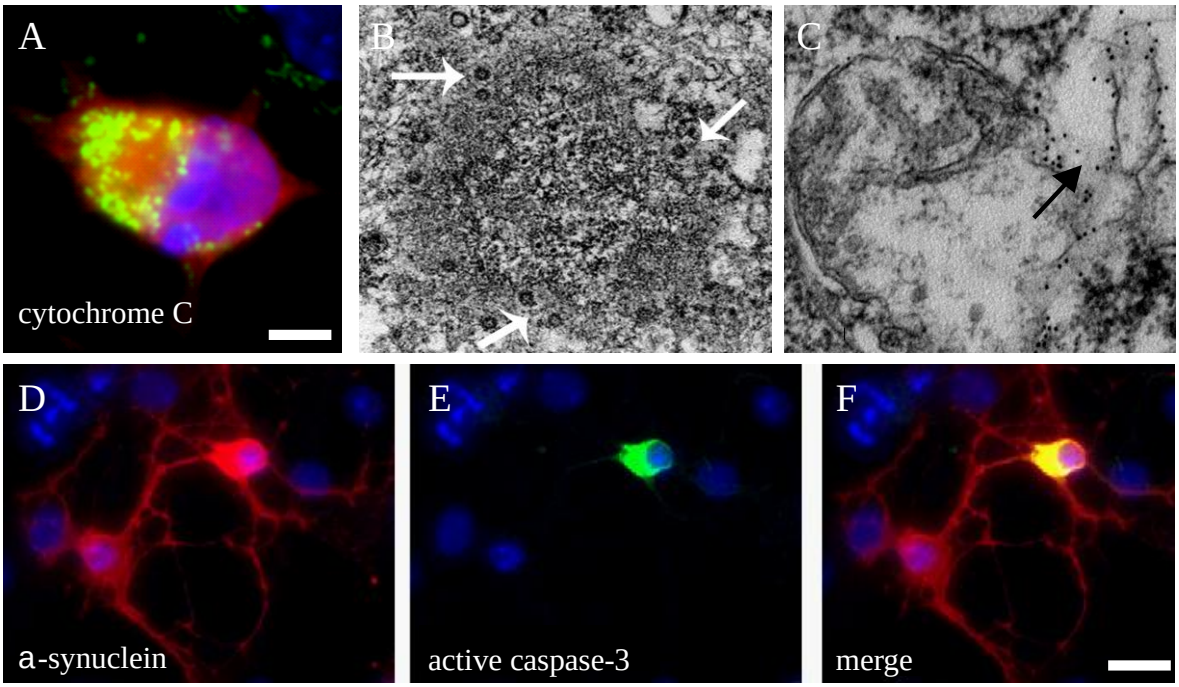


Figure 22: (A-C) The recruitment of mitochondria around the αSYN aggregates was shown by immunostaining with cytochrome c antibody (A, green) and electron microscopy (B, white arrows). *Scale bar, 5μm*. Anti-α-synuclein immunogold particles localized to the outer membrane of damaged mitochondria (C). (D-F) The accumulation of α-synuclein within the cytosol, prior the formation of microscopically visible α-synuclein aggregates (D) parallels the activation of capsase-3 (E, F). *Scale bar, 20μm*.

Quantitative analysis revealed that, when challenged with increasing doses of epoxomicin, primary α-synuclein transgenic oligodendrocytes were sensitized to caspase-3 activation (Figure 23A) and apoptotic chromatin condensation compared to eGFP transgenic oligodendrocytes. When left untreated, about 8% of α-synuclein- and eGFP-transgenic oligodendrocytes were positive for active caspase-3, reflecting spontaneous cell death. Proteasome inhibition with 0.1μM epoxomicin resulted in caspase-3 activation in 33.8% ± 2.1 of eGFP-overexpressing oligodendrocytes and 49.1% ± 0.7 of α-synuclein overexpressing cells. Hoechst staining revealed apoptotic morphology in about 35.3% ± 1.1 of these α-synuclein oligodendrocytes.

Because of the strong apoptosis-inducing effects of epoxomicin (Figure 23A), I studied the correlation between GCI formation and oligodendroglial apoptosis in LV-αSYN transduced cultures, in which inclusions form even in the absence of proteasome inhibitor. Transduction with the control vectors LV-eGFP and LV-βSYN activated an apoptotic cascade (as evidenced by active caspase-3 immunostaining) in 10-15% of the cells, most likely reflecting general toxicity of transduction of primary oligodendrocytes. Remarkably, a significantly greater proportion of the LV-αSYN transduced oligodendrocytes showed activation of caspase-3 (Figure 23B). Similar to oligodendrocytes isolated from transgenic (PLP)-α-synuclein mice, oligodendrocytes transduced with LV-αSYN were sensitized almost two-fold to proteasomal inhibition, even beyond the significant toxicity medited by lentiviral overexpression alone (Figure 23B).

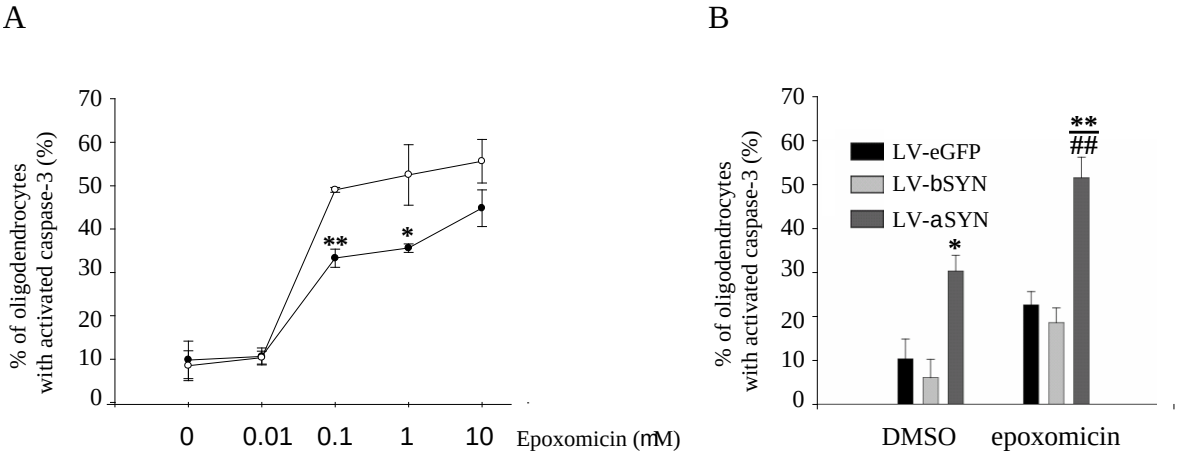


Figure 23: (A, B) Quantitative analysis depicts the dose-dependent activation of the apoptosis effector caspase-3 in primary oligodendrocytes. Oligodendrocytes were scored as active caspase-3 immunoreactive when the cytosol was intensely labeled. The graphs show the primary stained with O4. Parallel transgenic eGFP and α-synuclein cultures were exposed for 1 day to increasing concentrations of epoxomicin (A). Wild-type oligodendrocytes were transduced with LV-eGFP (black bars), LV-βSYN (light bars), and LV-αSYN (dark bars), and subsequently treated with 0μM or 10μM epoxomicin (B). Data are means +/- SEM from 3-5 independent experiments conducted in triplicate. Student *t* test; *P<0.05; **P<0.001 compared to eGFP, ##P<0.001 compared to solvent control.

7 Lentiviral Delivery of β-Synuclein Suppresses the Formation of α-Synuclein Inclusions and Apoptosis in Proteasome-Inhibited Transgenic Oligodendrocytes

Since α-synuclein aggregation seems to parallel caspase 3 activation on my MSA model, I decided to investigate whether anti-aggregative gene therapy could reduce α-synuclein mediated cytotoxicity.

β-synuclein is not only incapable of forming pathological fibrils, but actually inhibits α-synuclein aggregation in vitro (Hashimoto et al., 2001; Park and Lansbury, 2003; Uversky et al., 2002). When (PDGFβ)-α-synuclein mice were crossbred with transgenic mice

expressing β-synuclein throughout the brain, or when β-synuclein was administered using lentiviral vectors, a significant reduction of the number of neuronal α-synuclein inclusions was detected (Hashimoto et al., 2004; Hashimoto et al., 2001).

In order to establish the anti-aggregative and cytoprotective properties of LV-βSYN for oligodendrocytes, differentiated primary α-synuclein transgenic oligodendrocytes were transduced with LV-eGFP or LV-β-synuclein. After 2 days, cells were incubated for another 18h with 10μM epoxomicin or control solvent. 15G7 immunostaining analysis revealed that about 60% of the transgenic oligodendrocytes depicted cytosolic α-synuclein aggregates when transduced with LV-eGFP lentiviral or left untransduced. This percentage was decreased to 23.4 ± 4.7% when α-synuclein transgenic oligodendrocytes were transduced with LV-βSYN prior to the exposure to epoxomicin (Figure 24A).

While less than 10% of control α-synuclein oligodendrocytes were apoptotic, epoxomicin treatment increased this percentage to approximately 60%. In contrast, LV-βSYN transduction decreased the number of oligodendrocytes with activated caspase-3 down to 17.7 ± 3.8% (Figure 24B). Given the close correlation between the percentage of oligodendrocytes bearing inclusions and activated caspase-3, and the rescue mediated by LV-βSYN, GCI formation may be cytotoxic. Furthermore, LV-βSYN gene therapy could suppress oligodendrocyte pathology and possibly even putative oligodendrocyte dysfunction in MSA.

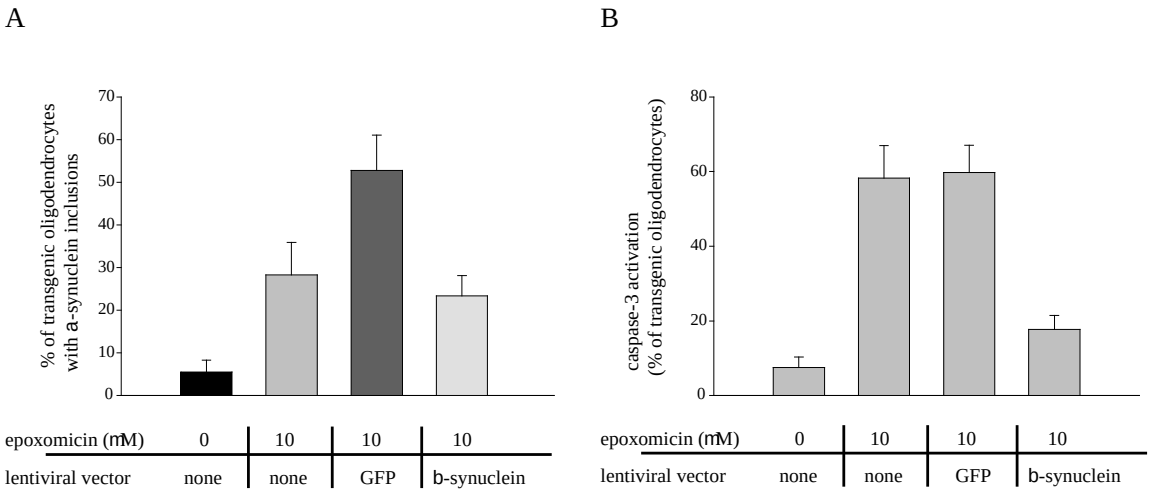


Figure 24: Primary (PLP)-α-synuclein transgenic oligodendrocyte cultures were incubated with no vector, LV-eGFP, and LV-βSYN, as indicated. After 2 days in culture, the cells were challenged or not (0μM) with 10μM epoxomicin for another day. Then cells were fixed and stained with anti-αSYN (A) and activated caspase-3 (B). Data are means +/- SEM from 3-5 independent experiments conducted in triplicate. Student *t* test; *P<0.05; **P<0.001.

8 Molecular Mechanisms Involved in Oligodendroglial Cell Death

8.1 Both the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways are involved in α-synuclein-sensitized oligodendrocyte apoptosis

To substantiate the causal role of caspases in the apoptosis of epoxomicin-treated α-synuclein oligodendrocytes, primary transgenic oligodendrocytes were pre-incubated for 20min with 100 μM of the broad-spectrum caspase inhibitor z-VAD-fmk and evaluated the percentage of apoptosis induced by 10μM epoxomicin. While 52.5% ± 5.5 of the transgenic α-synuclein oligodendrocytes displayed loss of cell processes and apoptotic chromatin condensation visualized by Hoechst staining after 24h proteasome inhibition,

pan-caspase inhibition strongly protected transgenic oligodendrocytes from cell death, bringing the percentage of apoptotic cells down to basal levels (Figure 25).

To further dissect the apoptosis pathways, specific inhibitors z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk against respectively caspase-3, caspase-8 and caspase-9 were employed. Consistent with its role as an executioner of apoptosis, caspase-3 was found to be critical to mediate apoptosis in epoxomicin-treated α-synuclein transgenic oligodendrocytes, because caspase-3 inhibition strongly suppressed apoptosis under these conditions (Figure 25). Inhibition of caspase-9 significantly reduced apoptosis, consistent with a mitochondrial apoptosis pathway. Unexpectedly, the percentage of cell death was dramatically decreased to about 3% in the presence of a caspase-8 inhibitor (Figure 25). Caspase-8 is a pivotal mediator of death receptor-triggered apoptosis (Thorburn, 2004). Thus, in addition to mitochondrial apoptosis pathways, ligand-induced cell death pathways might be of relevance to α-synuclein-sensitized oligodendrocyte death.

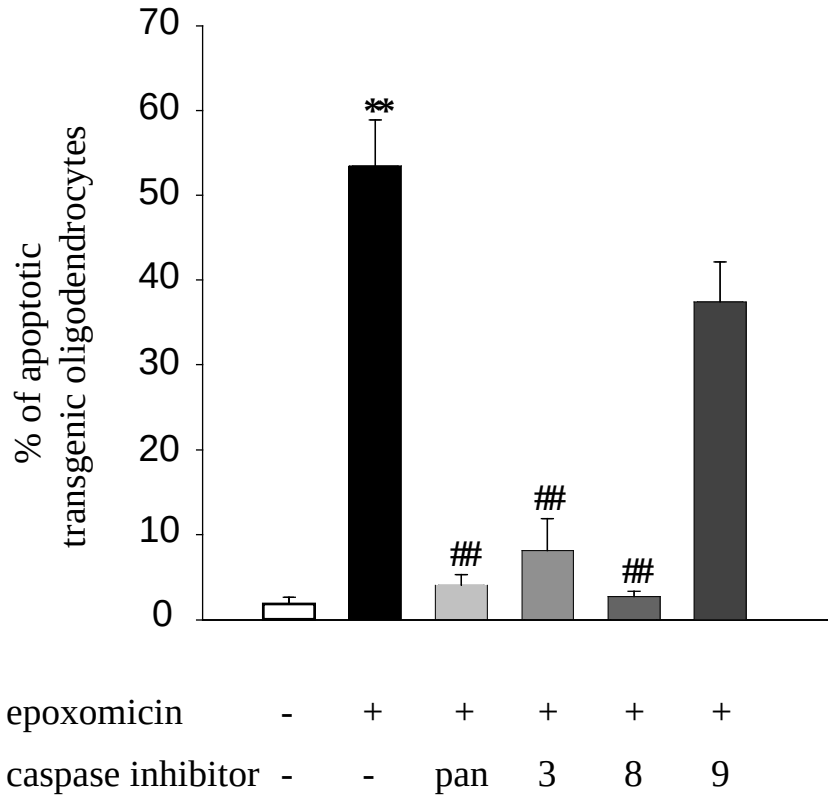


Figure 25: After 2 weeks in culture, primary α-synuclein transgenic oligodendrocytes were treated with the broad spectrum caspase inhibitor z-VAD-fmk or specific inhibitors z-DEVD-fmk, z-IETD-fmk and z-LEHD-fmk against caspases-3, -8 and -9. Control cultures were returned to BS medium in the absence of caspase inhibitors. After 20min, 10μM epoxomicin (or BS medium for control cultures) was added to the wells for a further 24h. Cells were immunostained for α-synuclein, active caspase-3 and counterstained with Hoechst nuclear dye. Quantitative analysis revealed that proteasome inhibition and subsequent α-synuclein accumulation elicited caspase-3 activation (I, **) and cell death (J, **), which were significantly blocked by caspase 1, 3 and 8 inhibitors (##). Data are means +/- SEM from 5 independent experiments conducted in triplicate. Student *t* test; **P<0.001 compared to control; ##P<0.001 compared to epoxomicin alone.

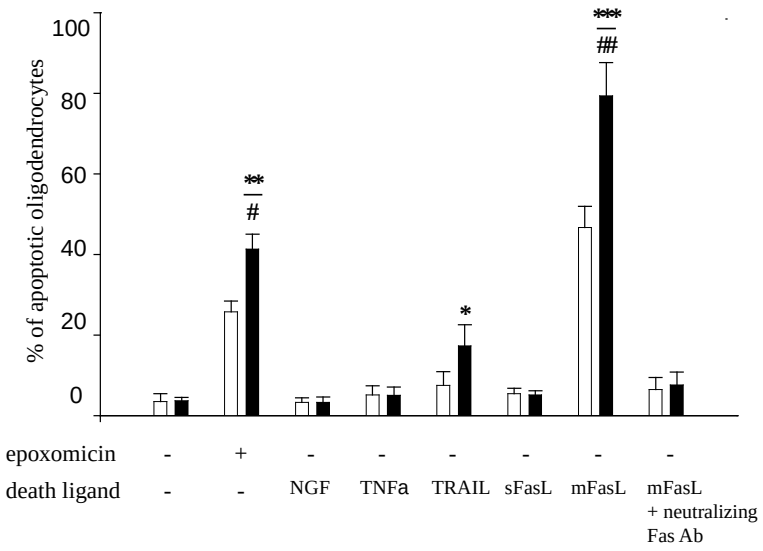
8.2 Expression of α-synuclein in oligodendrocytes specifically sensitizes to Fas-mediated apoptosis via Fas upregulation

The surprising efficacy of caspase-8 inhibitors to suppress α-synuclein-sensitized apoptosis in primary oligodendrocytes prompted me to systematically investigate the effects of death receptor ligands on transgenic oligodendrocytes. Differentiated primary oligodendrocyte cultures derived from eGFP and α-synuclein transgenic mice were treated for 24h with nerve growth factor (NGF, which can exert cytotoxicity via the p75 neurotrophin receptor), the pro-inflammatory tumor necrosis factor-α (TNF-α, which activates caspase-8 via recruitment of the TNF receptor associated death domain protein), TNF-Related Aptosis-Inducing Ligand (TRAIL) and Fas ligand (stimulating apoptosis through the recruitment of Fas associated death domain proteins). NGF and TNF-α treatment had no significant cytotoxic effect under these conditions, neither in primary eGFP oligodendrocytes nor in primary α-synuclein transgenic oligodendrocytes (Figure 26). TRAIL caused a moderate increase in the percentage of oligodendrocytes with apoptotic nuclei, especially in the α-synuclein transgenic cultures, but the cytotoxic effect of TRAIL was much lower compared to, e.g. epoxomicin treatment (Figure 26).

In contrast to the ineffective, soluble form of Fas ligand (Schneider et al., 1998), which did not efficiently stimulate apoptosis under these conditions, the active, membrane-bound form of Fas ligand stimulated apoptosis in 46.7 ± 5.24% of eGFP transgenic oligodendrocytes. α-synuclein transgenic oligodendrocytes were significantly sensitized to Fas-mediated apoptosis, with 79.3 ± 8.3% cells bearing apoptotic nuclei (Figure 26). The apoptotic potency of membrane-bound Fas ligand was completely blocked by neutralizing

with Fas antibody (Figure 26A, B), corroborating the specificity of α-synuclein sensitization to Fas-mediated apoptosis in cultured oligodendrocytes.

A



B

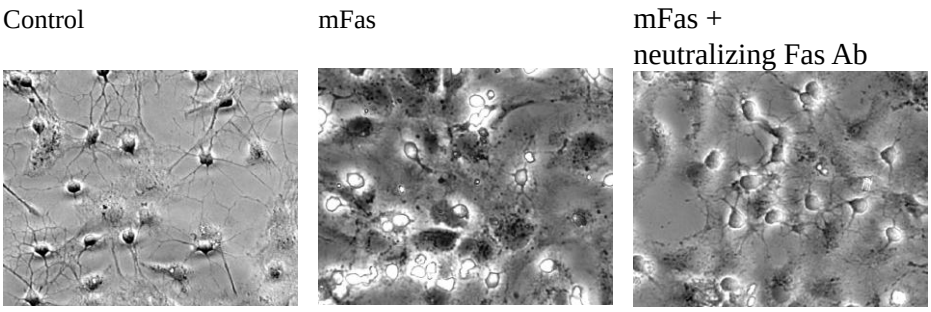


Figure 26: (A) eGFP and α-synuclein oligodendrocytes were treated 24h with DMSO, epoxomicin, NGF, TNFα, TRAIL, soluble Fas ligand (sFas), membrane-bound Fas ligand (mFas) or preincubated for 30min with Fas blocking antibody and challenged with mFas. Cells were then fixed, labeled with O4 and propidium iodide. mFas ligand treatment induced Fas trimerization and oligodendrocyte cell death, α-synuclein oligodendrocytes being significantly (*) more sensitive to Fas mediated cell death than eGFP oligodendrocytes. Fas ligand mediated cell death was reverted by preincubation with Fas-blocking antibody. Data represent mean percentage ± S.E.M of total transgenic oligodendrocytes from 3 independent experiments. Student *t* test *P<0.05, **P<0.001, ***P<0.0001 compared to untreated cultures; #P<0.005, ##P<0.001 compared to eGFP transgenic cultures exposed to the same challenge.

(B) Phase contrast imaging shows that pretreatment with Fas blocking antibody preserved oligodendrocyte morphology and prevented process loss.

The simplest mechanism by which oligodendrocytes would be sensitized to the apoptotic effects of Fas ligand is up-regulation of Fas itself. I determine by biochemical analysis Fas levels in wild-type and α-synuclein transgenic oligodendrocytes and found a great upregulation in a-synuclein transgenic cells (figure 27).

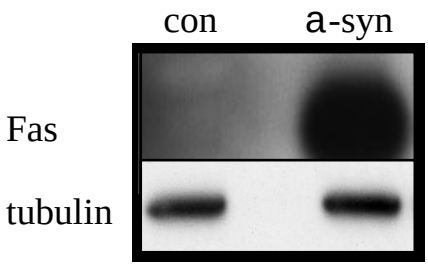


Figure 27: Primary wild-type and α-synuclein transgenic oligodendrocytes were cultured for 2 weeks and lysed. 30μg of cell lysates were electrophoresed and Western blotted, and probed with monoclonal Fas antibody.

8.3 Fas upregulation is observed in MSA

To investigate if Fas upregulation and Fas-sensitization observed in transgenic α-synuclein oligodendrocytes are relevant to MSA, I analyzed the expression of Fas in post-mortem human tissue. Remarkably, Western blots prepared from cerebellar white matter lysates of MSA patients contained more Fas than control samples (Figure 28). To prove that α-synuclein aggregation (GCI formation) specifically up-regulates the Fas receptor on oligodendrocytes, double-label immunostaining using antibodies against α-synuclein and Fas was performed on cerebellar white matter from human MSA brain. Immunohistochemistry confirmed that Fas was upregulated in MSA, and identified

oligodendrocytes as the cell type that induced the pro-apoptotic Fas receptor. In contrast to control samples, which were generally devoid of Fas, MSA white matter showed Fas receptors specifically associated with GCIs (Figure 28). Roughly 10-15% of GCI-bearing oligodendrocytes identified by α-synuclein staining were immunopositive for Fas. The punctuated pattern of Fas immunostaining on pathological oligodendrocytes is similar to the punctuated Fas staining of oligodendrocytes in multiple sclerosis lesions (D'Souza et al., 1996). It is tempting to speculate that this reflects Fas receptor clustering, which activates the death domain to induce apoptosis. In addition to oligodendroglial (GCI) pathology, MSA is also characterized α-synucleinopathy in neurons (NNIs). α-synuclein and Fas double-immunostained sections of MSA patients demonstrated that NNI-bearing neurons were strongly Fas-immunoreactive (Figure 28E). Thus, induction of the pro-apoptotic Fas receptor by α-synuclein aggregation is not restricted to oligodendrocyctes, but is also observed on neurons. It would be therefore of greatest importance to investigate whether LB positive neurons in PD and DLB also induce Fas, to determine whether Fas is a general key component of cytotoxicity in α-synucleinopathies

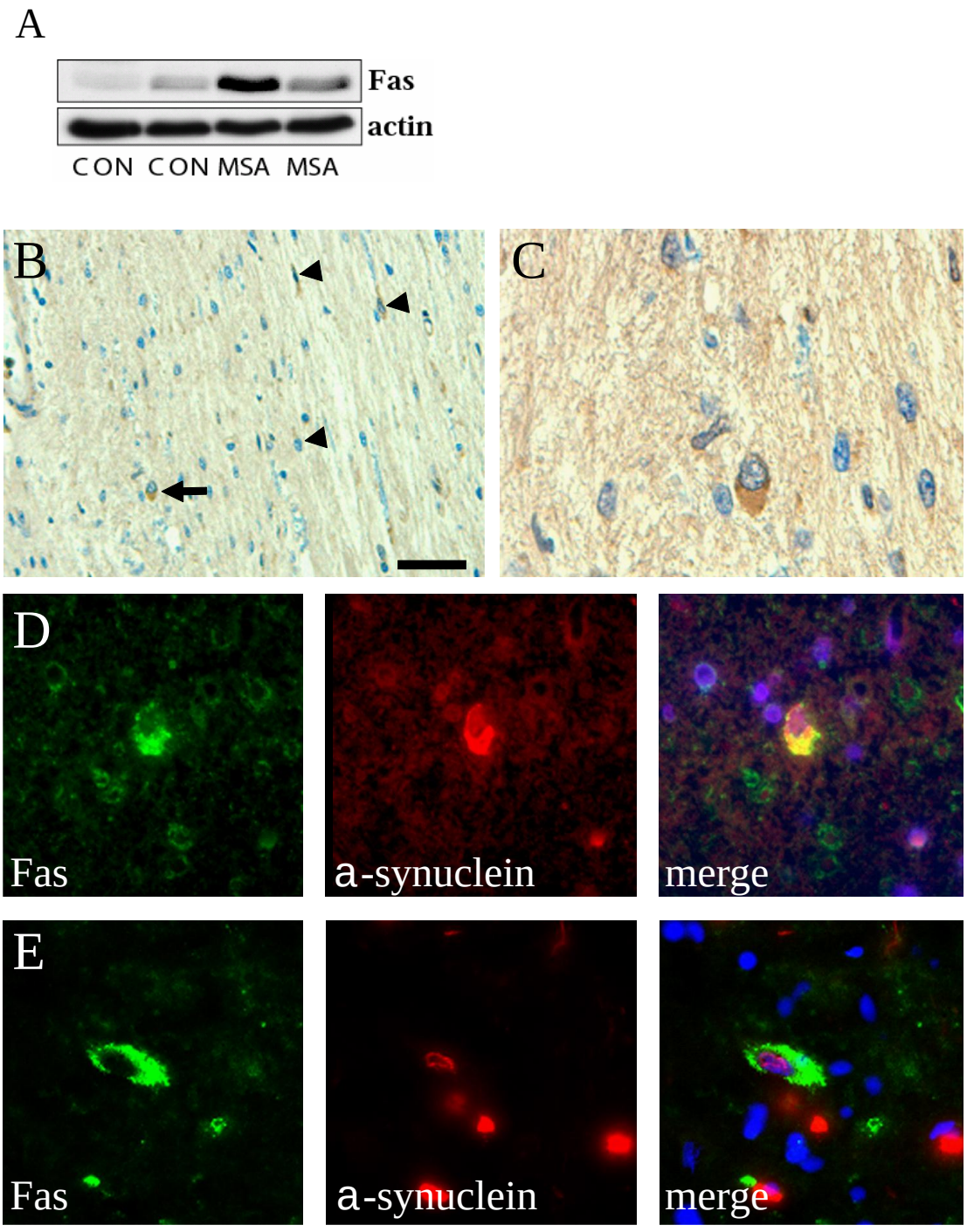


Figure 28: (A) Western blot prepared from lysates of cerebellar white matter of controls (lanes 1, 2) and MSA patients (lanes 3, 4) were sequentially probed with anti-Fas (upper panel) and anti-actin (lower panel) to confirm equal loading. (B-C) Immunohistochemical analysis of cerebellar white matter in human MSA brain reveals Fas-positive microglial cells and astrocytes (arrowheads) and staining of cells, which resemble GCI-bearin goligodendrocytes (arrow). (B) Higher magnification of

GCI-bearing oligodendrocytes. (D-E) Double-label immunofluorescence staining for Fas and αSYN of cerebellar white matter in human MSA brain reveals Fas-expression (green) co-localizes to a subset of oligodendrocytes with αSYN-positive GCIs (red). In addition, strong Fas labeling was observed in single neurons with nuclear αSYN-positive inclusions in the pons (E). Scale bar corresponds to 50μm (B) and 12.5 μm (C-E).

Chapter 5: Discussion

Recently, the dual roles of α-synuclein in neuroprotection and neurotoxicity were described (Chandra et al., 2005; da Costa et al., 2003). However, there is no clear picture of its biological activity, and the primary pathophysiological function of α-synuclein remains to be better defined. α-synuclein is genetically and pathologically linked to PD (Pfeiffer and Wagner, 1994; Polymeropoulos et al., 1997; Spillantini et al., 1997) and neurodegenerative disorders characterized by the presence of pathological brain inclusions that predominantly consist of filamentous, aggregated α-synuclein protein are collectively referred to as α-synucleinopathies (Spillantini et al., 1998a). While neurons are predominantly affected by pathological misfolding of α-synuclein in PD and DLB, oligodendroglial cytoplasmic α-synuclein inclusions comprise the hallmark for MSA.

1) Adverse effects of α-synuclein on oligodendrocyte viability

The primary importance of oligodendroglial dysfunction in the neurodegenerative process of MSA may also be supported by the observation that the distribution of cells undergoing apoptosis is comparable with that of the GCI pathology and also that apoptosis primarily affects oligodendroglial cells in MSA (Probst-Cousin et al., 1998).

Many toxin animal models of MSA have been developed over the years. Two of the most widely used toxin models to mimic the symptoms and striatonigral degeneration of MSA involve chemical administration to rodents of either MPTP (Ghorayeb et al., 2002) or 3-nitropropionic acid (Fernagut et al., 2002) (single toxin-double lesion models) or 6-hydroxydopamine and quinolic acid (double toxin-double lesion model) (Puschban et al.,

2000). Although both of these models are believed to induce MSA-like pathology and/or phenotype through oxidative mechanisms (Stefanova et al., 2005c), they did not recapitulate the presence of α-synuclein inclusions.

Both causes and effects of α-synuclein aggregation in MSA remain enigmatic as oligodendrocytes express undetectable levels of α-synuclein in the mature brain (Solano et al., 2000b). Thus, ectopic expression of α-synuclein could be causally linked to MSA pathology. Therefore, to more thoroughly and realistically study MSA in animals, transgenic mouse models were created. (PLP)-α-synuclein mice showed an accumulation of hyperphosphorylated α-synuclein within oligodendrocyte cell bodies (Kahle et al., 2002b). The pathologically phosphorylated transgenic α-synuclein was found to be detergent-insoluble. However, the transgenic expression of α-synuclein in mouse brain under the PLP promoter leads only to an early pathology as α-synuclein did not further mature to true "amyloid" fibrils. Possibly the (PLP) oligodendrocyte specific promoter employed did not mediate sufficiently high expression to generate a phenotype. Alternatively, oligodendrocytes may have a great capacity to suppress α-synuclein misfolding, either because of an abundance of small heat shock proteins (such as αB-crystallin) (Neri et al., 1997) or additional genetic or environmental risk factors may exacerbate oligodendroglial MSA pathology.

One such risk factor might be exposure to environmental toxins. In fact, the (PLP)-α-synuclein mice were sensitized to 3-nitropropionic acid, a mitochondrial toxin and resulted in MSA-like features including complex locomotor impairment and neuropathological changes such as striatonigral degeneration, olivopontocerebellar neuronal loss, astrogliosis, and microglial activation, along with the GCI-like pathology. Environmental toxins

exacerbate MSA pathology in the mouse (Stefanova et al., 2005b). These results support the idea of a "multi-hit" scheme for MSA pathogenesis in which mitochondrial dysfunction may play a role. Another pro-aggregative risk factor may be concomitant taupathy. When a (CNP) promoter was used employed to express human α-synuclein in transgenic mouse oligodendrocytes, no particular pathology and phenotype were observed in the young adults. When these animals were crossbred with mice that express (P301L)tau in oligodendrocytes, a subset of bigenic oligodendrocytes developed a fibrillar α-synuclein pathology, as demonstrated by thioflavin S staining (Giasson et al., 2003a). Such amyloid formation coincided with a limb phenotype. It is tempting to speculate that in oligodendrocyte cytosol, α-synuclein and tau synergistically form individual fibrils, as was shown *in vitro* (Giasson et al., 2003a). However, it has not been reported if demyelination and/or neurodegeneration occur in specific brain regions of these mice, and what molecular mechanisms contribute to the phenotype of (CNP)-α-synuclein x tau mice.

Earlier this year, a more human disease-like transgenic mouse was created by Yazawa *et al.* Human α-synuclein is expressed in oligodendrocytes under the control of the (CNP) oligodendrocyte-specific promoter (Yazawa et al., 2005). These mice exhibit some age-dependent neurodegeneration, and α-synuclein-positive aggregates are present in oligodendrocytes, supporting the view that α-synuclein pathology in oligodendrocytes may cause neurodegeneration (Yazawa et al., 2005). However, these α-synuclein inclusions are not thioflavin S positive as they are in the human diseased brain. The CNP-a-synuclein mice lack neuronal cell loss in many brain regions commonly affected in MSA, such as substantia nigra. These studies indicate that α-synuclein overexpression alone is not adequate for truly replicating the complexities of MSA pathology and highlight the

importance of both genetic predisposition (ectopic α-synuclein expression in oligodendrocytes) and epigenetic factors (environmental toxins, aging) causes in MSA (Stefanova et al., 2005). Future studies will likely examine whether substantia nigra and other brain regions are more vulnerable to toxic insults such as mitochondrial, as was recently demonstrated in mice that overexpress α-synuclein in oligodendroglia via the PLP promoter (Stefanova et al., 2005).

By developing a transgenic mouse that overexpresses human α-synuclein in oligodendrocytes via the oligodendrocyte specific MBP promoter, Shults and colleagues (Shults et al., 2005) have effectively recapitulated several of the key functional and neuropathological features of MSA. Not only do mice exhibit GCI formation in oligodendrocytes, they also display signs of neurodegeneration. The sequence of these events suggests that oligodendroglial abnormalities are the primary cause of MSA and drive neurodegeneration. This work further establishes a critical role for α-synuclein in MSA pathology and neurodegeneration. It supports the prediction that an overabundance of α-synuclein in oligodendroglia is a key event in MSA pathogenesis.

When I started this work, it existed only the (PLP)-α-synuclein genetic model, which failed to fully recapitulate MSA pathology (Kahle et al., 2002b) and the consequences of α-synuclein expression, as well as the molecular mechanisms leading to its aggregation, had never been studied in primary oligodendrocyte cultures. The goal of my PhD research project was to gain insight into the mechanisms by which α-synuclein aggregation causes oligodendroglial pathology and how this process can be modulated. I focused mainly on a biochemical and cell biological analysis of α-synuclein fibrillogenesis and toxicity in

oligodendrocyte. For this purpose, I developed a cellular model of oligodendroglial MSA pathology by isolating oligodendrocyte progenitors from transgenic (PLP)-α-synuclein mice.

Immunostaining of primary transgenic oligodendrocyte cultures with the human specific monoclonal 15G7 antibody revealed that the transgenic α-synuclein is diffusely distributed throughtout the cytosol. Possibly, (PLP) α-synuclein expression in primary oligodendrocytes under basal conditions was not robust enough to induce α-synclein aggregation and oligodendrocyte degeneration, similar to what was documented for transgenic (PLP)-α-synuclein mice *in vivo* (Kahle et al., 2002b). Proteasome inhibition of (PLP)-α-synuclein transgenic oligodendrocytes increased cytosolic α-synuclein concentrations and induced α-synuclein aggregation. Initially, small α-synuclein aggregates formed and then appeared to coalesce and mature to a single large perinuclear GCI-like inclusion, similar findings were reported in an adenoviral COS-7 cell culture model (Lee and Lee, 2002). Special emphasis should be placed on the process of formation of the large inclusions form. In fact, it remains unknown whether this maturation follows an aggresome-like mechanism by which small aggregates would be transported to the microtubule organizing center (Johnston et al., 1998). Thus, interfering with the microtubular transport system could help elucidating the maturation mode of the large α-synuclein aggregates. The tubulin depolymerizing drug nocodazole is widely used to block aggresome formation (Lee and Lee, 2002), but its use in primary (PLP)-α-synuclein oligodendrocytes was hampered by its toxicity. Thus, it remains to be shown if the formation of GCIs involve aggresomal mechanisms.

The use of proteasome inhibitors to induce MSA-like pathology raises the issue of potential artifacts due to general protein turnover impairment by proteasome inhibition and makes it difficult to determine whether high α-synuclein levels could be sufficient to induce its aggregation. An alternative approach to highly express α-synuclein in primary oligodendrocytes cultures is to transduce wild-type oligodendrocytes with lentivirus harboring α-synuclein constructs. By transducing wild-type mouse oligodendrocytes with lentiviral vectors harboring the aggregation-prone α-synuclein, the non-amyloidogenic β-synuclein, and control eGFP, high-level protein expression were achieved. Unlike eGFP and β-synuclein, which evenly distributed throughout oligodendrocytes, α-synuclein often formed perinuclear inclusions, reminiscent of α-synuclein staining in MSA patients (Lantos, 1998). In summary, my data provides evidence that α-synuclein, when expressed at sufficiently high concentrations, may spontaneously form GCI-like aggregates.

The nature of the large α-synuclein aggregates was characterized using established markers of GCI pathology. Immunostaining with antibodies against ubiquitin revealed that α-synuclein inclusions were positive ubiquitin, a well known GCI marker (Kato et al., 1991). Finally, the specific accumulation of strictly pathologically phosphorylated and oxidized α-synuclein was shown by immunofluorescence with a specific antibody raised against phosphorylated serine 129 and nitrated α-synuclein (Duda et al., 2000; Giasson et al., 2000; Fujiwara et al., 2002).

The granulo-filamentous appearance of electron-dense inclusions is typical for GCIs (Kato et al., 1991), and these fibrils are labeled with anti-α-synuclein immunogold particles

(Arima et al., 1998; Spillantini et al., 1998a). The fibrillar "amyloid" conformation of aggregated α-synuclein in my models were confirmed by thioflavin S staining and the presence of immunogold labeled α-synuclein fibrils in the inclusions.

Detergent-resistant α-synuclein is a biochemical marker of MSA autopsy samples and (PLP)-α-synuclein transgenic mice (Kahle et al., 2002b). Conversion of detergent-insoluble α-synuclein to fibrillar "amyloid" appears to be age- and gene dependent, at least in neuronal α-synucleinopathy mouse models (Giasson et al., 2002; Lee et al., 2002; Neumann et al., 2002). Biochemical experiments using the relatively mild urea extraction protocol was performed on primary transgenic α-synuclein and eGFP oligodendrocyte cultures and revealed pathological insolubility of a significant fraction of the transgenic α-synuclein after proteasome inhibition, in contrast to eGFP protein. The characteristic ubiquitination of α-synucleinopathy inclusions were also visualized in my model by Western probing extracts from transgenic oligodendrocytes with antibodies against ubiquitin.

Once the primary culture models of oligodendroglial α-synuclein fibrillization were fully characterized, the adverse effects of α-synuclein on oligodendrocyte viability were studied. These pathological deposits evidently impose a challenge to the oligodendrocytes, and it is reasonable to assume that the observed co-localization of chaperone proteins in the α-synuclein inclusions formed in the epoxomicin-treated transgenic oligodendrocytes as well as in GCIs of human patients reflects a defense mechanism against protein misfolding. Hsp70 has been previously shown to suppress α-synuclein aggregation in cell culture (McLean et al., 2002) and in neurons of transgenic mice (Klucken et al., 2004), and

neurotoxicity in transgenic *Drosophila* (Auluck et al., 2002). The finding of Hsp70 and its co-chaperone Hsp40 in α-synuclein inclusions formed in oligodendrocyte cultures suggests that these molecular chaperones are recruited to these pathological structures. Furthermore, αB-crystallin, a reference marker of GCIs, was also present in transgenic oligodendrocyte α-synuclein inclusions, thereby providing evidence for the recruitment of this chaperone protein to oligodendroglial α-synuclein inclusions, as suggested in a recent report using C6 glioma cells (Pountney et al., 2005). The small heat shock protein αB-crystallin is thought to be a major chaperone in oligodendrocytes, which is instrumental to maintain microtubular integrity of these cells (Goldbaum and Richter-Landsberg, 2004). However, when proteolytic stress exceeds the chaperone capacity, disturbances of the microtubular network may accrue. It is possible that p25α and 14-3-3, both of which have been found in GCIs (Kawamoto et al., 2002; Kovacs et al., 2004) and in proteasome inhibitor treated α-synuclein transgenic oligodendrocytes, reflect such microtubular damage in a manner that remains to be fully established.

α-synuclein fibrillization within oligodendrocytes is cytotoxic as shown by the deterioration of the ramified morphology that characterizes well-differentiated oligodendrocytes. How the formation of α-synuclein aggregates is mechanistically linked to cellular toxicity is a matter of intense debate (Wanker, 2000). Several reports presented evidence that aggregate formation may even be a protective mechanism (Tanaka et al., 2004; Taylor et al., 2003). Mainly two, non-exclusive, ideas have been put forward for how α-synuclein aggregation may cause cellular dysfunction. In one model, aggregated α-synuclein proteins are thought to engage the ubiquitin proteasome system in a non-

productive manner, causing a partial inhibition of proteasome-dependent cell regulation (Bence et al., 2001; Kovacs et al., 2004; Snyder et al., 2003). In the other model, cell toxicity results from the ability of the aggregates to sequester factors essential to cell viability, such as components of the protein quality control system. The underlying mechanisms of both proposed toxicity mechanisms and their contribution to the pathology of MSA remain to be elucidated. Inhibition of the proteasome system upon α-synuclein expression was observed in α-synuclein transgenic oligodendrocytes compared to eGFP oligodendrocytes. The impairment of the proteasome system observed in culture was not sufficient to induce α-synuclein aggregation but may nevertheless be of pathomechanistic significance, considering the long duration of the disease and the postmitotic nature of oligodendroglial cells. Preliminary experiments done on MSA brain samples showed a great decrease of the chymotryptic activity in patients compared to controls, supporting the involvement of the ubiquitin proteasome system in MSA pathogenesis. The models I developed should prove useful to test whether α-synuclein aggregation is likely a cause leading to inhibition of the proteasome degradation. The lentiviral α-synuclein system would serve to provide insight into α-synuclein toxicity mechanisms that affect ubiquitin-proteasome function.

2) Molecular mechanisms of oligodendroglial degeneration

No matter how the apparent critical concentration for α-synuclein fibril formation is reached (accumulation of transgenic α-synuclein after proteasome inhibition or high-level over-expression with lentiviral vectors), oligodendrocytes are sensitized to apoptotic stimuli. In my primary oligodendrocyte culture models of MSA, α-synuclein fibrillization was accompanied by activation of caspase-3 and chromatin condensation visualized with

Hoechst nuclear staining. Two main caspase activation pathways, receptor-mediated sequential activation of caspase-8 and cytochrome-*c* dependent nonreceptor mediated caspase-9 activation, are known to be involved in caspase 3-induced apoptosis (Lavrik et al., 2005b). Ultrastructural examination of the dying oligodendrocytes revealed mitochondrial damage, and anti-α-synuclein immunogold particles were found in the vicinity of damaged mitochondria (Figure 22C). Thus, I expected the intrinsic (mitochondrial) apoptosis pathway to be responsible for α-synuclein-sensitized oligodendrocyte death. To determine which type of caspase pathway functions in α-synuclein induced apoptosis, I dissected the molecular pathways of apoptosis in this system using specific caspase inhibitors. Interestingly, inhibition of caspase-9, the major effector of the mitochondrial apoptosis cascade, was less efficient blocking cytotoxicity in my model than inhibition of caspase-8 (Figure 25), which is known to be activated by cell death receptors. However, additional experiments to confirm that caspase-9 and caspase-8 inhibitors work equally well should be conducted.

I investigated in greater molecular details how α-synuclein inclusions trigger the apoptotic cascade via the extrinsic apoptosis pathway coupled to caspase-8 in α-synuclein-expressing oligodendrocytes. In a systematic analysis of death receptor agonists, Fas ligand was the most efficient apoptosis inducer in primary oligodendrocyte cultures. α-synuclein-induced caspase-8 activation suggests the possible involvements of Fas and FasL, which act together in receptor-mediated apoptosis (Ferrer et al., 2000). Importantly, α-synuclein expression significantly sensitized oligodendrocytes to Fas-mediated apoptosis, when compared to eGFP transgenic oligodendrocytes (Figure 26A). Oligodendrocytes can express Fas on their membrane surface under pathological

conditions (D'Souza et al., 1996; Elovaara et al., 1999). The involvement of Fas in α-synuclein toxicity was confirmed by the elevated levels of Fas in cultured α-synuclein transgenic oligodendrocytes and in cerebellar white matter of MSA patients. These data suggests that overexpressed α-synuclein up-regulates membranous Fas expression.

The Fas/Fas ligand (FasL) system is believed to contribute to the suppression of inflammatory processes in the healthy brain (Choi and Benveniste, 2004). Normally only few peripheral T lymphocytes invade the brain. These are stringently controlled by a number of immunosuppressants, among them FasL. In the case of multiple sclerosis, however, a strong auto-inflammatory reaction to myelin occurs, and the oligodendrocytes become a target of Fas-mediated apoptosis (D'Souza et al., 1996). In the present study I provide evidence that Fas not only participates in the acute autoimmune damage of oligodendrocytes in multiple sclerosis lesions, but also occurs in the chronic disease MSA. The observed up-regulation of the pro-apoptotic Fas receptor α-synuclein transgenic primary oligodendrocyte cultures, and in cerebellar white matter, one of the most severely affected brain regions of MSA patients, could enhance the vulnerability of oligodendrocytes (27A and 28). Interestingly, transfection of α-synuclein into IM-9 myeloma cells has been recently reported to induce the Fas gene and increase Fas expression on the cell surface (Kim et al., 2004). Additional biochemical and histochemical studies are warranted to elucidate the intracellular cascade of events triggered by α-synuclein and mediating Fas induction in this disorder showing slow progression over many years. For example, histochemical co-localization studies in MSA patients may provide information regarding the coupling between Fas expression and

activation of the extrinsic pathway, as evidenced by caspase-8 cleavage and Bid truncation (Lavrik et al., 2005a).

Collectively, the results of this dissertation suggest that α-synuclein overexpression in oligodendrocytes influences the aggregation of α-synuclein protein, alter the conformation of α-synuclein protein, and promote the stability of α-synuclein fibrils, which are all relevant to the pathogenesis of MSA. These data provide extensive evidence and support for the role and implication of α-synuclein protein in the pathogenesis of MSA and may hopefully aid in the development of therapies for MSA patients and patients of synucleinopathies in addition to the creation of prophylactic treatments and remedies for the worldwide population that is at risk of developing this disorder. Figure 29 summarizes the data collected from Chapters 4 in the attempt to understand the mechanism of α-synuclein fibrillogenesis and its consequences on glial pathology. Increased expression of α-synuclein protein most likely leads to altered function, structure, and conformation, which may also promote the dysfunction of the chaperone and ubiquitin-proteasome system, as described in the introduction. The generation of misfolded, aggregated α-synuclein protein may obstruct cellular trafficking, disrupt cell morphology and homeostasis, and interfere with other cellular functions, which all may lead ultimately to cell death. Further understanding of the correlation between α-synuclein levels and Fas upregulation/apoptosis will provide an insight into the molecular basis of the disease.

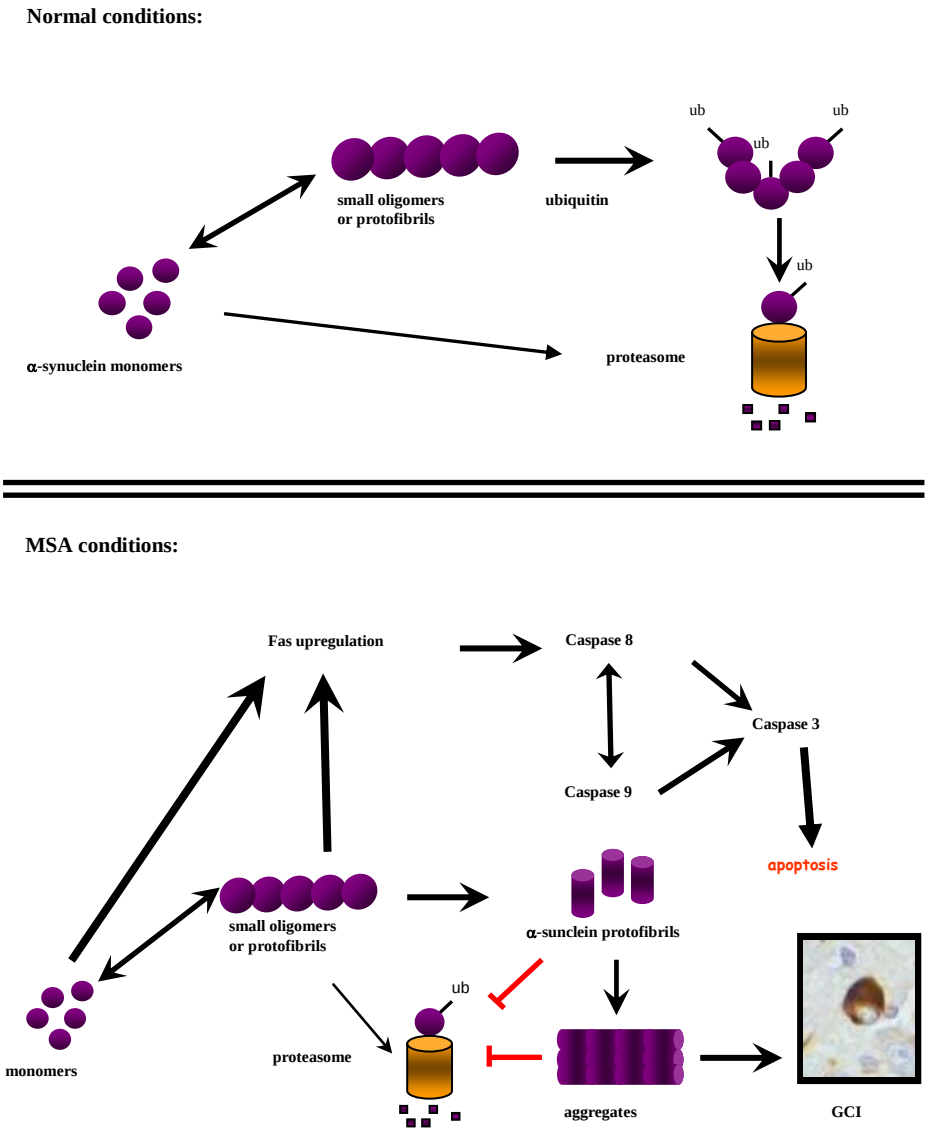


Figure 29: Proposed model for the implications of α-synuclein expression on the pathogenesis of MSA. Monomeric α-synuclein naturally undergoes conformational and structural changes from random coil to β-pleated sheet and to small oligomers. Chaperone proteins may aid in refolding these oligomers back to monomers. Otherwise, the small oligomers or protofibrils might become ubiquitinated and targeted for proteasome degradation. However, under pathological conditions if the small oligomers become polymeric and form protein aggregates, they will form GCIs and will not be degraded by the proteasome. Furthermore, α-synuclein is involved in apoptosis mechanism. α-synuclein expression also up-regulates membranous Fas expression which then sensitizes to caspase-8 activation.

3) Prophylactic approaches againt MSA

The present study provides evidence that the α-synuclein may be one of the primary causes of MSA pathology and offers new targets for pharmacotherapeutic intervention.

These results suggest that α-synuclein pathology precedes disease phenotype, as Fas upregulation precedes α-synuclein aggregation, therefore confirming the importance of disease detection before the onset of symptoms. Antagonizing the α-synuclein-Fas pathway could be tested in the (CNP)-α-synuclein mice, which show neurodegeneration even in the absence of amyloid-like GCI formation. If Fas immunotherapy prevents neurodegeneration in these mice, it may offer opportunities for therapeutic approaches to MSA.

A causal relationship of α-synuclein aggregation and cytotoxicity can be derived from the finding that LV-β-synuclein prevents α-synuclein aggregation and oligodendrocyte death to the same extent. Although it remains to be rigorously proven if it is the visible GCI or some partially folded α-synuclein "protofibrils" that kill oligodendrocytes, the beneficial effects of LV-β-synuclein on cultured oligodendrocytes open a possibility to treat oligodendroglial pathology. It will be interesting to test if β-synuclein-based suppression of GCI formation maintains the functions of oligodendrocytes (myelination, ion homeostasis) and neurodegeneration in transgenic mouse models of MSA (Kahle et al., 2002; Yazawa et al., 2005). The mechanisms by which β-synuclein suppresses α-synuclein aggregation and toxicity were not defined in my thesis. Further experiments would be needed to determine at what stage of the α-synuclein toxicity pathway β-synuclein interferes. Additionally, it would be necessary to clarify how β-synuclein

interferes with the aggregation and cell toxicity processes, protecting oligodendrocytes against the detrimental effects of α-synuclein accumulation. In fact it remains unclear whether β-synuclein exerts its protective function by physically interacting with α-synuclein and prevent the conformational changes leading to the formation of β-pleated sheets (Hashimoto et al., 2001). Alternatively, β-synuclein may mediate its protective function by regulating pro-survival and apoptotic pathways. Hashimoto et al., reported β-synuclein may directly interact with Akt, suggesting that this signaling pathway could be a potential therapeutic target for neurological conditions associated with parkinsonism and α-synuclein aggregation (Hashimoto et al., 2004). It was also shown that β-synuclein triggers drastic reduction of p53 expression and transcriptional activity (da Costa et al., 2003).

β-Synuclein might function to ameliorate the proteasomal inhibition caused by aggregated α-synuclein and thereby limit proteasomal inhibition. A previous study by Snyder et al., suggests that although β-synuclein has no direct effect on proteasomal activity, β-synuclein is able to modulate α-synuclein binding to the proteasome and thereby antagonizes the inhibition of the proteasome by aggregated α-synuclein (Snyder et al., 2004). Altogether these data supports the use of β-synuclein or β-synuclein derived peptides for treatments of MSA and other synucleinopathies.

Molecular chaperones of the Hsp70/Hsp40 family block the formation of amyloid-like fibrils in a cell culture model of Huntington's disease (Klucken et al., 2004; Schaffar et al., 2004). Interestingly, increased expression of molecular chaperones of the Hsp70/Hsp40 system has been shown to suppress the neurotoxicity of α-synuclein proteins (Auluck et

al., 2005; Dedmon et al., 2005). It would be of great interest to examine the effects of molecular chaperones on α-synuclein aggregation in my cell culture models. In order to study how Hsp70 can modulate α-synuclein aggregation, α-synuclein oligodendrocytes could be transduced with lentiviral vector coding for Hsp70, and the number of α-synuclein inclusions could be scored. Along the same lines, stimulation of the stress response by a geldanamycin, a naturally occurring benzoquinone ansamycin that induces Hsp70, may prevent and/or revert α-synuclein aggregation and toxicity in oligodendrocytes as it was previously shown in *Drosophila* (Auluck et al., 2005).

4) Benefits and limitations of the *in vitro* models of MSA

The primary oligodendrocyte culture models of MSA developed in this study are a unique tool to investigate in great details the molecular mechanisms of α-synuclein aggregation and oligodendroglial death in MSA. Furthermore, the cellular accessibility and microenvironmental control they offer are a big advantage to identify potential therapeutic compounds in MSA. It was shown in this study that when β-synuclein is in direct contact with oligodendrocytes it prevents α-synuclein aggregation and toxicity; however, the effects seen in vitro must be confirmed or substianted in animal models and patients. In fact, the lack of a blood-brain barrier in culture, a major factor in determining a chemical's access to the brain, limit the extrapolation of this result to the *in vivo* condition.

Primary oligodendrocytes are isolated from normal *in vivo* homeostatic mechanisms. Refining these models by co-culturing primary neurons and α-synuclein overexpressing

oligodendrocytes, would not only restore the synergistic interaction between all cell types present in the CNS but would also enable us to analyse oligodendrocyte dysfunction and myelination deficiencies in α-synuclein oligodendrocytes and to assess neurodegeneration observed in the patients.

MSA is a movement disorder, and animal models of MSA which combine authentic oligodendroglial and neuronal pathology with neurological dysfuntion would prove extremely useful to investigate the causal relationship between locomotor disorders and α-synuclein expression. Along with CNP-α-synuclein mice (Yazawa et al., 2005), the MBP-α-synuclein mice (Shults et al., 2005) provide the means to examine the potential downstream effects of α-synuclein overabundance in oligodendroglia. Such analysis will likely reveal toxic mechanisms of neuronal cell death in MSA, thereby providing novel targets for MSA therapeutics. Hopefully, other diseases in which glial deficits lead to neuronal cell loss will also benefit from such advances.

Finally, α-synuclein is not normally expressed in oligodendrocytes of a mouse brain (Yazawa et al., 2005) or a human brain (Solano et al., 2000; Miller et al. 2005). Clearly, a future advance in MSA research will be determining the cause for ectopic overabundance of α-synuclein in GCI-containing oligodendrocytes. Both *in vitro* and *in vivo* MSA systems offer complementary approaches to assess the cause of the abnormal expression of α-synuclein in oligodendrocytes in the disease, and would allow us to act before symptoms occur by determining risk factors as well as drug targets.

Chapter 6: CONCLUSION AND SIGNIFICANCE

Two major findings arise from my PhD work and contribute to the understanding of MSA:

1. High expression levels of α-synuclein in oligodendrocytes are sufficient to induce formation of GCIs.
2. α-synuclein expression in oligodendrocytes leads to upregulation of the pro-apoptotic Fas receptor. This cell culture finding was confirmed in MSA patients, providing novel insight to the mechanisms by which α-synuclein induces cytotoxicity.

These results suggest α-synuclein expression in oligodendrocytes may be the primary cause of oligodendrocyte dysfunction and death in MSA. Fas upregulation may contribute to the damage of oligodendrocytes in the chronic disease MSA and opens new avenues for the development of therapeutic approaches against oligodendroglial pathology. An understanding of the correlation between α-synuclein expression and Fas upregulation in oligodendroglial α-synucleinopathy would offer new targets for therapeutic intervention. The models of α-synuclein overexpression in oligodendrocyte culture I developed should prove useful in clarifying these issues and may allow the development and testing of compounds that could prevent oligodendrocyte degeneration.

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CURRICULUM VITAE

Name :Gwenaëlle FILLON
Place of birth : Dreux, France
Date of birth : 28.03.1977
Nationality : French
Marital status : single

EDUCATION

2002-present Preparation of a PhD in biology at the Ludwig Maximilian University in Munich, Germany

1998-2001 Completion of the three year bioengineer's degree in Biotechnology at the "Ecole Superieure de Biotechnologie de Strasbourg" (ESBS; European School of the Upper Rhine Universities) passed with high honors high honors
Preparation of the DEA (Diplome d´Etudes Approfondies) in molecular and cell biology in the Louis Pasteur University of Strasbourg, passed with high honors

1996-1998 Completion of the DEUG (Diplome d'Etudes Universitaires Generales) in Biology and Geology at the University of Orleans, France, passed with high honors

1995-1996 One year post-A-level preparatory courses at the INSA from Toulouse (France)

1995 Baccalaureat (equivalent to A level) in science (Physics and Chemistry), passed with distinction

FORMAL TRAINING

2002-present PhD in biochemistry supervised in the **Alzheimer's and Parkinson's Institute** (Munich, Germany) lead by Professor Christian Haass, under the supervision of Dr. Philipp Kahle in collaboration with Professor Catherine Lubetzki, Salpêtrière hospital, **INSERM U495** (Paris, France): Establishment of a glial cell culture model of Multiple System Atrophy

2001-2002 Research associate, **Elan Pharmaceuticals** (South San Francisco, CA, USA): Participant in immunotherapy development program against Alzheimer's disease

2001 8-month internship at **Elan Pharmaceuticals** (South San Francisco, CA, USA): Antibody mediated phagocytosis of amyloid-beta plaques

2000 **CNRS**, immunotechnology laboratory (1 month, Strasbourg, France)
Screening of peptides recognized by the E6 oncoprotein from HPV (Papillomavirus involved in uterus cancer)

1996-1999 Training course in the department of research and development from **HORIBA** (5 weeks, Kyoto, Japan): Trials to optimize a blood cell counter
Microbiological genetics (Basel, Switzerland)
Plant molecular genetics (Freiburg, Germany)
Biotechnology/chemical engineering (Basel and Karlsruhe)
Training course in molecular genetic, **C.S.I.C**, Barcelona, Spain: Preparation of a Bac clone for the sequencing of Arabidopsis thaliana chromosome III bottom arm

The present study was prepared from March 2002 until August 2005 in the laboratory of Prof. Christian Haass, Biochemistry, Adolf-Butenandt-Institute of the Ludwig-Maximilians-University of Munich.

Parts of this study have been published and presented recently:

SCIENTIFIC PUBLICATIONS

Fillon, G and Kahle PJ (2005). *α-Synuclein Transgenic Mice: Relevance to Multiple System Atrophy*. Movement Disorders. Vol. 20, Supplement 12.
Fillon, G et al., (2005). *Overexpression of α-Synuclein in Oligodendrocytes Causes Formation of Glial Cytoplasmic Inclusions and Sensitizes to Fas-Dependent Apoptosis*. Submitted.
Fillon, G et al., (2005). α-Synuclein Mediated Proteasome Inhibition in a Primary cell culture model of MSA. In preparation.

SCIENTIFIC PRESENTATIONS

2005 Society for Neuroscience Meeting, Washington, USA
2005 SFB 596, Meeting of the Movement Disorders Research Focus, September 2005, Munich
2005 Euroglia, VII European Meeting on Glial Cell Function in Health and Disease, Amsterdam, The Netherlands
2005 SFB 596, Meeting of the Movement Disorders Research Focus, May 2005, Munich
2005 4th German-Scandinavian Meeting on Movement Disorder, Gothenburg, Sweden
2005 4th German-Scandinavian Meeting on Movement Disorder, Gothenburg, Sweden
2004 Society for Neuroscience Meeting, San Diego, USA
2004 Neuropark, Catholic University of Leuven, Belgium

SCIENTIFIC POSTERS

2004 SFB 596, Munich, Germany
2003 SFB Symposium, Molecular Mechanisms of Neurodegeneration**,** Wildbad Kreuth, Germany

REFERENCES