AN INSIGHT INTO ALPHA-SYNULCIEN'S BIOLOGICAL FUNCTION AND ITS PATHOGENESIS IN NEURODEGENERATIVE DISEASE

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DEDICATION

I would like to thank and dedicate this work to my mother, father and brother.

AN INSIGHT INTO ALPHA-SYNULCIEN'S BIOLOGICAL FUNCTION AND ITS PATHOGENESIS IN NEURODEGENERATIVE DISEASE

by

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AN INSIGHT INTO ALPHA-SYNULCIEN'S BIOLOGICAL FUNCTION AND ITS PATHOGENESIS IN NEURODEGENERATIVE DISEASE

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The discovery of two missense mutations (A53T and A30P) in α-synuclein that are genetically linked to Parkinson's disease, together with α-synuclein being the major component in Lewy bodies, has generated extensive interest in α-synuclein as a key component in neurodegenerative diseases. In recent years modeling this disease in transgenic mice and flies has lead to new understandings of α-synuclein function and pathogenesis in neurodegeneration. In the current study we analyzed transgenic mice overexpressing human α-synuclein and human α-synuclein mutations (A53T & A30P) to;

First establish these transgenic mice as a model for degenerative diseases; second to identify potential contributing factors in neurodegeneration; third to decipher a potential function of α-synuclein.

We first established that transgenic mice expressing human variants of α-synuclein developed an age dependant motor dysfunction with symptom logy characteristic of Parkinson's disease. Immunohistological studies revealed the presence of α-synuclein inclusions and a loss of motor neurons. Biochemical analysis identified a 4-5 fold increase in ubiquitin with altered expression of proteasomal subunits, characteristic of proteasomal impairment. In addition, we identified a significant increase in amyloid β-peptides.

Protein quantification of apolipoprotein E (ApoE) a protein that has been associated with the development of Alzheimer's disease, demonstrated a 5-15 fold increase in symptomatic transgenic mice. Ablation of ApoE in α-synuclein transgenic mice by genetic crosses revealed a delayed onset for motor dysfunction and an overall increase in survival. ApoE deficient transgenic mice displayed a decrease in ubiquitin and amyloid β-peptides. This study illustrates ApoE, ubiquitin and Aβ- peptides contribute to the onset and progression of the neurodegeneration in transgenic α-synuclein mice.

Genetic crosses of transgenic α-synuclein with a csystine string protein-α (CSPα) knockout mouse revealed a potential function for α-synuclein. CSPα deficient mice develop an early age neurodegenerative disease that is lethal at 3-4 months. Transgenic expression of human α-synuclein prevented the deleterious effects of CSPα deficiency. Immunofluorescence studies illustrated α-synuclein function in a cell autonomous manner. Biochemical analysis demonstrated CSPα deficient mice have impaired SNARE complexes

that are partially reverted by transgenic α-synuclein. This study illustrates a protective function of α-synuclein in preventing neurodegeneration.

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Chandra S., Gallardo G., Fernández-Chacón R., Schlüter O.M., and Südhof, T.C. (2005). α-Synuclein Cooperates with CSPα in Preventing Neurodegeneration. Cell 123, 383-396.

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ApoE	apolipoprotein E
Aβ	amyloid beta
Co-IP	co-immunoprecipitation
CpxI	complexin I
CpxII	complexin II
CNS	central nervous system
CSF	cerebral spinal fluid
CSPα	cysteine sting protein α
hA53Ttg	A53T-mutant human α-synuclein
hA30Ptg	A30P-mutant human α-synuclein
HSC70	heatshock chaperone protein 70
htg	wildtype human α-synuclein
KO	knockout
L-DOPA	L-dihydroxyphenylalanine
LRRK2	leucine-rich repeat kinase 2
MAO	monoamine oxidase
MPTP	N-Methyl-4phemyl-1,2,3,6-tetrahydropyridine
mtg	mouse α-synuclein
JPD	juvenile parkinsonism
NAC	non-amyloid β-protein component
PAGE	polyacrylamide gel electrophoresis

PD	Parkinson’s disease
PINK1	PTEN-induced kinase-1
SNAP	soluble NSF attachment protein
SNAP-25	synaptosome-associated protein of 25 kDa
SNARE	soluble NSF attachment receptor element
SNARE core complex	SNARE complex containing only SNARE motifs
SN	substania nigra
Syb 2	synaptobrevin 2
Syt 1	synaptotagmin 1
Synt	syntaxin 1A
TH	tyrosine hydroxylase
UPS	ubiquitin protease system
UCH-L1	ubiquitin carboxy-terminal hydroxylase L1
VCP	valosine-containing protein

Chapter I: Introduction

1.1 Parkinson's disease history

In 1817, James Parkinson published his landmark essay entitled 'An Essay on the Shaking of Palsy' describing a disease characterized by involuntary tremulous motion and a decrease in muscular strength. Several years later Jean-Martin Charcot redefined this syndrome and named it Parkinson's disease (PD). Parkinson's disease (PD) is a chronic neurodegenerative disease characterized by a progressive movement disorder known as parkinsonism syndrome. Parkinsonism syndrome is clinically defined as resting tremors, slowness of movement, bradykinesia, rigidity and gait instability (Linazasoro, 2007). Non-motor symptoms may include autonomic dysfunction, sleep disturbance and cognitive impairment. PD accounts for approximately 75% of parkinsonism syndrome cases and is the most common neurological disorder second to Alzheimer's (Linazasoro, 2007).

Currently there are two classifications for PD, idiopathic and familial induced. Idiopathic PD consists of a late-onset of symptoms, without any obvious family history, postmortem pathology reveals the presence of neurodegeneration, astrocytic gliosis, and Lewy bodies. Idiopathic PD affects 1% of population over 65 with an average age of onset between 60-65 and male to female frequency of 1.5-2.0:1 (Lester and Otero-Siliceo, 2006;Accolla et al., 2007; Lester and Otero-Siliceo, 2006).). The identification of several PD-associated genes has lead to a category of familial PD, which makes only a small fraction of PD cases. However, the role of genetics in idiopathic PD still remains unresolved due to either causative genes having a low penetrance or the disorder is a combination of genetic predisposition and environmental factors. The latest studies in monozygotic twin have reported over 50% disease concordance, versus 22% concordance

in dizygotic pairs regardless of age-onset. These recent studies suggest a genetic contribution to PD is greater than what was once believed.

The main neuropathological features of PD consist of eosinophillic cytoplasmic inclusions that were first described in 1912 by Friedrich Lewy. These inclusions are now known as Lewy bodies and have become a hallmark of PD pathology. Electron microscopy and biochemical studies have further characterized Lewy bodies as abnormal filaments of misfolded proteins (Brown et al., 1998). Early studies reported an increase of these Lewy bodies in the substania nigra (SN) of PD patients, and were the earliest indication of the SN involvement in PD (Tretiakoff Thesis Univ. Paris 1919). Although, the distribution of Lewy bodies is not exclusive to the SN they are found throughout many regions including lower brain stem, stiatum, hypothalamus, motor nucleus of the vagus nerve, nucleus basalis, locus coeruleus, cerebral cortex, olfactory bulb, autonomic nervous system and more recently the spinal cord (Forno, 1996). Of these regions cell loss has been reported in the locus coeruleus, cerebral cortex and spinal cord motor neurons. Braak *et al*. (2003) have recently proposed that the development and progression of PD has distinct neuropathological stages. The Initial stage is in asymptomatic patients with neuronal inclusions originating at the lower brain stem that progressively spread toward the midbrain, limbic and cerebral cortex. The clinical symptoms of PD manifest at the later stages once inclusions appear in striatum and SN. Lewy bodies have also been associated with other diseases including Alzheimer's disease, dementia with Lewy bodies, multiple system atrophy and diffuse Lewy body disease (Lansbury and Lashuel, 2006).

In the early 1950s Montagu and Carlsson discovered dopamine as the major neurotransmitter that controlled extrapyramidal motor function leading to major advances in the treatment and understanding of PD. This was followed by studies illustrating dopamine deficiency in the corpus striatum and SN of brains from PD patients (Ehringer, H and Hornykiewicz, 1960). In addition, Poirier and Sourkes demonstrated the connection between SN and striatum, and suggested a loss of dopamine cells in the SN directly leads to dopamine deficiency in the striatum (Poirier and Sourkes, 1965) Support for this hypothesis came when additional studies confirmed PD symptoms manifest after 50% of dopaminergic neurons in the SN are lost (Marsden, 1990). Although dopamine failed to be a proven treatment for PD due to its inability to pass the blood brain barrier the discovery of L-dihydroyphenylalanine (L-DOPA) a precursor of dopamine that effectively crosses the blood-brain barrier lead to the first treatment for PD (Cotzias et al., 1969; Lloyd and Hornykiewicz, 1973; Poskanzer, 1969).

Initial reports describing PD treatment with L-DOPA described dramatic improvements in motor dysfunction and mental cognition. Although, L-DOPA proved to be a valued treatment and an essential tool for the diagnosis of PD, it had its limitations. One such limitation is the decrease in L-DOPA efficacy as the disease progresses. More importantly long term use may lead to the development of choreic dyskinesias in approximately 50% of patients (Foster and Hoffer, 2004). Both limitations are attributed to L-DOPA's relatively short half life. This has lead to combinational treatment that includes inhibitors of catechol-O-methyl-transferase (COMT) which metabolizes L-DOPA to clinically useless 3-O-methldopa. Therapeutic design has also targeted inhibiting the breakdown of dopamine mediated by monoamine oxidase (MAO).

Specific inhibitors of MAO increase endogenous dopamine levels providing symptomatic benefits (Savitt et al., 2006). Dopamine agonists have also been developed which provide potential advantages in comparison to L-DOPA treatment, for they are not dependant on conversion to an active form and have a longer half-life which prevents the adverse effects of long term treatment (Savitt et al., 2006). Despite these advancements in PD therapy, current treatments are unable to ameliorate late-stage symptoms, in large due to, the inability to prevent the progressive neurodegeneration of dopaminergic and nondopaminergic neurons. This has lead to an expansion of research that focuses on understanding the mechanism of neuronal cell death and the causality of the disease.

Early studies suggested environmental factors lead to the development of PD. For example the occurrence of postencephlatic PD following the influenza pandemic of 1918 implicated infectious agents as an environmental factor. In the early 1980s, N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) an analogue of synthetic heroin was mistakenly synthesized. MPTP is a highly lipophilic proneurotoxin that selectively degenerates the nigrostriatal dopaminergic pathway. Following systemic administration addicts developed L-DOPA-responsive parkinsonian syndrome (Langston et al., 1983). This is mediated by the conversion of MPTP to MPP+ that is selectively taken into dopaminergic neurons via dopaminergic transporter. Within dopaminergic neurons MPP+ accumulates in the inner mitochondria membrane inhibiting complex 1 resulting in cell loss.

Following the discovery of MPTP, numerous neurotoxins have since been identified**.** Bertarbet *et al.* 2000 identified a naturally occurring lipophilic compound, known as rotenone that is commonly used in many insecticides and inhibits

mitochondrial complexes I and II of mammalian cells. Chronic infusion of rotenone into rats leads to motor dysfunction and selective neurodegeneration of SN. Immnohistological studies revealed fibrillar cytoplasmic inclusion with characteristics of Lewy bodies. A second natural occurring compound with MPTP+ like properties are isoquinllines, that have been isolated from cerebral spinal fluid (CSF) and brains of postmortem PD patients (Makino et al., 1988; Niwa et al., 1987; Yamakawa and Ohta, 1997). Direct injection of isoquinllines into the striatum of rats induced parkinsonism like symptoms (Naoi et al., 1996). Morphological analysis revealed a decrease in tyrosine hydroxylase positive neurons within the SN. In addition, β-carbolines that are synthesized from tryptophan and contain MPP+ properties have also been isolated from CSF of PD affected patients (Matsubara et al., 1995). In-vivo activities of Beta-carbolines in animal models have not been reported. The discovery of MPTP and these other neurotoxins has lead to the development of animal models for PD, that has enabled fundamental understanding of neurodegeneration within SN-striatum system. Furthermore, the specificity for these neurotoxins to target the mitochondria has suggests mitochondrial dysfunction may play an important role in PD pathogenesis (Di Monte, 2003).

An enormous impact on understanding the pathogenesis of PD began with the description of an autosomal-dominant inherited form of PD in an Italian-American family in 1990 by Golbe *et al*. This was the first report of familial PD with parkinsonism symptoms and the presence of Lewy bodies. In 1996 and 1997 two landmark studies by Polymeropoulos *et al.*, first mapped the genetic defects responsible for the Italian-American familial PD to chromosome 4q21-23 followed by the identification of a

missense mutation (A53T) in the α-synuclein gene. It was further reported that the A53T missense mutation in α-synuclein was responsible for PD in 3 non-related Greek families. Since then, 4 additional chromosome regions have been linked to PD and 6 genes have been identified including synuclein, Parkin, UCH-L1, PINK 1, DJ-1, and LRRK2

1.2 Genetics of Parkinson's disease

PARK 1 (α-synuclein)

Shortly after the initial discovery of the A53T point mutation in α-synuclein that leads to early onset PD, Spillantini *et al.* 1997 reported that the major component of Lewy bodies was α-synuclein. In 1998, a second mutation (A30P) in α-synuclein was reported in a German kindred (Kruger et al., 1998). Clinical features of the A53T point mutation exhibited an early onset of symptoms with an aggressive pathogenecity and deterioration that included dementia (Spira et al., 2001). The A30P mutation resembled clinical features similar to idiopathic PD. Both mutations revealed an extensive amount of Lewy body deposits characteristic to idiopathic PD. More recently a third mutation (E46K) and gene triplication of α-synuclein have been identified in early onset familial PD (Golbe and Mouradian, 2004; Singleton et al., 2003).

Of three members of syuclein family α, β and γ, only α-synuclein has been associated with neurodegenration. Expression of α-synuclein wild type A30P and A53T mutants in yeast lead to cytoplasomic inclusions, inhibition of phospholipase D, vesicle trafficking alterations and inhibited growth (Outeiro and Lindquist, 2003). In addition, wild type and A53T α-synuclein concentrated at the plasma membrane in contrast to

cytoplasomic localization for A30P α-synuclein. This inability of A30P α-synuclein to localize at plasma membranes had previously been demonstrated in lipid binding assays, suggesting the pathogenic properties may differ from the wild type and A53T α-synuclein mutation (Jo et al., 2000; Perrin et al., 2001). Overexpressiong of α-synuclein mutations and wildtype in Drosophlia illustrated dopaminergic cell loss and the presence of synuclein inclusion (Feany and Bender, 2000). Experiments in this model identified phosphorylation of Ser129 of α-synuclein were necessary for neuronal cell loss. Later studies reported antibodies generated specifically to phosphor-Ser 120 α-synuclein displayed intense labeling to Lewy bodies of PD brains (Fujiwara et al., 2002). *In vitro* studies demonstrated phosphor-Ser 120 α-synuclein promoted fibril formations believed to be important in pathogenesis of α-synuclein. The findings of inclusions and fibril forming properties of α-synuclein suggest missfolding of the protein may initiate neuronal cell loss. This notion is further supported by co-expression of wild type and mutant variants of α-synuclein with molecular chaperone Hsp70 in Drosophila prevented dopaminergic cell loss (Auluck et al., 2002).

The ability of α-synuclein to form fibrils lies in a central hydrophobic region which tends to self associate and is absent in the β and γ isophorms (Bodles et al., 2001; Giasson et al., 2001). Both A30P and A53T α-synuclein mutations have increased rates of self-assembly and fibrillization in comparison to wildtype. These fibrils consist of a transition from α-helices random-coil conformation to β-sheets. Accumulation of synuclein β-sheets are believed to form fibrils becoming heavily insoluble polymers that are the major constituent in formation of Lewy bodies (Conway et al., 2000; Serpell et al., 2000). In support of α-synuclein aggregation as a key component in

neurodegeneration, studies have demonstrated pesticides, metals and oxidizing conditions all promote α-synuclein aggregation (Di Monte, 2003). *In vitro* studies have further demonstrated α-synuclein aggregation is concentration dependant which leads to a potential explanation for how gene triplication of wild type α-synuclein leads to PD (Conway et al., 2000; Serpell et al., 2000). Understanding the genetics and nongenitic factors that contribute to α-synuclein aggregation is imperative in elucidating its pathological role in PD.

Although much effort has been placed on α-synuclein pathogenesis its normal function has remained elusive and largely controversial. α-Synuclein is predominantly cytoplasmic were it is natively unfolded with little ordered secondary structure and is heavily concentrated at presynaptic terminal (Maroteaux et al., 1988; Weinreb et al., 1996). It is also associated with phospholipids of vesicles and membranes via its amphipathic N-terminal region were it forms into two approximately equally long α-helices (Bussell et al., 2005). This ability of synuclein to bind phospholipids suggests it may play a role in lipid metabolism (Castagnet et al., 2005; Golovko et al., 2005). Studies of α-synuclein depletion in astrocytic cultures demonstrated a decrease in fatty acid incorporation. α-Synuclein knockout mice (KO) have reportedly, decreased brain palmitate uptake and altered palmitate metabolism (Golovko et al., 2005). Neuronal culture studies have illustrated α-synuclien may alter phospholipase C and phospholipase D enzyme activity altering lipid mediated signaling (Narayanan et al., 2005). Further studies in α-synuclein KO mice indicated a decrease in synpatic vesicles with a decrease in synaptic response following repetitive stimulus (Cabin et al., 2002; Murphy et al., 2000).

PARK 2 (PARKIN)

In 1998, Kitada *et al*. reported a mutation located in the long arm of chromosome 6q25.2-q27 in Japanese family that lead to autosomal recessive juvenile parkinsonism (JPD). Positional cloning analysis revealed a protein with similarities to ubiquitin at the amino terminus and a RING-finger motif at the carboxy terminus. This newly identified gene was named Parkin. A large variety of mutations in Parkin gene have since been reported including missense and nonsense mutations, deletions and rearrangements (Cabin et al., 2002; Munoz et al., 2000; Peng et al., 2003). Mutations in Parkin account for 77% of JPD cases with symptoms onset before the age of 20. Parkin mutations are further found in 49% of PD cases and 18% of idiopathic cases under the age of 45. Clinical features include dyskinesia, dystonia, with a slow progression and are responsive to L-DOPA treatment (Lucking et al., 2000). In contrast to α-synuclein's pathology, Parkin lacks the presence of classical Lewy bodies. Although, recent studies have revealed α-synuclein and ubiquitin immunoreactive positive inclusions in a subset of patients with PD related to a Parkin mutation (Pramstaller et al., 2005).

Parkin is a 465 amino acid protein localized both pre- and post-synapticly in neurons throughout the central nervous system. Shirmura and colleges demonstrated Parkin functions as an E3 ubiquitin ligase by binding UBCH7 an E2 ubiquitin-conjugating enzyme (Shimura et al., 2000). Mutations in Parkin that result in disruption of its E3 activity may lead to the accumulation of target proteins. Potential substrates targeted for degradation by Parkin have been identified including α-synuclein (Chung et al., 2001; Oluwatosin-Chigbu et al., 2003). Yet, analysis of Parkin KO mice displayed no

accumulation of α-synuclein or inclusions (Goldberg et al., 2003). Other studies reported Parkin KO mice displayed altered dopaminergic-related behaviors without any detection of neurodegeneration (Itier et al., 2003). Drosophila Parkin mutants displayed decreased overall mass, locomotor defects, reduced life span and muscle degeneration related to mitochondrial dysfunction and oxidative stress (Greene et al., 2003; Pesah et al., 2004).

PARK 5 (UCH-L1)

The ubiquitin proteasome system (UPS) is a major biochemical pathway for degradation of normal and abnormal protein that otherwise may accumulate resulting in cell death (Leroy et al., 1998; Sherman and Goldberg, 2001). Identification of various mutations in Parkin protein that is associated with UPS and the presence of cytoplasmic inclusions containing both ubiquitin and α-synuclein suggest UPS impairment in PD pathogenesis. Further support for this hypothesis is the discovery of a missense mutation (I93M) of ubiquitin carboxy-terminal hydroxylase L1 (UCH-L1) in a German family with PD (Leroy et al., 1998). The age of onset was between 49 and 51with less than 50% penetrance. UCH-L1 PD demonstrated classical symptoms with the presence of Lewy bodies that are immunoreactive to UCH-L1 protein.

UCH-L1 belongs to a family of deubiquitinating enzymes that generate mono-ubiquitin from polyubiquitin chains that are produced following protein degradation which aid in recycling ubiquitin (Larsen et al., 1998). In vitro studies of I93M UCH-L1 demonstrated a 50% reduction in catalytic activity implicating a loss of function which may lead to impaired UPS and accumulation of abnormal proteins. Although the natural substrates for UCH-L1 have not been identified transfected cells have demonstrated α-

synuclein as a potential target (Liu et al., 2002). Mutations in UCH-L1 in mice exhibited neurodegeneration and hind limb paralysis that were characterized as Gracile Axonal Dystrophy syndrome (Kurihara et al., 2001). GAD mice display axonal degeneration within gracile tract of the spinal cord and medulla oblongata ultimately leading to motor ataxia (Saigoh et al., 1999). The absence of SN degeneration and inclusions in UCH-L1 mice, suggested lost of function is not sufficient or is not the only factor involved in PD development consistent with its low penetrance.

The mechanism of UCH-L1 mutation leading to neurodegeneration remains unclear but supports UPS dysfunction in PD pathogenesis. *In vivo* studies have demonstrated systemic administration of inhibitors specific for UPS in rodents display motor deficit, cytoplasmic inclusions and selective SN cell loss (McNaught et al., 2004). Inhibition of UPS in cultured neurons resulted in formation of α-synuclein-immnuoreactive inclusions (Rideout et al., 2001). Inducible PC12 cell lines of mutant forms of α-synuclein resulted in impairment of proteasome activity leading to cell death (Tanaka et al., 2001). In conclusion, the presence of α-synuclein, ubiqutin and other proteasomal subunits in Lewy bodies suggest UPS impairment results in accumulation of these proteins that may aid in the pathogenesis of PD.

PARK 6 (PTEN-induced kinase-1)

Valente *et al.* 2001 reported a mutation in an Italian family localized to chromosome 1p35-36 and identified a mutation in the PTEN-induced kinase-1 (PINK1) gene that leads to PD. PINK1 mutations are second only to Parkin mutation as the most-common cause of autosomal recessive PD, reportedly with 20 mutations being identified

(Hatano et al., 2004). It is associated with both early and late-onset ranging from 32 to 48 years of age, with typical features of idiopathic PD (Lester and Otero-Siliceo, 2006). No pathology has been determine in PTEN1 PD patients but it is present in Lewy bodies from idiopathic PD postmortem (Gandhi et al., 2006).

PINK1 is a 581 amino acid protein ubiquitously expressed throughout the CNS. (Silvestri et al., 2005). It is highly homologous to calmodulin-dependent protein kinase 1 with an N-terminal targeting motif for mitochondria membranes. *In- vitro* studies have demonstrated that PINK1 contains a catalytic serine-threonine kinase domain with autophosphorlylation properties. Most mutations identified are within or near the serine-threonine protein kinase domain and have shown reduced catalytic activity *in-vitro* (Beilina et al., 2005). This presumed lost of function combined with its localization to mitochondria, implicate that both may play a role in PD pathogenesis. In support of this idea, mitochondrial dysfunction related to PIINK1 mutations have been demonstrated in Drosophila models (Clark et al., 2006; Park et al., 2006). PINK1 loss of function mutants leads to male sterility, muscular and dopaminergic degeneration with enlargement and fragmentation of the mitochondria christae. More importantly a link between Parkin function and PINK1 was illustrated by Parkin overexpression rescuing PINK1 deficient phenotype. This suggests Parkin and PINK interaction is vital for proper mitochondrial function. In addition, culture cell studies have demonstrated protective properties of PINK1 from mitochondria mediated apoptosis induced by inhibition of the UPS (Petit et al., 2005). Overexpression of various PINK1 mutants that disrupt its kinase activity failed to elicit these protective effects, implying it kinase activity is necessary for it

functions. Currently it is unknown if these properties and interaction of PINK1 are analogous in a mammalian based model which has yet to be reported.

PARK 7 (DJ1)

A second locus in the chromosome 1p36 linked to early onset autosomal recessive PD in an Italian family was identified as a mutation in DJ1 gene (Bonifati et al., 2002; van Duijn et al., 2001). DJ1 mutations are speculated to account for 1-2% of early onset PD cases (Hedrich et al., 2004). In addition to classical parkinsonism symptoms an association with psychiatric symptoms have also been reported (Dekker et al., 2003). The pathology of DJ1 PD patients has not been published nor has DJ1 been shown to be a component of Lewy bodies of idiopathic PD.

DJ1 was initially discovered as a mitogen dependant oncogene product involved in the Ras-related signal transduction pathway (Nagakubo et al., 1997). It is expressed in a variety of tissues and is enriched in the CNS. Within neurons, DJ1 is primarily cytoplasmic but a small fraction is also localized to the mitochondria (Zhang et al., 2005). DJ1 shares structural similarities to cysteine proteases and contains a large amount of residues that are readily oxidized. These residues propose a function as a protective mechanism from oxidative stress were by DJ1 acts as a redox dependant chaperone (Canet-Aviles et al., 2004). DJ1 proteins isolated from idiopathic PD patients brains contain oxidative damage suggesting that high levels of oxidative stress may contribute PD pathogenesis (Choi et al., 2006). *In vitro* studies have demonstrated extensive oxidation of DJ1 inactivates it chaperone capabilities suggesting a lost function in PD etiology (Zhou et al., 2006). These studies lead to the development of the DJ1 knockout

mouse that displayed parkinsonism like motor symptoms and SN dopaminergic dysfunction without cellular loss (Goldberg et al., 2005). Biochemical analysis revealed DJ1 attenuates dopamine D2-receptors signaling and DJ1 mutants display increased reuptake of dopamine resulting in motor impairment. Drosophila DJ1 mutants have increased sensitivity to oxidative stress but exhibit no motor dysfunction nor neurodegeneration (Yang et al., 2005). The potential function of DJ1 as a redox dependant chaperone for oxidative stress is not conclusive and further investigation is required. Additionally, it remains unclear what role DJ1 plays in relation to its mitochondria localization.

PARK 8 (leucine-rich repeat kinase 2)

Mutations in the leucine-rich repeat kinase 2 (LRRK2) gene have recently been linked to autosomal dominant late onset-PD by two independent groups (MacLeod et al., 2006; Zimprich et al., 2004). Since then many LRRK mutations have been identified and have been estimated to accounted for 5%-10% of late onset familial PD and 1.5% of idiopathic PD cases (Di Fonzo et al., 2005; Gilks et al., 2005; Nichols et al., 2005). Population studies reported a higher frequency of LRRK2 mutations in North Africa Arabs (37% of familial and 41% of idiopathic PD cases) and Ashkenazi Jews (30% of familial PD and 13% of idiopathic PD) (Lesage et al., 2006; Ozelius et al., 2006). The penetrance of these mutations mimics that of idiopathic PD, in which it is age dependant increasing from 17% at age 50 to 85% at age 70 for all populations (Kachergus et al., 2005). Clinical features of LRRK2 PD are indistinguishable from typical idiopathic late onset PD. Post mortem analysis of four patients from a single familial cohort carrying a

common LRRK2 mutation revealed both the presence and absence of Lewy bodies (Goldwurm et al., 2007).

LRRK2 is a large gene containing 51 exons which encodes a 2,527 amino acid protein that consist of multiple conserved domains. LRRK2 conserved domains include a N-terminal leucine-rich repeat domain, a Roc GTPase domain followed by its associated C-terminal of Roc (COR) domain, a tyrosine like kinase domain, and a C-terminal WD40 domain (Manning et al., 2002; Mata et al., 2006; Skipper et al., 2005). The presence of the WD40 domain which mediates protein-protein interaction suggests that LRRK2 may act as scaffold for assembly of multiprotein signaling complexes in addition to its predicted protein kinase and GTPase activities. Purified LRRK2 protein demonstrates both GTPase and kinase activity with the ability to autophosphorylate. The PD associated LRRK2 mutations G2019S and I2020T are located at the N-terminal boundaries of the activation segment of the kinase domain. In-vitro studies demonstrate G2019S and I2020T mutation enhance kinase activity (Gloeckner et al., 2006; West et al., 2005). A third mutation assocated with PD, R1441C lies in the GTPase domain which also increases kinase activity (West et al., 2005). Although these three mutations tend to suggest a gain of function of LRRK2 may play a role in the pathogenesis of PD, *in-vivo* models have yet to be reported.

Conclusion

Nearly two centuries from its original description much knowledge has been gained of PD from the components of Lewy bodies to the degeneration of dopaminergic neurons of the SN. The identification of neurotoxin that mimic the disease not only implicated environmental factors as a causality of PD but paved the road for the development of the first animal models. More importantly, the discovery of familial PD associated genes has put genetics and biology at the forefront for understanding the pathogenesis of PD. Mutations in Parkin, PINK1 and DJ1 that are all located in the mitochondria implicate mitochondrial dysfunction and potentially oxidative stress as major contributors for PD development. The identification of mutations in UCHL1, Parkin and α-synuclein all support the hypothesis of misfolded proteins that accumulate and impair proteasomal function leading to neuronal cell death. Yet, several questions remain: Is there a common pathway that leads to PD or is it a combination of mitochondria dysfunction and impairment of proteasome, what predisposition does genetics play in development of idiopathic PD, is there a combination between environmental factors and genetics involved in PD development, what contribution does the neuroinflammatory response play, are there specific proteins targeted by UCHL-1 and Parkin proteins, and what is the function of α-synuclein?

Chapter 2

Characterization of the α-synuclein transgenic mouse

Abstract

The recent findings of mutations in α-synuclein that have been linked to early onset Familial Parkinson's disease have implicated α-synuclein in the pathogenesis of neurodegeneration. Animal models expressing human α-synuclein have recently been developed that display characteristics of Lewy bodies and develop age dependant motor deficits. In this study, we analyzed neuorpathological effects of human α-synuclein, murine α-synuclein, A30P and A53T mutants of α-synuclein under the control of mThy-1 promoter. High levels of neuronal transgenic synuclein expression were achieved in the cortex, thalamus, basal ganglia and spinal cord motor neurons. Transgenic mice expressing human wild type and mutant α-synucleins developed an age-dependent motor dysfunction with rigidity, dystonia, decreased muscle strength and abnormal gait. Immunohistological studies revealed extensive neurodegeneration, gliosis and presence of synucleinopathies, with the motor neurons being more susceptible. Biochemical analysis revealed α-synuclein becomes insoluble in symptomatic mice with an increase in oligomerization of α-synuclein. Transgenic mice expressing murine α-synuclein did not develop any motor deficits or pathology, despite expressing similar levels as the human wild type and mutant α-synculein transgenics. Thus, these mice models provide a means for studying pathological role of α-synuclein and have the potential to be used in developing new therapeutic strategies.

2.1 Introduction

Synuclein was discovered with an antiserum against purified cholinergic synaptic vesicles from the electric organ of Torpedo in 1988 (Maroteaux et al., 1988). In 1993, Nakajo and colleagues identified a brain specific bovine protein which later was cloned in rats and termed phosphoneuroprotein 14 (PNP-14) (Nakajo et al., 1993). PNP-14 is now known as β-synuclein and the original Tropedo synuclein is designated as γ-synuclein. The third member, α-synuclein was originally isolated as a precursor protein provisionally named non-amyloid β-protein component (NAC) of the Alzheimer's disease plaques (Ueda et al., 1993). This was the first indication of α-synuclein involvement in neurodegenerative diseases.

Synucleins are encoded by different genes ranging in size from 120-140 amino acids. All three members contain a repeated KTKEGV consensus sequence in the N-termninal with an acidic stretch toward the C-terminal. α and β synucleins are 62% identical in amino acid sequence and share a conserved C-terminal region, however α-synuclein contains 11 residues (positions 73-83) that are absent in the β isoform. This stretch of 11 amino acids lie within hydrophobic region believed to promote fibrillization of α-synuclein (Giasson et al., 2001). Both α and β synucleins are extensively expressed throughout the central nervous system. α-synuclein is concentrated within nerve terminals distributed in cerebral cortex, caudate nucleus, putamen, substantia nigra pars reticulata, locus coeruleus and laminae I and II of the spinal cord (Brett et al., 2002; Klos et al., 2006; Neumann et al., 2004). α-synuclein is intensely expressed in the oculomotor, facial, hypoglossal and ambiguous nuclei. γ-synuclein shares only 55% homology with α-synuclein, lacking the conserved C terminal region and is predominantly expressed in

spinal cord and peripheral nervous system (Lavedan, 1998). Mammalian γ-synuclein was later identified as a human breast cancer susceptibility gene (Ji et al., 1997). Originally termed breast cancer-specific gene 1 (BCSG1), it was identifdied by direct-differnetial cDNA sequencing illustrating high expression of BCSG1 in breast cancer cDNA library. Sequence alignment of BCSG1 to the ***NAC*** and *Torpedo* synuclein demonstrated high homology, and eventually was determined to be gamma synuclein. *In situ* analysis demonstrated BCSG was absent in normal or benign breast cancer lesions and highly expressed in advanced infiltrating breast cancer. Overexpression of BCSG in cancer cells in vitro, increased motility and invasiveness, *in vivo* studies demonstrated increased metastasis (Jia et al., 1999).

In 1997, the first genetic missense mutantion linked to PD was identified as a substitution of alanine at postion 53 with threonine (A53T) in α-synuclein (Polymeropoulos et al., 1997). Within the same year, α-synuclein was identified as the major component of abnormal intracellular aggregates known as Lewy bodies from postmortem PD patients (Spillantini et al., 1997). One year later a second mutation, alanine to proline at amino acid 30 (A30P) was linked to PD in a German family (Kruger et al., 1998). Since then a third mutation at amino acid 46, a switch from acidic glutamate to a polar lysine was identified in a Spanish family with autosomal dominant PD. More recently, gene triplication of wild type α-synuclein has also led to early onset PD (Singleton et al., 2003). This discovery suggests expression levels of wildtype α-synuclein is not only sufficient for development of PD but may also determine the age of onset.

In attempts to study intraneuronal aggregation of α-synuclein *in vivo*, transgenic mouse models using neuronal specific promoters to over express human wild type, A53T and A30P α-synuclein have been developed. These studies have demonstrated motor deficits resembling PD in mice expressing wild type and mutant synucleins (Giasson et al., 2002; Gomez-Isla et al., 2003; van der Putten et al., 2000). Transgenic mice that over express mouse endogenous α-synuclein do not elicit any phenotype, suggesting this phenotype is specific for the human isoform. Among several promoters used for overexpression of α-synuclein is the platelet derived growth factor receptor ß (PDGFRß). These PDGFRß α-synuclein-transgenics had no apparent loss of dopaminergic neurons within SN, yet dopaminergic terminals in the caudo-putamen region had a 25-50% decrease at 12 months of age (Masliah et al., 2000; Rockenstein et al., 2002). Transgenic lines expressing highest levels of α-synuclein had intraneuronal protein accumulation at 3 months and a decrease in tyrosine hydroxylase (TH) fibers within caudoputman region. Analysis of α-synuclein oligomers suggested A53T mutant α-synuclein mouse line had increased amount of oligomers compared to wild type. This is similar to the PrP promoter driven mouse α-synuclein-transgenic, except, unlike the PDGFß lines, PrP α-synuclein A30P mutant and human wild type α-synuclein transgenics did not develop any motor dysfunction (Lee et al., 2002). To address whether over expression of α-synuclein is toxic specifically in SN neurons, Richfield and colleages created transgenic mice using a TH promoter to express wild type and mutant forms of α -synuclein. Despite considerable amounts of α-synuclein within SN neurons, no degeneration or motor deficits where reported. These studies further confirmed that higher expressing lines elicited a stronger and an earlier onset for the phenotype. This suggested that using a

stronger promoter such as the Thy-1 could elicit a greater neuropathology. Thy-1 α-syn transgenic mice have a greater distribution of expression throughout the brain and spinal cord, including SN and motor neurons (Rockenstein et al., 2002; van der Putten et al., 2000). Analysis of these transgenic mice illustrated axonal degeneration at neuromuscular junction with a breakdown and segmentation of myelin (van der Putten et al., 2000). Pathology indicated both brain and motor neurons contained an increase in gliosis and Lewy-like inclusions that were ubiquitin immuno-reactive. Reportedly, α-synuclein A53T transgenic mice have an increased amount of insoluble α-synuclein within the spinal cord and cerebellum in comparison to human wild type α-synuclein. Subcellular fractionation studies identified abnormal accumulation of A30P α-synuclein in neuronal cell bodies and neurites within the brain but did not affect synucleins anterograde transport to the synapses (Kahle et al., 2000).

Despite numerous studies performed *in vitro* and in transgenic models, little is known about the direct correlation of α-synuclein and motor deficits. Although cell cultures transfected with α-synuclein elicit apoptotic events, no neuronal cell loss has been demonstrated in transgenic models. The appearance of Lewy-like bodies and an increase in gliosis within the spinal cord raises the possibility that motor dysfunction is in part due to spinal cord neuronal degeneration. In order to better evaluate this possibility we will characterize Thy-1 promoter α-synuclein expressing transgenic mice with an emphasis on biochemical and pathological role or α-synuclein in the spinal cord.

2.2 Methods

2.2.1 Generation, breeding and analysis of transgenic mice.

Transgenic mice that express wildtype or mutant α-synucleins under the control of the Thy-1 promoter (Andra et al., 1996) were produced as described (Missler et al., 2003). For analysis of α-synuclein transgenics, littermate offspring from matings of transgenic mice with wildtype controls were observed for 16 months. Phenotypes were scored based the length of time mice griped to a wire and on the ability of the mice to move normally on their hindlimbs that are first affected by neurodegeneration in these mice (Fernagut and Chesselet, 2004). The grip analysis is performed by placing a mouse on a 0.5 cm wire mesh and inverting it. On average, control nontransgenic mice grip for an excess of 1 min. A30P transgenic mice grip strength declines as a function of time. Onset time for a phenotype was set at a 50% decrease in grip strength.

2.2.2 Morphological Studies

Immunofluorescence and FluoroJadeB were performed on cryosections as described briefly. Anesthetized mice littermates were perfuse with ice cold .1 M PBS followed by ice cold 4% paraformaldehyde in phosphate buffer. Following perfusion, the spinal cords were removed and postfixed by immersing in 4% paraformaldhyde overnight. Cryosection (20μm) were permeablilized with Tris-buffer containing .1% Triton X-100 and 3% BSA for blocking of nonspecificity. Sections were incubated with primary antibody for synuclein, ubiquitin, and GFAP diluted in blocking solution overnight at 4°C. Following a series of washes section were incubated with fluorescence-labeled secondary antibody and mounted onto slides with Vectashield (Vector Laboratories).

2.2.3 Protein Biochemistry.

To determine the relative amounts of soluble vs. insoluble α-synuclein in transgenic mice, whole brain and spinal cord from 12-14 month old littermate mice was homogenized in PBS buffer, solubilized in 1% NP-40, 0.1% SDS, and 1% DOC (RIPA buffer), and centrifuged (71,000 g for 45 min). Equal concentrations from each sample were analyzed by quantitative immunoblotting.

2.3 Results

2.3.1 Transgenic synuclein overexpressed ubiquitously throughout the CNS.

We generated transgenic mice that overexpress wildtype human α-synuclein (htg), A30P-mutant human α-synuclein (hA30Ptg), A53T-mutant human α-synuclein (hA53Ttg), or wildtype mouse α-synuclein (mtg) under control of the murine Thy-1 promoter. The transgenic mice produced identical levels of α-synuclein overexpression except for the A53T-transgenic mice which exhibited ~2 fold higher α-synuclein levels (Fig. 2.1A). Immunocyto-chemistry showed that the various transgenic α-synucleins were expressed in similar pan-neuronal patterns throughout the brain (Fig. 2.1B).

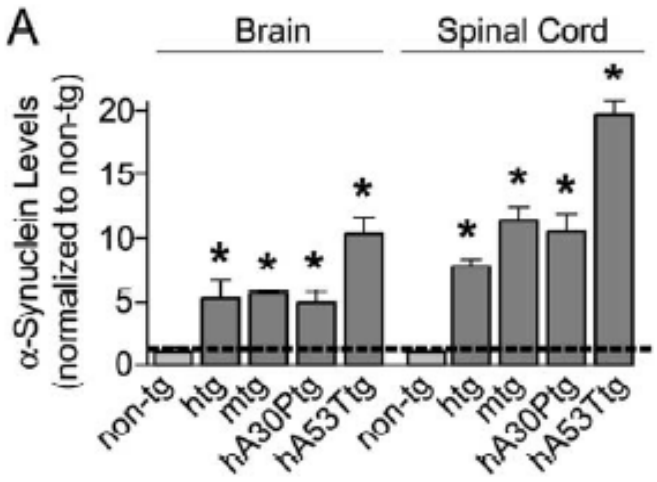

Figure 2.1 Quantification of α-synuclein expression from brain and spinal cord

A. Levels of α-synuclein in the brain and spinal cord of transgenic mice expressing wildtype human α-synuclein (htg), wildtype murine α-synuclein (mtg), and A30P- (hA30Ptg) and A53T-mutant human α-synucleins (hA53Ttg). Levels are expressed as fold of α-synuclein in transgenic over non-transgenic control mice (non-tg). Statistical significance was assessed by Student's t-test as indicated (*=$P<0.05$) comparison refer to levels between transgenic and non-transgenic mice; the difference between the various transgenics was not statistically significant).

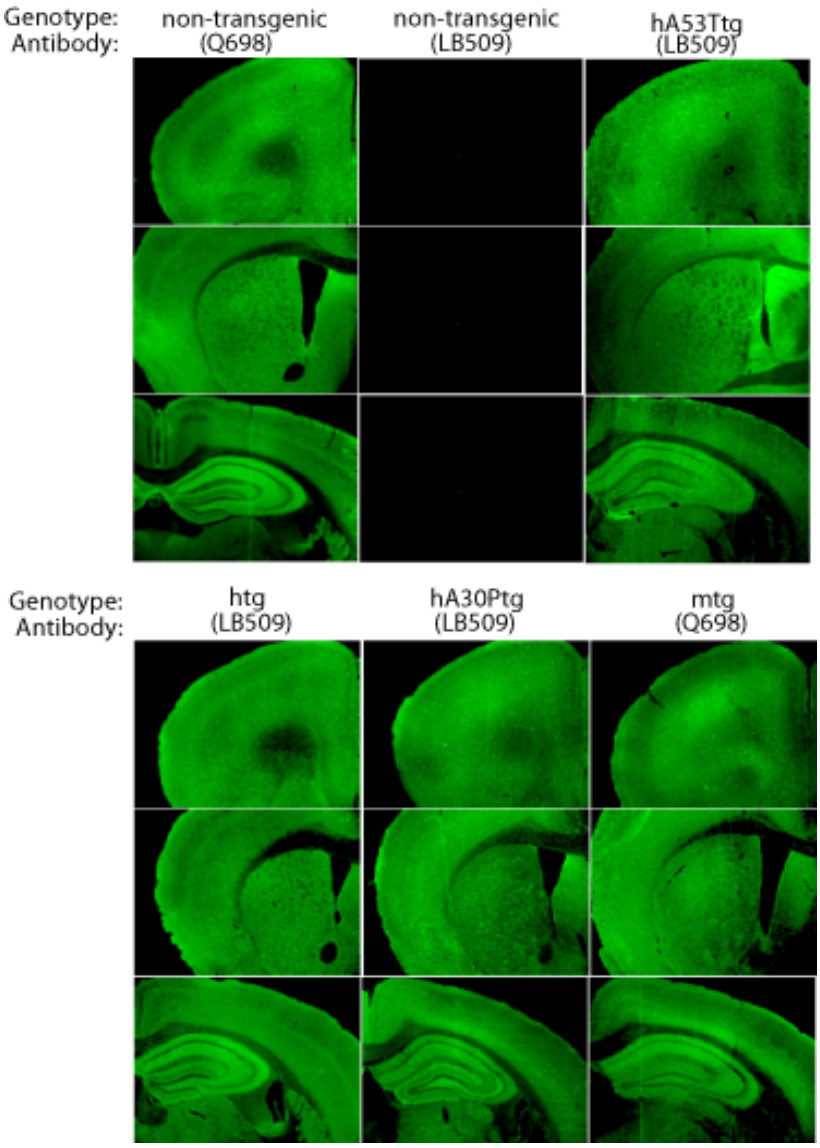

Figure 2.1B Distribution of transgenic α-synucleins in mouse brains.

Cryostat sections of brains from mice with the indicated genotypes were examined using indirect immunofluorescence labeling with an antibody that is specific for transgenic human α-synuclein and does not stain non-transgenic brains (LB509), and an antibody that recognizes both transgenic and endogenous mouse α-synuclein (Q698). For all transgenics, cortex (top panels), thalamus and basal ganglia (middle panels) and hippocampus (bottom panels) were probed. Calibration bars (0.4 mm) apply to all panels.

2.3.2 Late-onset neurodegeneration in transgenic mice for human but not mouse α-synuclein

Consistent with previous reports (reviewed in Fernagut and Chesselet, 2004), we observed a partial penetrant, age-dependent neurodegenerative disorder in transgenic mice that express wildtype or mutant human α-synucleins. Mice expressing wildtype human α-synuclein exhibited only a modest neurodegenerative phenotype (~15% incidence after 16 months). However, mice containing transgenic A30P- or A53T-mutant human α-synuclein displayed a more severe phenotype (>70% incidence; Fig. 2B).

Transgenic α-synuclein induced a loss of motor neurons (shown for mice expressing human A30P-mutant α-synuclein in Figs. 2.2C and 2.2D). Strikingly, mice overexpressing murine α-synuclein did not develop a neurodegenerative phenotype (Fig. 2B; tested in 5 independent lines [data not shown]), in spite of the fact that the transgenic expression levels of mouse and human α-synuclein were similar (Fig. 1A).

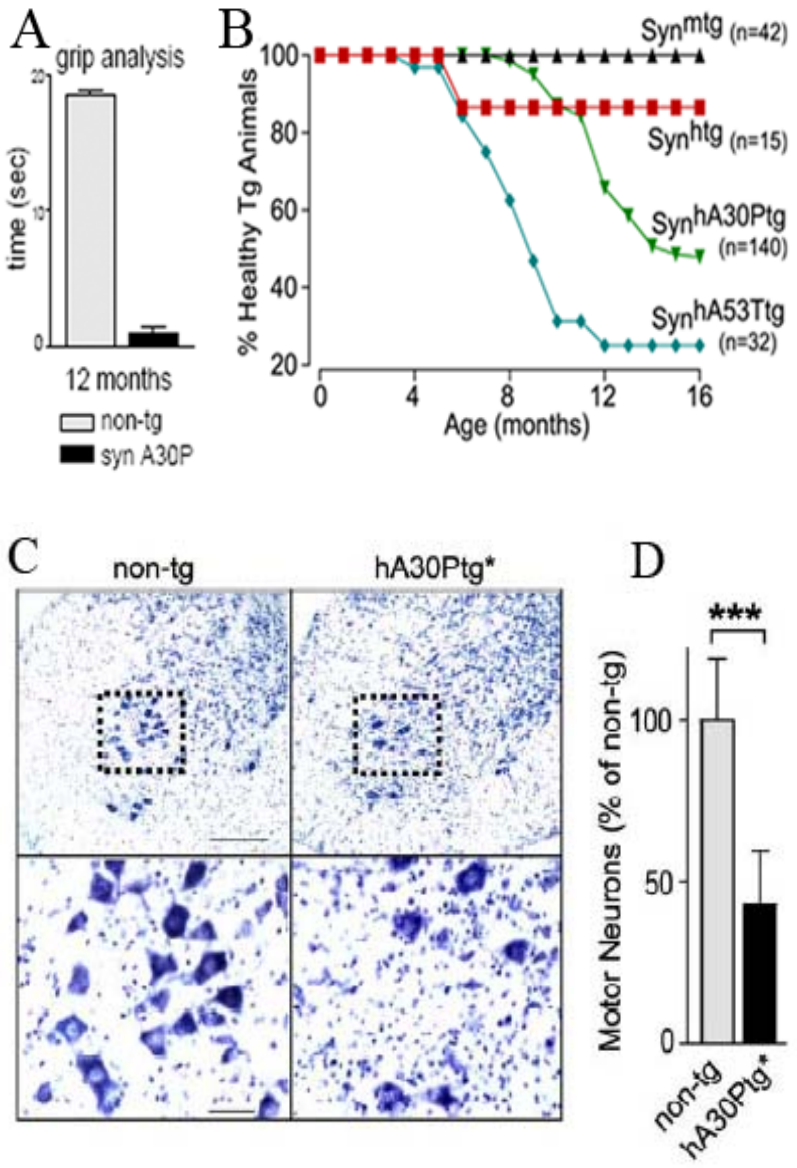

Figure 2.2 Loss of motor neurons at the onset of motor deficits in transgenic mice expressing human A30P α-synuclein

A. Grip strength was measured as a function of time to determine onset of disease. B. Age-of-onset of symptoms in transgenic mice expressing various α-synucleins. C. Representative Nissl-stained spinal cord sections from littermate nontransgenic controls and symptomatic A30P-transgenic littermate mice (calibration bar: top, 0.2 mm; bottom,50 um). Genotypes of the mice used for these sections are indicated on top. D. Quantitation of motorneurons in Nissl-stained sections from nontransgenic control and

symptomatic A30P α-synuclein transgenic littermate mice. Statistical significance was assessed by Student's t-test as indicated (***=P<0.001; n=3; age of mice 12-16).

2.3.3 Transgenic synuclein mice display neuropathology including inclusions, gliosis and insoluble synuclein aggregates

To determine if transgenic mice developed any neuropathological inclusions, cross section of spinal cords from hA30Ptg symptomatic mice were analyzed. hA30Ptg mice displayed inclusions immunoreactive for synuclein and ubiquitin (Fig.3A-B; anti-ubiquitin data not shown). These synuclein inclusions were accompanied with gliosis that is visualized as an increase in expression of astrocytic protein GFAP (Fig 3C-D). Overexpression of A30P-mutant synuclein leads to neurodegeneration and cell death of the motor neurons, as illustrated by selective staining of degenerated neurons with FluoroJadeB (Schmued and Hopkins) (Fig. 3 E-F). The neurodegeneration was determined to be mediated by a neucrotic mechanism which is evident by the absence of an apoptotic marker (TUNEL stain) (Fig. 3 G-I). This neuropathology correlated with the formation of insoluble α-synuclein aggregates (Fig. 4).

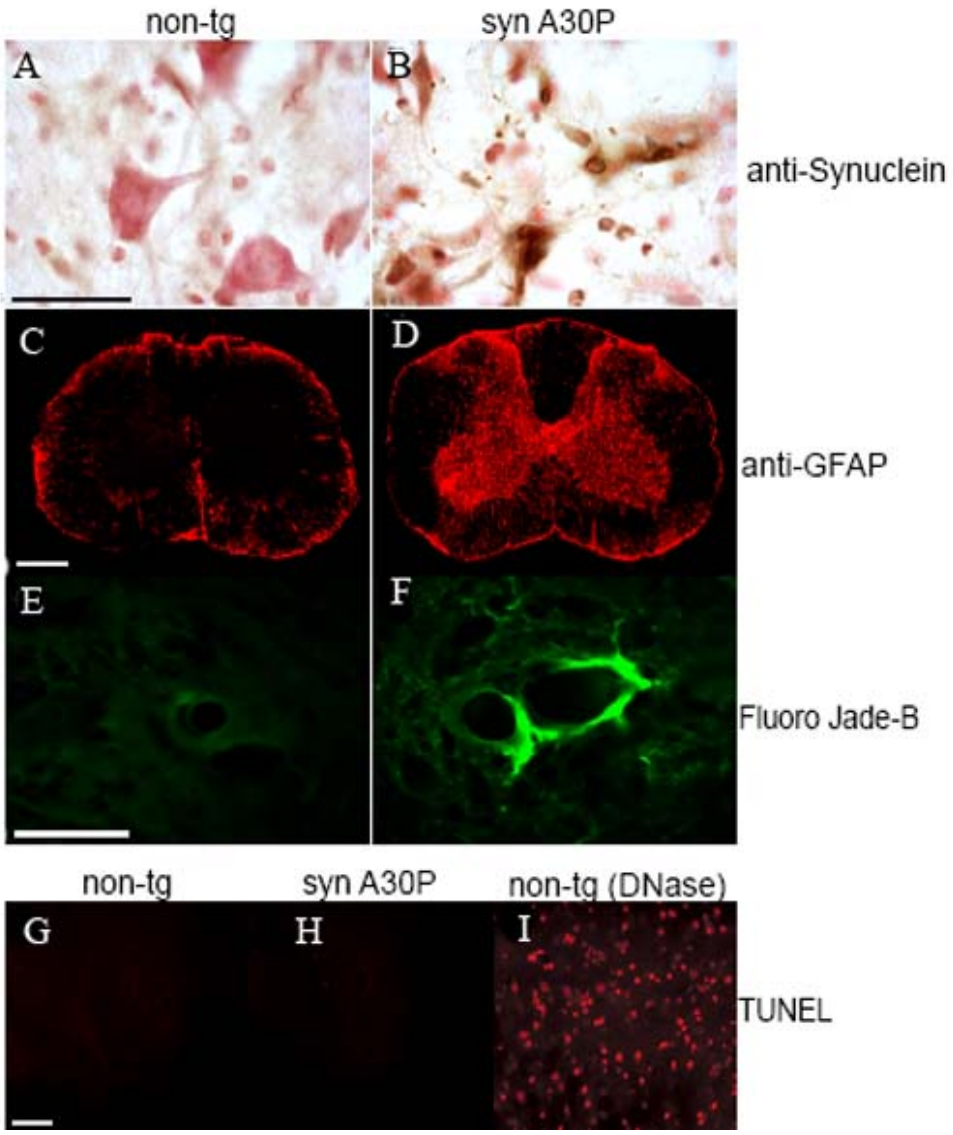

Figure 2.3 Pathology of symptomatic mice expressing A30P mutant α-synuclein

A-B Abnormal accumulation of α-synuclein in spinal cord sections from A30P α-synuclein transgenic mice. Nontransgenic control mice (non-tg) do not stain for A30P α-synucelin. Sections were stained by immunohistochemistry with synuclein antibody (LB509); calibration bar=50um.C-D Immunofluorescence of nontransgenic control and A30P α-synuclein transgenic spinal cord sections illustrating gliosis in A30P transgenic mice (sections stained with GFAP antibody; calibration bar=0.1mm). E-F Staining of spinal cord sections from A30P α-synuclein transgic and from littermate wildtype control mice with Fluoro Jade B. Cryosections were stained as described (Fornai, F., *et al.*, Parkinson-Like Syndrome Induced by Continuous MPTP Infusion: Convergent Roles of the Ubiquitin-Proteasome System and α-Synuclein.

Proc. Acad. Sci. U.S.A., **102**, 3413-3418 [2005]) Fluoro-Jade B is a high affinity fluorescent marker for the localization of neuronal degeneration. *Brain Res*. **874**, 123-130 [2000]). G-I TUNEL staining assay for the detection of apoptotosis. Calibration bar = 50. Genotypes of the mice used for these sections are indicated on top (n=3 age of mice 12-14 months)

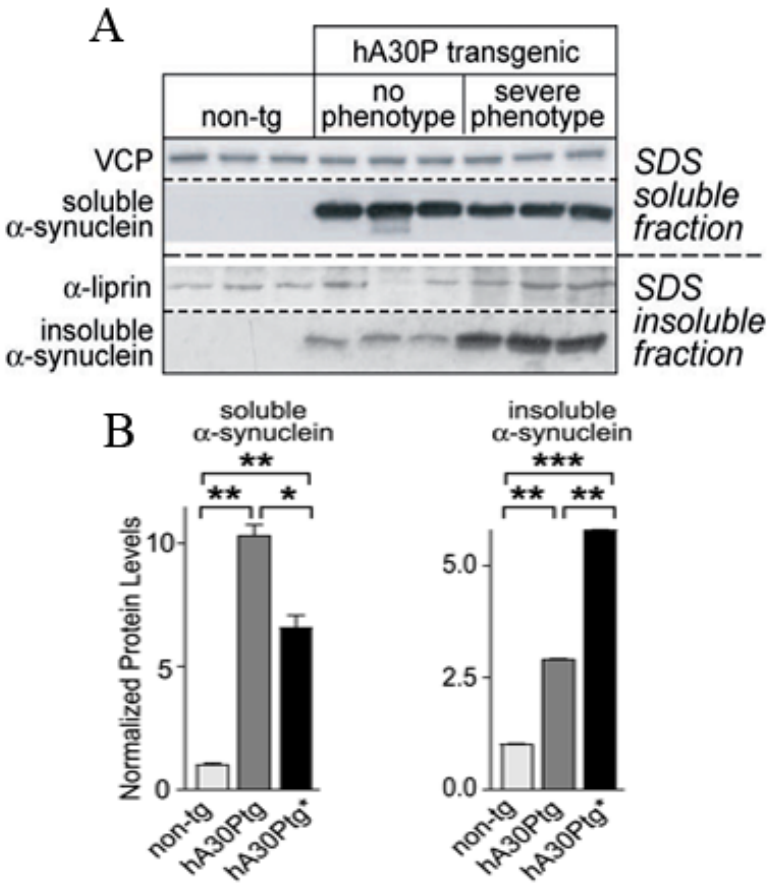

Figure 2.4 α-synuclein solubility is altered in symptomatic mice expressing A30P mutant α-synuclein

A. Representative immunoblot of samples, analysed in triplicate, from spinal cord of littermate non-transgenic control mice, non-symptomatic A30P-mutant α-synuclein transgenic mice, and symptomatic A30P-mutant α-synuclein transgenic mice. Samples were prepared by resuspending spinal cord homogenates in RIPA buffer, and separating soluble and insoluble materials by centrifugation (71,000 g for

45 min). Blots were probed with antibodies to α-synuclein and to VCP and α-liprins as loading controls for soluble and insoluble materials, respectively, and visualized by ECL. B. Quantitation of SDS-soluble and SDS-insoluble α-synuclein in non-symptomatic (hA30Ptg) and symptomatic (hA30Ptg*) A30P-transgenic mice, normalized to the levels observed in non-transgenic littermate control mice. Statistical significance was assessed by Student's t-test as indicated (*=P<0.05; **=P<0.01; ***=P<0.001)

2.4 Discussion

In the present study, we show transgenic mice expressing α-synuclein human wildtype, A30P-mutant human α-synuclein, and A53T-mutant α-synuclein via Thy-1 promoter developed an aged dependant neurodegenerating disease. The A53T mouse line had the highest expression of α-synuclein with the highest pentrants for the disease and the earliest onset for motor impairments. This result confirms previous studies demonstrating that in transgenic mice A53T-mutant α-synuclein induces a stronger phenotype and is dosage dependant (reviewed in (Fernagut and Chesselet, 2004). In contrast transgenic mice expressing mouse synuclein did not develop any abnormalities, although they displayed similar levels and distribution of transgene expression as to the human synucleins mouse lines. The lack of a pathogenic effect by wildtype mouse α-synuclein suggests that the neurodegeneration induced by human α-synuclein does not reflect a general physiological function of α-synuclein.

Biochemical and immunohistological studies of our α-synuclein transgenics determined that motor impairments are primarily due to a loss of motor neurons from the spinal cord (Fig 2). This is in contrast to previous studies of α-synuclein transgenics lines that have not reported any neuronal cell lose within the CNS. Perhaps, this inability was due to the use of lower expressing promoters used in these

studies. This notion is further supported by recent studies illustrating gene triplication of human wildtype synuclein in PD afflicted patients (Singleton et al., 2003). Further more, we demonstrated a large amount neurodegeneration within the spinal cord leading to a decrease in motor neurons in a non-apoptotic fashion (Fig 3). This implies neuronal cell lost may be mediated by neucrosis or an inflammatory response which is evident by presence of extensive gliosis. (McGeer et al., 1988; McGeer and McGeer, 1995).

In solution α-synuclein exists in a random coiled conformation with the propensity to form β-sheets. In the presence of phospholipids α-synucelin folds into α-helices thought to be important for proper function of α-synuclein (see Chapter 3). Several studies have demonstrated α-synuclein β-sheets may form oligomers capable of becoming insoluble aggregates, believed to be important in the formation of inclusions (Giasson et al., 2001). We illustrated α-synuclein solubility is altered becoming detergent insoluble in spinal cords of symptomatic mice. This finding is consistent with accumulation of fibrillar α-synuclein found in Lewy bodies believed to be toxic. We further illustrate the presence of inclusion that are immunoreactive for both synuclein and ubiquitin protein in spinal cords of symptomatic mice. These analyses establish our mouse model as an important tool for studying α-synuclien pathogenesis in neurodegenerative diseases.

Chapter 3

A MOLECULAR PATHWAY OF NEURODEGENERATION LINKING α-SYNUCLEIN TO AMYLOID-β AND APOE

ABSTRACT

Pathogenic aggregates of α-synuclein are thought to contribute to the development of Parkinson's disease (PD), and α-synuclein containing inclusion bodies are present in PD and other neurodegenerative diseases, including Alzheimer's disease (AD). Moreover, α-synuclein mutations are found in cases of familial PD, and transgenic overexpression of α-synuclein causes neurodegeneration in mice. The molecular mechanisms involved, however, remain unknown. Here we show that in transgenic mice, α-synuclein induced neurodegeneration involves the ubiquitin/proteasome system, causes a massive increase in ApoE levels, and produces accumulation of insoluble mouse Aβ-peptides into plaques. Surprisingly, ApoE was not protective but injurious for this neurodegeneration as deletion of ApoE delayed the neurodegeneration caused by α-synuclein, and suppressed the accumulation of Aβ-peptides. Our data reveal a molecular link between central pathogenic mechanisms implicated in PD and AD, and suggest that intracellular α-synuclein is pathogenic at least in part by activation of extracellular signalling pathways involving ApoE.

INTRODUCTION

Extensive studies revealed a pathogenic role for a-synuclein in several types of neurodegeneration, both in human disease and in animal models2 (Fernagut and Chesselet, 2004) The pathogenic activity of α-synuclein involves formation of a-synuclein aggregates and is specific for human α-synuclein as no involvement of human α- or β-synuclein in disease was found, and as transgenic mouse α-synuclein does not cause a phenotype in mice. α-synuclein is not only involved in Parkinson's disease in which its possible pathogenic role was first identified, but also in multiple other neurodegenerative diseases, most prominently dementia with Lewy bodies, and multiple system atrophy. Strong evidence implicates α-synuclein aggregation as a major pathogenic mechanism in these diseases, but the molecular pathway that mediates disease remains unknown. Moreover, genetic evidence suggests that common mechanisms underlie neurodegeneration in PD and AD, for example in that both are dependent on ApoE (Harhangi et al., 2000; Huang et al., 2004). Transgenic mice that overexpress various forms of α-synuclein (wild-type human and mouse α-synuclein; A53T- and A30P-mutant human α-synuclein) develop a neurodegenerative disease that originates within the spinal cord. This localization of spinal cord degeneration is in part due to a higher expression of transgenic α-synuclein within the spinal cord in comparison to the brain. Although the neurodegeneration caused by transgenic α-synuclein can debilitate the transgenic mice by causing hindlimb paralysis, its onset is late, and its penetrance is incomplete. In the present study, we investigated whether ApoE would have protective effects or detrimental effects in transgenic mice that overexpressed human α-synuclien mutations.

3.2 Methods

3.2.1 Antibodies. The following antibodies were used: 1. antibodies to synaptic proteins as previously described (Rosahl et al., 1995; Chandra et al., 2005; Ho et al., 2006); 2. antibodies to proteasome subunits and ApoE (generous gifts of Drs. G. DeMartino and J. Herz, respectively; both UT Southwestern, Dallas TX); and 3. commercial antibodies to Lmp7 (Biomol), α-synuclein (BD Transduction Lab #610786), ubiquitin (Santa Cruz P401), clusterin (Chemicon G190), GFAP (Chemicon MAB360), and $A\beta_{42}$ (Chemicon AB5078P and Upstate #05-831).

3.2.2 Generation, breeding and analysis of transgenic mice and KO mice.

The following mice were used for the present study: 1. transgenic mice overexpressing human or mouse wild-type or mutant α-synuclein (Chandra et al., 2005); 2. ApoE KO mice (Jackson Laboratories strain B6.129P2-*Apoe*^{tm1Unc}/J; (Piedrahita et al., 1992) and 3. Wallerian degeneration slow (WLD) mutant mice (strain C57BL/601aHsd from Harlan, UK; (Hall, 1993). Mouse husbandry with timed matings and analysis of age-dependent phenotypes were performed as described (Chandra et al., 2005); all analyses were done with littermate mice derived from heterozygous matings, and with mice containing a single transgenic α-synuclein allele. Briefly, α-synuclein transgenics were analyzed as the offspring of matings of α-synuclein transgenic with wild-type mice; the effect of the ApoE KO on the α-synuclein induced neurodegeneration was examined in the offspring of matings of doubly heterozygous α-synuclein transgenic/ApoE KO

mice with singly heterozygous ApoE KO mice; and the effect of the WLD mutation on the α-synuclein induced pathology was tested in the offspring of matings of between singly heterozygous α-synuclein transgenic and WLD mice. All analyses were performed in α-synuclein KO mice that lacked endogenous α-synuclein to exclude compounding effects of endogenous α-synuclein except for the WLD analyses in which the WLD heterozygous mutant mice contained a normal complement of α-synuclein genes, and the offspring were thus heterozygous for the α-synuclein KO. Grip strength tests were used to analyze the time course of the phenotype development. Grip strength tests were performed by placing a mouse on a 0.5 cm wire mesh, inverting the mesh, and measuring the length of time a mouse held onto it (wild-type = >1 min). A30P-mutant α-synuclein transgenic mice exhibit an age-dependent decline in the time a mouse holds onto the mesh; the phenotype onset was defined as a 50% decrease in the average time a mouse held onto the mesh. This onset time is also accompanied by abnormal hind limb movement and abnormal limb clasping behavior, considered as initial stages of disease (Boillee et al., 2006; Mangiarini et al., 1996). Weight analyses were obtained by weighing each mouse weekly beginning at 7 months of age.

3.2.3 Morphological Studies. All immunohistochemistry analysis was performed on cryosections from spinal cords. Briefly, anesthetized mice were perfused with 4% paraformaldhyde followed by decalcification in Cal-Rite solution (Richard-Allan Scientifics). Sections (30 μm) were incubated in blocking solution (3% BSA, 0.1% Triton X-100 in PBS) for 1 h followed by an overnight incubation at 4 °C with antibodies to α-synuclein, GFAP, Aβ42 polyclonal and Aβ42 monoclonal. Following 3 washes of 5 min

each in PBS, sections were incubated with Alexa Fluor 488- or Alexa Fluor 563-coupled secondary antibodies for 3 h at room temperature, and visualized in a fluorescence microscope. For measurement of amyloid deposits, TIFF images were captured with a SPOT camera in a Olympus BX-51 microscope, followed by quantitation by MetaMorph imaging software (Molecular Devices, Union City, CA). The threshold value was set at 100-256, and applied to all images followed by measurement of individual plaques within the spinal cord of each section. Motor neuron quantifications were performed with Nissl stained sections, and calculated with Image J program. FluoroJade B stain (Chemicon) was perform as described in (Schmued and Hopkins, 2000).

3.2.4 Protein biochemistry. Protein quantitations were performed by quantitative immunoblotting using ^{125}I-labeled secondary antibodies and phosphoImager detection with internal controls run on the same blots to normalize the signal in order to control for differences in blotting efficiency and protein concentration as described (Rosahl et al 1995), with 25 μg protein/lane. The following proteins and fractions were analyzed: 1. Proteasome subunits, clusterin, and ApoE as well as synaptic proteins were measured in total spinal cord, whole brain, or sciatic nerve homogenates obtained in Tris buffer (50 mM Tris-NaOH pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF and proteinase inhibitors). 2. Ubiquitin conjugates were measured in total spinal cord homogenates, and in soluble and insoluble proteins derived as pellets or supernatants after spinal cord was homogenized in Tris buffer containing 1% Triton X-100 and 0.1% SDS was centrifuged at 71,000g for 45 min. 3. α-Synuclein was measured in three fractions: the Triton-soluble supernatant was obtained after homogenizing whole spinal cord in Tris buffer containing

1% Triton X-100 and 2 mM MgCl (solubilized for 2 h at 4 °C), and centrifuging the homogenate at 15,000 x g at 4 °C for 30 min. The SDS-soluble and –insoluble fractions were obtained from the pellet of the first centrifugation that was re-extracted for 1 h at 4 °C with RIPA buffer (Tris buffer containing 1% Triton X-100, 0.1% SDS, and 0.5% deoxycholate) containing 2 mM $MgCl_2$. The solubilized pellet was re-centrifuged at 15,000 x g at 4 °C for 30 min, and the supernatant was termed SDS-soluble fraction and final pellet SDS-insoluble fraction.

3.2.5 Aβ ELISAs. To determine the relative amounts of $A\beta_{40}$ and $A\beta_{42}$, spinal cords from aged matched littermate mice were homogenized in 20 mM Tris pH 8.5 with proteinase inhibitors and centrifuged at 135,000 x g for 1 h at 4 °C. The supernatant was termed soluble fraction; the pellet was re-extracted with RIPA buffer for 1 h at 4 °C, followed by centrifugation at 100,000 x g for 1 h at 4 °C, resulting in the 'insoluble fraction' (Dewachter et al., 2000); the supernatant from this centrifugation was further analyzed because prior quantitations revealed that it contains only very low levels of Aβ peptides (<1% of the insoluble fraction). $A\beta_{42}$ and $A\beta_{42}$ quantitations from each fraction were obtained by sandwich ELISA (IBL Co., Ltd.; cat# 27711, 27730).

3.3 Results

3.3.1 Transgenic synuclein mice have increase total ubiquitin and altered proteasomal subunits.

To gain insight into the pathogenic mechanisms by which transgenic overexpression of α-synuclein promotes neurodegeneration, we examined transgenic α-synuclein mice. In these studies, we compared littermate mice that are either wild-type or transgenic for A30P-mutant α-synuclein (a mutation that causes PD in human patients (Kruger et al., 1998), and that are either asymptomatic or exhibit spinal cord neurodegeneration typical for these mice (e.g., see Masliah et al., 2000; Fernagut and Chesselet, 2004). These mice characteristically exhibit in spinal cord sections a loss of motor neurons, intense staining with FluoroJade B dye typical for degenerating neurons, and mild gliosis (chapter 2 Fig. 2.3). However, we did not detect a significant increase in TUNEL staining, suggesting that the α-synuclein induced neurodegeneration does not involve apoptosis (chapter 2 Fig. 2.3). Quantitative immunoblotting with ^{125}I-labeled secondary antibodies and phosphoimager detection revealed that the levels of ubiquitinated proteins were increased several fold in asymptomatic transgenic mice expressing mutant α-synuclein, and further increased in symptomatic mice (Figs. 3.1A and 3.1B). Moreover, the levels of proteasome subunits changed dramatically: most were increased (up to >4-fold for Lmp7), although the pa28 subunit was decreased (pa28; Fig. 3.1B). Again, the increase in asymptomatic transgenic mice was much lower than in symptomatic mice (Fig. 3.1B). A systematic analysis of 18 other neuronal proteins, however, failed to reveal a significant increase (data not shown). These data demonstrate that the neurodegeneration caused by transgenic overexpression

of A30P-mutant α-synuclein involves a specific activation of the ubiquitin-proteasome system (UPS); similar results were obtained for independent transgenic lines expressing wild-type, A30P-mutant, or A53T-mutant human α-synuclein (data not shown).

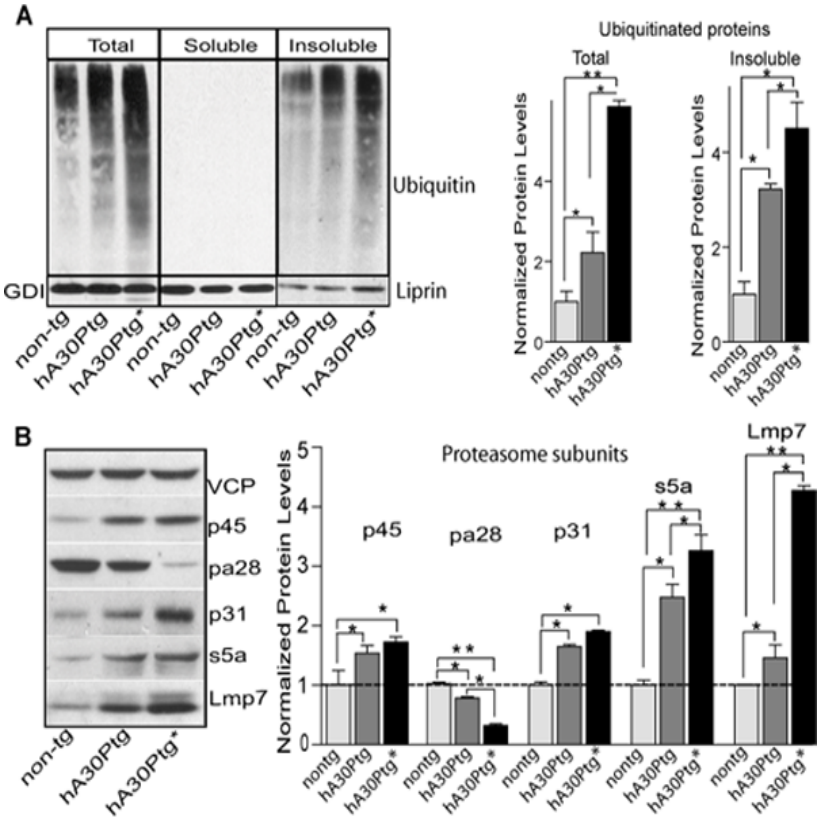

Figure 3.1 Transgenic mice expressing human α-synuclein have increased total ubiquitin and altered expression of proteasome subunits

(A) Representative immunoblots of total, SDS soluble and SDS insoluble ubiquitin from nontransgenic control mouse (non-tg), nonsymptomatic A30P α-synuclein (hA30Ptg) and symptomatic A30P α-synuclein transgenic mouse (hA30Ptg*). (B) Relative levels of total and SDS insoluble ubiquitin from nonsymptomatic A30P α-synuclein and symptomatic A30P α-synuclein transgenics normalized to nontransgenic control mice. (C) Representative immunoblots of selected proteasome subunits p45, pa28,

p31, s5a, and Lmp7, with valosin-containing protein (VCP) as loading control. (D) Levels of selected proteasome subunits in nontransgenic, nonsymptomatic and symptomatic A30P α-synuclein transgenic mice. Data shown represent age matched littermates 12-13 months n=3. Statistical significance for B and C was assessed by Student's t-test (*=P<0.05; **=P<0.01).

3.3.2 Asymptomatic and symptomatic transgenic α-synuclein mice have increased ApoE and clusterin

We next tested whether the changes in the UPS in the α-synuclein transgenic mice might reflect a specific neurodegenerative process by analyzing ApoE and clusterin (a.k.a. ApoJ), lipid transport proteins implicated in Alzheimer's disease (reviewed in Holtzman, 2004), and GFAP that exhibits reactive increases due to gliosis in neurodegenerative diseases. We observed a massive increase in ApoE (~4 fold) and clusterin levels (~3 fold) in spinal cord in transgenic mice expressing A30P-mutant α-synuclein (Fig. 2B). GFAP levels, in contrast, were increased only moderately (<2 fold). We also analyzed sciatic nerve because disruptions in axonal transport may contribute to neurodegenerative pathogenesis in PD and AD (reviewed in Roy et al., 2005), and observed an even more dramatic increase in ApoE in sciatic nerve (10-20 fold) than in spinal cord (~4 fold), but could not detect any clusterin or GFAP (Fig. 2C). In contrast to spinal cord or sciatic nerve, the ApoE and GFAP levels increased only moderately in brain (Fig. 2A), consistent with the primary focus of the neurodegeneration in spinal cord where the highest transgenic α-synuclein levels are observed. Importantly, A30P-mutant α-synuclein expressing mice that were asymptomatic had a significantly lower level of ApoE and clusterin than symptomatic mice; in fact, ApoE was not significantly increased in asymptomatic mice in either the

spinal cord or the sciatic nerve, suggesting that the ApoE increase reflects the disease process (Figs. 2B and C). To ensure that these patterns of proteins changes reflect an α-synuclein induced change and not a particular transgene-induced alteration, we additionally examined a second transgenic line expressing A30P-mutant α-synuclein, and a third transgenic line expressing A53T-mutant α-synuclein. In both additional transgenic lines that exhibit a similar neurodegeneration as our standard A30P-mutant α-synuclein expressing line we observed the same dramatic increase in ApoE levels in the spinal cord and sciatic nerve but not in brain (Figs. 2), thus independently confirming the specificity of these findings

3.3.3 Transgenic α-synuclein mice have an increased insoluble Amyloid β40-42 peptide

Increases in ApoE are characteristic of neuronal injury (Haasdijk et al., 2002). Since ApoE and Aβ-aggregates derived from APP are both linked to AD (Yu et al., 2000), we measured the levels of endogenous mouse $A\beta_{40}$ and $A\beta_{42}$ in the spinal cords of various transgenic mice, using APP KO mice as a negative control. We detected no major change in the levels of soluble $A\beta_{40}$ and $A\beta_{42}$, but a major increase (2-5 fold) in insoluble $A\beta_{40}$ and $A\beta_{42}$ that was specific for transgenic mice expressing human α-synuclein (Fig. 2D). We found increases in insoluble $A\beta_{40}$ and $A\beta_{42}$ in symptomatic transgenic mice that expressed A30P- or A53T-mutant α-synuclein, but not in mice with a comparable degree of overexpression of transgenic mouse α-synuclein, consistent with a lack of pathogenicity of mouse α-synuclein.

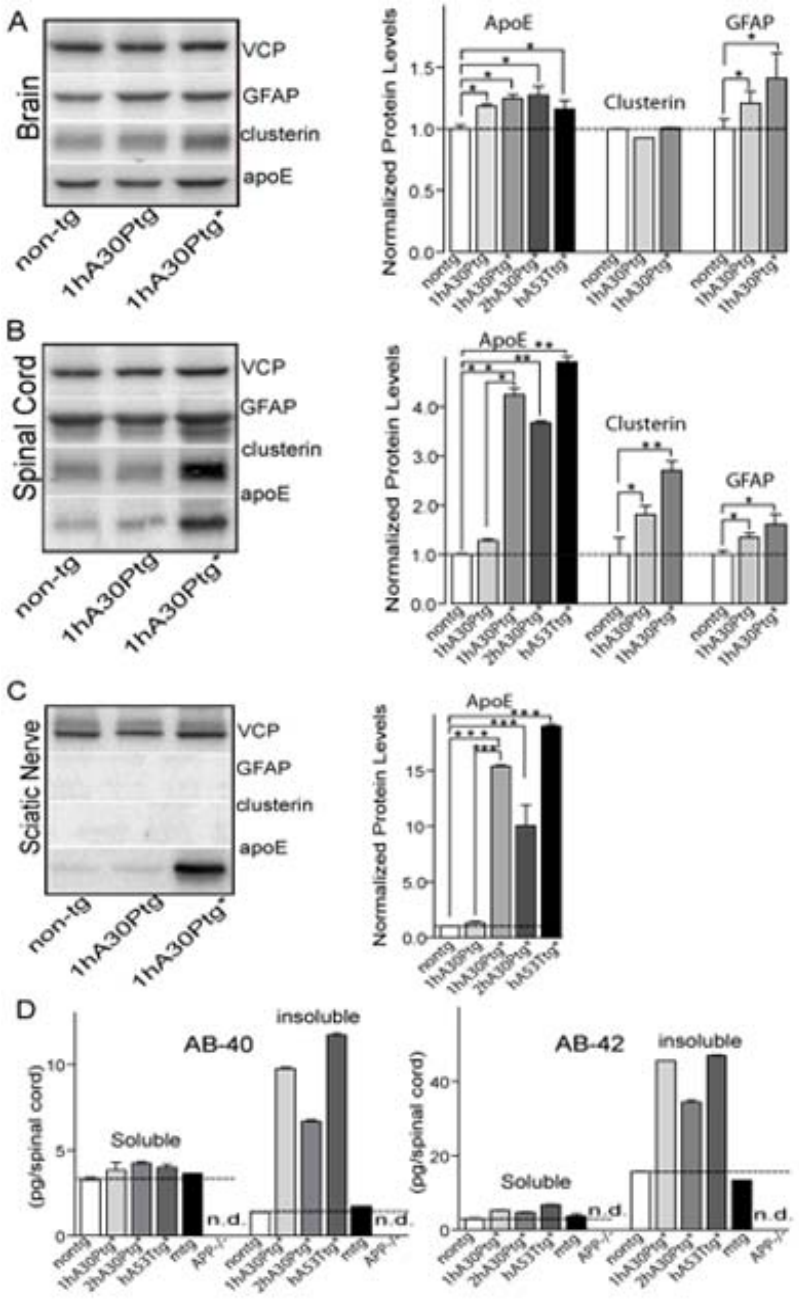

Figure 3.2 Effects of transgenic mutant α-synuclein on the levels of apoE, clusterin and Amyloid-β40-42 (A-C) Representative immunoblots of glial fibrillary acid protein (GFAP), clusterin and Apolipoprotein E (apoE) from brain, spinal cord and sciatic nerve homogenates from nontransgenic, nonsymptomatic A30P α-synuclein and symptomatic A30P α-synuclein transgenic mice. Graphs depict relative levels of GFAP, clusterin and apoE in nontransgenic and nonsymptomatic transgenic A30P α-synuclein (hA30ptg), two separate lines of symptomatic A30P α-synuclein transgenic (1hA30Ptg*,

2hA30Ptg*) and symptomatic A53T α-synuclein transgenic mouse (hA53ttg*). All proteins were normalized to nontransgenic age matched littermate 12-13 months; n=3. (D) Levels of Amyloid-β40-42 (Aβ) from soluble and insoluble spinal cord fractions determined by ELISA. All human α-synuclein transgenic mice were symptomatic normalized to nontransgenic aged match littermates mice 12-13 months; n=3. In addition, levels of Aβ40 and Aβ42 from murine α-synuclein transgenic mice (mtg) and amyloid precursor protein knockout mice (APP-/-); age of mice 15 months n=3.

3.3.4 ApoE deficient transgenic α-synuclien mice have a delayed onset and an increase in survival

The large changes in ApoE and Aβ levels in α-synuclein transgenic mice exhibiting neurodegeneration is consistent with two alternative hypotheses: 1. Intracellular α-synuclein activates a pathogenic extracellular signaling loop that involves ApoE and that contributes to neurodegeneration; 2. Intracellular α-synuclein causes neurodegeneration which leads to a secondary increase in ApoE and Aβ-peptides without a role in the pathogenic process itself. To differentiate between these hypotheses, we tested the effect of deleting ApoE on the neurodegeneration caused by transgenic α-synuclein overexpression.

Deletion of ApoE strongly delayed but did not abolish the neurodegeneration induced by transgenic α-synuclein (Figs. 3A1-A3). This effect was specific because the Wld allele which protects neurons from Wallerian degeneration (reviewed in Coleman, 2006) did not ameliorate the pathogenesis induced by transgenic α-synuclein (Fig. 3A4). The mice that contain transgenic α-synuclein but lack ApoE and still develop a phenotype develop the same loss of motor neurons as their ApoE-containing phenotype α-synuclein transgenic mice (Fig. 3B). Deletion of ApoE depresses the increase in

protein ubiquitination induced by A30P-mutant α-synuclein, but has little effect on the changes in proteasome subunits in phenotypic α-synuclein transgenic mice (Figs. 4A-D).

3.3.5 ApoE deficient decrease total ubiquitin and increases α-synuclein solubility

One characteristic of mutant human α-synuclein is that it selectively aggregates into SDS-insoluble inclusion bodies (Duda et al., 2000; McAllister et al., 2005)). We thus determined the effect of ApoE on α-synuclein solubility in symptomatic mice expressing A30P-mutant α-synuclein (Figs. 4E and 4F). Importantly, deletion of ApoE significantly increased the amount of soluble α-synuclein but decreased the amount of insoluble α-synuclein; most strikingly, α-synuclein aggregates in the SDS-insoluble fraction were significantly decreased in the ApoE-deficient mice. These data suggest that deletion of ApoE has direct effect on α-synuclein aggregation, which is presumably intracellular.

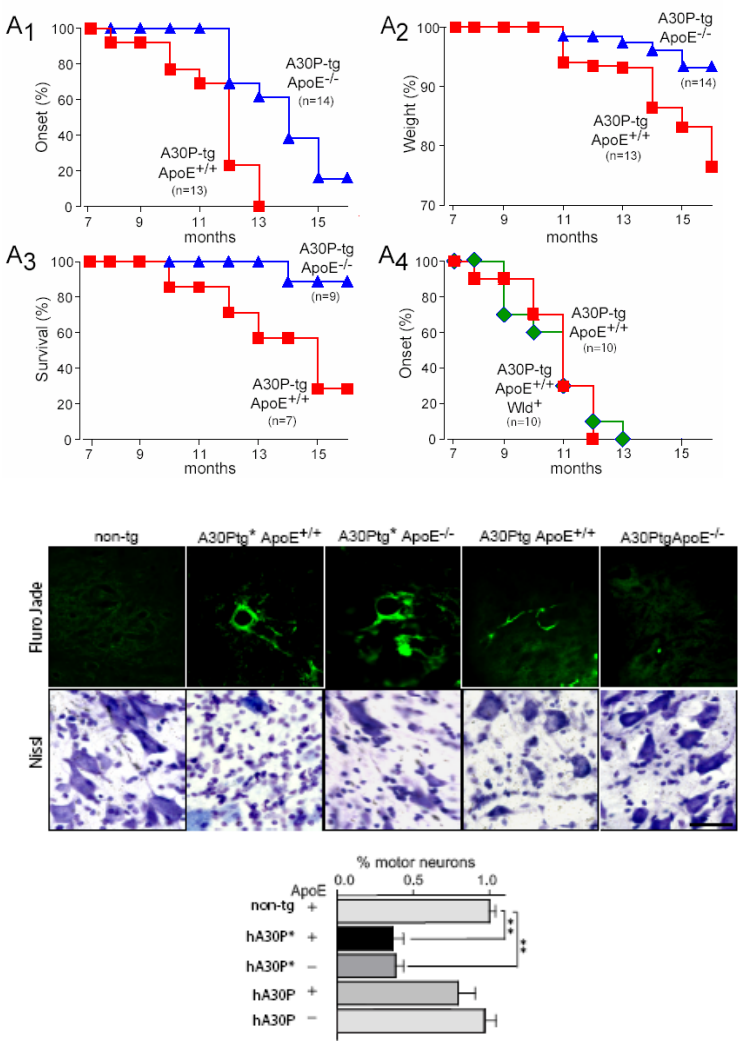

Figure 3.3 Effect of apoe deletion on disease onset, growth and survival of A30P transgenic synuclein mice.

A1) Age-of-onset of symptoms in transgenic mice expressing A30P α-synuclein with and without apoe. A2). Onset of symptoms of A30P transgenic mice breed to Wallerian degeneration slow mice (WLD). A3-4) Weight and survival of apoE wild type or apoe KO A30P transgenic mice as a function of age. B). Spinal cord sections staining for neurodegeneration (Fluro Jade B), and motor neurons (Nissl) from control non transgenic mice (non-tg), symptomatic A30P transgenic mice with and without apoE (A30P-tg apoE$^{+/+}$*, A30P-tg apoE $^{-/-}$*), and non symptomatic A30P transgenic mice with and without apoe (A30P-tg apoE$^{+/+}$, A30P-tg apoE $^{-/-}$); calibration bars =50um n=3. Graph depicts quantitation of motor neurons in Nissl-stained sections from nontransgenic control, symptomatic and nonsymptomatic A30P transgenic mice

expressing or lacking apoE. Genotypes are indicated at the bottom of the graph. Data shown are means ± SEMs asterisks indicate statistical significance at $p<0.01$; for B-D n=3; nonsymptomatic mice compared at 10 months and symptomatic mice ages 13-14 months.

3.3.6 ApoE deletion in α-synuclein transgenic mice display decreased Amyloid-β40-42 levels and deposition.

To explore the unexpected actions of extracellular ApoE on intracellular α-synuclein further, we also examined the effect of ApoE on the levels and aggregation of Aβ-peptides in the transgenic mice. Immunofluorescence labeling revealed that the increase in Aβ-peptides observed in A30P-mutant α-synuclein expressing mice (Fig. 2D) corresponds to a specific observation of Aβ-aggregates in spinal cord neurons (Fig. 5A). These aggregates were observed with two independent Aβ-antibodies, were absent from non-transgenic mice, and did not coincide with GFAP-positive structures. Quantitation showed that the Aβ-positive aggregates were significantly depressed, but not completely abolished, by deletion of ApoE (Fig. 5B). Consistent with this observation, measurements of $A\beta_{40}$ and $A\beta_{42}$ by ELISA revealed that the increase in both peptides was significantly depressed by deletion of ApoE (Fig. 5C). As shown above, the Aβ quantitations were specific because no detectable Aβ was detected in APP KO mice.

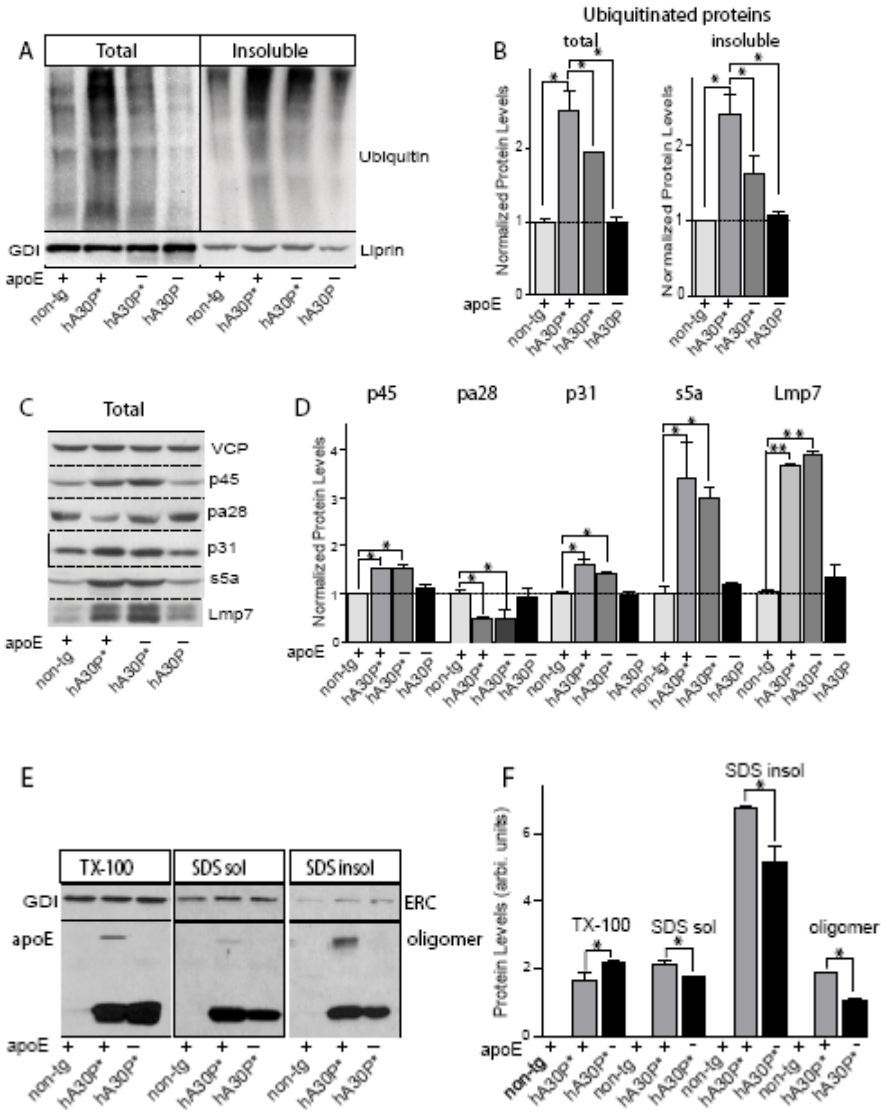

Figure 3.4. Deletion of apoe decreases ubiquitin and insoluble synuclein oligomers

A). Representative immunoblot of ubiquitin from total and insoluble fractions from control non transgenic mice (non-tg), symptomatic A30P transgenic mice with and without apoE (A30P-tg apoE$^{+/+}$*, A30P-tg apoE $^{-/-}$*), and non symptomatic A30P transgenic mice (A30P-tg apoE $^{-/-}$); age of mice 10-12 months n=3. B) Graphs depict relative levels of total and insoluble ubiquitin normalized to non-tg. C) Representative immunoblots of selected proteasome subunits and VCP. D) Graphs depict relative levels of the various proteasome subunits; age of mice 10-13 months, n=3. E) Representative immunoblot of synuclein

solubility from 1% triton (TX-100), RIPA buffer (SDS sol) and resultant insoluble pellet (SDS insol). F) Graphs depict arbutary protein quantification of synuclein solubility.

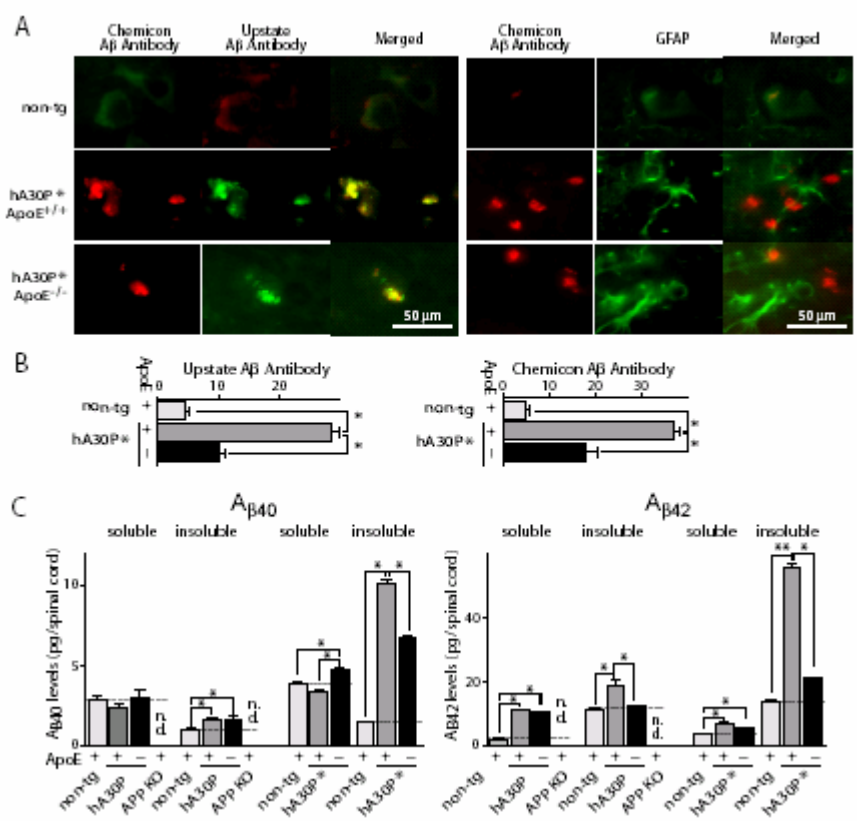

Figure 3.5. Deletion of apoE decreases Amyloid beta 40-42 in A30P transgenic mice. A) Immunofluorescense of nontransgenic control and A30P α-synuclein transgenic spinal cord sections with or without apoE illustrating Aβ42 deposits (sections stained with Aβ42 polyclonal (Chemicon AB5078P) Aβ42 monoclonal (Upstate #05-831) antibodies and GFAP antibody (Chemicon MAB360; calibration bar=0.1mm) B) Amyoid deposits quatification from two anti-Aβ42 antibodies determined by MetaMorph software (Molecular Devices) C) Qauntification of Amyloid-β40-42 (Aβ) from soluble and insoluble spinal cord fractions determined by ELISA. Genotypes are indicated at the bottom of the graphs. Nonsymptomatic mice are age matched littermates at 10 months, n=3. Symptomatic mice are aged match littermates 10-14 months n=6. APP KO mice age 15 months. Data shown are ± SEM *=P<0.05; **=P<0.01

3.4 Discussion

Accumulating evidence demonstrates that α-synuclein is neurotoxic, and that this neurotoxicity is exerted by misfolding and aggregation of α-synuclein. Using transgenic mice overexpressing α-synuclein mutants, we now show that the neurodegeneration induced by α-synuclein not only involves, as do other forms of neurodegeneration, activation of the UPS, but also a dramatic increase in the levels of ApoE and of insoluble, presumably aggregated endogenous mouse Aβ-peptides. To our knowledge, this is the first time that endoenous mouse Aβ was found to be involved in a neurodegenerative process. The linkage between α-synuclein, ApoE, and Aβ is mechanistically important because deletion of ApoE suppresses α-synuclein induced neurodegeneration, although it did not totally prevent it. The connection between intracellular α-synuclein and extracellular ApoE and Aβ is surprising considering the distinct subcellular locales of these proteins. It suggests an unanticipated general signaling loop in neurodegeneration that operates across the plasma membrane, and could be a fundamental mechanistic event that is shared by multiple types of neurodegeneration.

A potential mediator of this fundamental mechanism of neurodegeneration is ApoE. Previous studies have illustrated ApoE is not normally expressed in neurons in mouse brain, but can be dramatically increased in neurons in response to neuronal injury (Xu et al., 2006). ApoE is also increased in SOD transgenics mice and ablation of ApoE resulted in an increase in survival (Haasdijk et al., 2002). In humans, the E4 allele of ApoE is injurious for a number of neurodegenerative conditions, whereas there is no evidence that the other alleles of ApoE are (Mahley et al., 2006). Studies in transgenic animals have suggested mouse ApoE behaves in a similar fashion as to the humans E4

allele (Holtzman et al., 2000). The toxicity of ApoE is thought to be mediate by interaction with Aβ, altering its clearance and deposition (Bales et al., 1997; Ye et al., 2005). Mutant APP transgenic mice display elevated levels of mouse ApoE that correlated with the increased Aβ levels, whereas the lack of mouse ApoE dramatically reduced Aβ deposition (Bales et al., 1997; Kuo et al., 2000).

The evidence shown in the present study demonstrates that the pathogenic process involves the gradual elevation of ApoE in presymptomatic mice, to a dramatic increase in symptomatic mice that correlates with increased Aβ levels and deposition. Presymptomatic mice also display elevated levels of total ubiquitin which is suggestive of proteasomal impairment. Previous studies have demonstrated α-synuclein exist in two confirmations, a random coiled confirmation and a amphiopathic α-helical conformation upon binding to phospholipids believed to be important in its biological function. Overexpression of mutant α-synuclein in transgenic mice may lead to an abundance of unfolded α-synuclein that ultimately impairs proteasomal function resulting in neuronal injury and elevated levels of ApoE. These elevated levels thus influence the clearance and deposition of Aβ that may aid in pathogenesis of neuordegeneration.

Chapter 4

Transgenic α-Synuclein prevents neurodegeneration of Cysteine String Protein-α deficent mice

Abstract

Cysteine string protein alpha (CSPα) is a presynaptic protein containing a DNA-J domain distinctive to Hsp40-type cochaperaones. Genetic studies have demonstrated deletion of CSPα leads to early onset of neurodegeneration and lethality. Much like CSPα, α-synculein is a presynaptic protein in which mutations or gene triplications are linked to familial Parkinson's disease. In our current study, we demonstrate that overexpression of α-synuclein prevents neurodegeneration and lethality of CSPα deletion. Genetic analyses revealed triple knockouts of α and β synuclein and CSPα accelerated lethality and neurodegeneration. CSP-deficient mice developed retinal degeneration. Immunohistochemical analyses of transgenic α-synuclein- CSPα knockout mice revealed an absence of synuclein transgene expression in the retina, which coincides with the lack of rescue of retinal degeneration. Furthermore, mutant A30P synuclein transgenic mice were incapable of preventing CSP deletion lethality. Protein quantification of CSPα deficient mice identified decreased expression of HSC70, HSP70, endogenous synuclein and SNAP25. Transgenic synuclein reversed the HSC70 and HSP70 decreases but did not rescue the levels of SNAP25. Biochemical analyses demonstrated impaired SNARE complex assembly in CSP deficient mice. This impairment was reverted by transgenic synuclein and required phospholipid-binding by the synuclein variants. This study demonstrates a potential function for α-synuclein in preventing neurodegeneration via SNARE-mediated and/or phospholipid-binding pathways.

Introduction

Nerve cells communicate with other neurons by the release of neurotransmitters from their presynaptic terminals following the arrival of an action potential. Upon release, neurotransmitters activate either excitatory or inhibitory signaling cascades by binding to their receptors on the postsynaptic membrane. Presynaptic nerve terminals are specialized secretory machines capable of repeated rounds of neurotransmitter release (Katz 1969 Liverpool, united Kingdom: Liverpool University Press). Release is mediated by vesicle exocytosis, followed by rapid vesicle endocytosis enabling recycling of vesicles. SNARE proteins are the major components that regulate and facilitate vesicle fusion during exocytosis (Jahn, 2004; Sudhof, 2004). Core complexes that are formed by SNARE proteins assemble at the vesicle-membrane junction, which forces them into close proximity and initiates exocytosis.

In order for vesicles to be recycled, SNARE core complexes must be rapidly disassembled to allow endocytosis. To maintain continuous synaptic vesicle release and recycling at high frequency for extended periods of time, nerve terminals require protective mechanisms to prevent misfolding and aggregation of synaptic proteins. Chaperone proteins at presynpatic terminals may prevent this misfolding and aggregation of proteins that are detrimental to neuronal survival.

Synaptic vesicles contain cysteine-string protein-α (CSPα), a membrane associated protein that contains a DNA-J domain typical for Hsp40-type co-chaperones (Gundersen and Umbach, 1992; Zinsmaier et al., 1990). Initial studies suggested that CSPα may function in regulating the activity of presynaptic Ca^{2+} channels (Gundersen and Umbach, 1992), yet other studies report CSPα interacts with the SNARE proteins

syntaxin and synaptotagmin to regulate exocytosis (Nie et al., 1999). Perhaps a more suitable function for CSP is as a synaptic co-chaperone. *In vivo* and *in vitro* studies have demonstrated that CSPα assembles with HSC70 and a small tetratricopepetide repeat protein (SGT) that catalyzes chaperone activity (Tobaben et al., 2001). CSP localization to vesicles, in combination with this co-chaperone activity, suggests that it may function in preventing the accumulation of misfolded proteins and maintaining the integrity of synaptic transmission. Drosophila mutants for CSPα display progressive neurodegeneration, paralysis and premature lethality, supporting the notion that CSPα is a synaptic chaperone. In addition, electrophysiological studies revealed a heat-sensitive phenotype resulting in impaired synaptic transmission (Bronk et al., 2005). In mice, CSPα-deficient animals are relatively normal at birth, but subsequently develop a rapidly progressive neurodegeneration that alters synaptic transmission after 2-3 weeks, and kills the mice after 1-3 months (Fernandez-Chacon et al., 2004).

Much like CSPα, α-synucleins are presynaptic proteins associated with synaptic vesicles (Maroteaux et al., 1988). α-Synuclein has also been associated with neurodegeneration in which point mutations and gene triplication have been liked to familial PD (Golbe and Mouradian, 2004). Pathological studies suggest that misfolding of α-synuclein may result in formation of insoluble inclusions called Lewy bodies that are detrimental to neurons (Braak et al., 2003). In mice and in Drosophila, overexpression of human α-synuclein induces a progressive neurodegenerative phenotype with motor deficits. Extensive studies have demonstrated that α-synuclein plays a central role in neurodegenerative diseases, yet the normal function of α-synuclien remains largely elusive. Single and double α-, β-synuclein KO mice are viable and fertile and display

very subtle changes in dopamine levels with no changes in short- or long-term synaptic plasticity nor synaptic vesicle pools (Chandra et al., 2004). These studies demonstrate that synucleins do not participate in the basic execution of nerve terminal functions, but may perform a function that becomes important only under exceptional circumstances, such as injury. In the present study, we provide evidence for such a function. We demonstrate transgenic synuclein prevents the lethality of CSP KO mice. Double KOs of α-synuclein and CSPα accelerate the lethality of the single CSPα KO. We further demonstrate that CSPα deficient mice develop extensive neurodegeneration throughout the CNS, and transgenic synuclein prevents this neuronal cell death. In addition, we illustrate transgenic α-synuclein elicits its protective effects in a cell autonomous fashion via a phospholipid-binding mechanism. CSPα KO's displayed a loss of proper SNARE complexes, and transgenic α-synuclein partially reverted this defect. This study reveals a potential function of α-synuclein in protecting nerve terminals from abnormalities that result in cell death.

4.2 Methods

4.2.1 Generation, breeding and analysis of transgenic mice and KO mice.

Transgenic mice that express wildtype or mutant α-synucleins under the control of the Thy-1 promoter (Andra et al., 1996) were produced as described in Missler et al., 2003. For analysis of α-synuclein transgenics, littermate offspring from matings of transgenic mice with wildtype controls were observed for 16 months. Phenotypes were scored based on the ability of the mice to move normally on their hindlimbs, which are the first affected by neurodegeneration in these mice (Fernagut and Chesselet, 2004).

Double heterozygous α-synuclein transgenic/CSPα KO mice (Fernández-Chacón et al., 2004; $CSP^{+/-}Syn^{tg}$) were bred with $CSP^{+/-}$ mice to obtain littermate $CSP^{+/+}$, $CSP^{+/-}$, and $CSP^{-/-}$ mice that either express or lack an α-synuclein transgene (see Fig. 2A). All analyses of the effects of α-synuclein transgenes on CSPα KO mice were performed in littermates to exclude background effects. Heterozygous CSPα KO mice were crossed with α-/β-synuclein KO mice (Chandra et al., 2004) to produce α/β-synuclein double KO mice ($\alpha/\beta\text{-}Syn^{-/-}$) that are heterozygous for the CSPα KO, which were then crossed with each other to generate littermate $\alpha/\beta\text{-}Syn^{-/-}CSP^{+/+}$, $\alpha/\beta\text{-}Syn^{-/-}CSP^{+/-}$, and $\alpha/\beta\text{-}Sn^{-/-}CSP^{-/-}$ mice. For the weight and survival analyses of mice, offspring from heterozygous matings (Fig. 2A) were tagged 4-6 days after birth and weighed on alternate days until six weeks, after which measurements were conducted weekly until 3 months. For analyses of $\alpha/\beta\text{-}Syn^{-/-}CSP^{-/-}$ mice, animals were weighed at P8, P15, P20 and P22. Survival analyses were started at 10 days to avoid artifacts due to in experienced mothers.

4.2.2 Behavior analysis. The righting-up reflex is performed by forcing a mouse to lie on its left or right side (Fernández-Chacón et al., 2004). Force-plate actometry (Fowler et al., 2001) was performed with six-week old mice on a force plate (28 cm x 28 cm) for 6 min.

4.2.3 Morphological Studies. Immunofluorescence, FluoroJadeB, and α-bungarotoxin labeling experiments were performed on cryosections (for immunofluorescence and FluoroJadeB labeling experiments) or whole mounts (for NMJ analyses) essentially as described (Missler et al., 2003; Lin et al., 2001; Fornai et al.,

2005). NMJ bouton sizes were calculated with the ImageJ program using the particle analysis module.

4.2.4 Protein Biochemistry. 1. To determine the relative amounts of soluble vs. insoluble α-synuclein in transgenic mice, whole brain and spinal cord from 12-14 month old littermate mice were homogenized in PBS buffer, solubilized in 1% NP-40, 0.1% SDS, and 1% DOC (RIPA buffer), and centrifuged (71,000 xg for 45 min). Equal concentrations from each sample were analyzed by quantitative immunoblotting. 2. For quantitation of total brain proteins, homogenates from 6 week old littermate mice (unless stated otherwise) of the denoted genotypes were analyzed by quantitative immunoblotting using ^{125}I-labeled secondary antibodies and PhosphorImager detection (Molecular Dynamics) with GDP-dissociation inhibitor (GDI) and vasolin-containing protein (VCP) as internal standards (Rosahl, et al., 1995). 3. Brains from littermate mice (6 week old) were homogenized in 50 mM HEPES-NaOH pH 7.2, 100 mM NaCl, 4 mM EGTA, 1 mM DTT and 2 mM $MgCl_2$, extracted with 1% Triton X-100 for 2 h at 4 °C, and the solubilized proteins were precleared with protein A for 30 minutes at 37 °C. SNAREs were immunoprecipitated using polyclonal antibodies to syntaxin 1 (U6251, 30 µl/ml precleared supernatant) or synaptobrevin (P939, 30 µl) for 1 h at 37 °C, and analysis of co-immunoprecipitation of synaptic proteins was performed by quantitative immunoblotting using ^{125}I -labeled secondary antibodies.

Miscellaneous. All analyses were done blindly without knowledge of the mouse genotype. All data are presented as means ± SEM. All statistical analyses are based on a two-tailed t-test.

4.3 Results

4.3.1 Transgenic α-synuclein prevents the lethal neurodegeneration in CSPα KO mice

. To study the relationship between the late-onset neurodegeneration caused by transgenic α-synuclein (Chapter 2) and CSPα chaperone activity (Fernandez-Chacon et al., 2004), we crossed transgenic α-synuclein and CSPα KO mice, producing mice that are homozygous for the CSPα deletion and hemizygous for the α-synuclein transgene (Fig. 4.1D). In these breedings, all comparisons were made between littermate mice to limit potentially confounding background effects. Unexpectedly, we found that transgenic α-synuclein expression abolished the fulminant neurodegeneration observed in CSPα KO mice.

Homozygous CSPα KO mice appear to be comparatively normal at birth, but exhibit a relative loss of body weight after postnatal day P20, and begin to perish after 1 month; no CSPα KO mouse survived beyond 4 months of age (Fernandez-Chacon et al., 2004; Fig. 4.1E). Transgenic expression of human or mouse wildtype α-synuclein, or of human A53T-mutant α-synuclein, prevented the weight loss, and completely abolished the lethality of CSPα KO mice (Figs. 4.1D and E). In contrast, transgenic A30P-mutant human α-synuclein was unable to suppress the CSPα KO phenotype (Figs. 4.1D and 1E),

even though A30P-mutant α-synuclein was expressed at the same level and in the same pattern as wildtype human and mouse α-synuclein (Chapter 2 Fig. 1).

The neurodegeneration caused by the CSPα deficiency leads to gliosis that is visualized as increased expression of the astrocyte protein GFAP. The gliosis was also reversed by transgenic wildtype α-synuclein (Fig. 4.2A-B), whereas transgenic A30P-mutant α-synuclein again was inactive (data not shown). Deletion of CSPα results in neuronal cell death, as documented by selective staining of degenerated neurons with FluoroJadeB again; this phenotype was reversed by transgenic wildtype α-synuclein (Figure 4.3). In addition to its effects on central synapses, the deletion of CSPα causes a progressive deterioration of the neuromuscular junction (Fernandez-Chacon et al., 2004). This phenotype manifests at the light microscope level as a decrease in the average size of the neuromuscular junction (Fig. 4.2B), and again was fully reversed by transgenic wildtype α-synuclein (Fig. 4.2C).

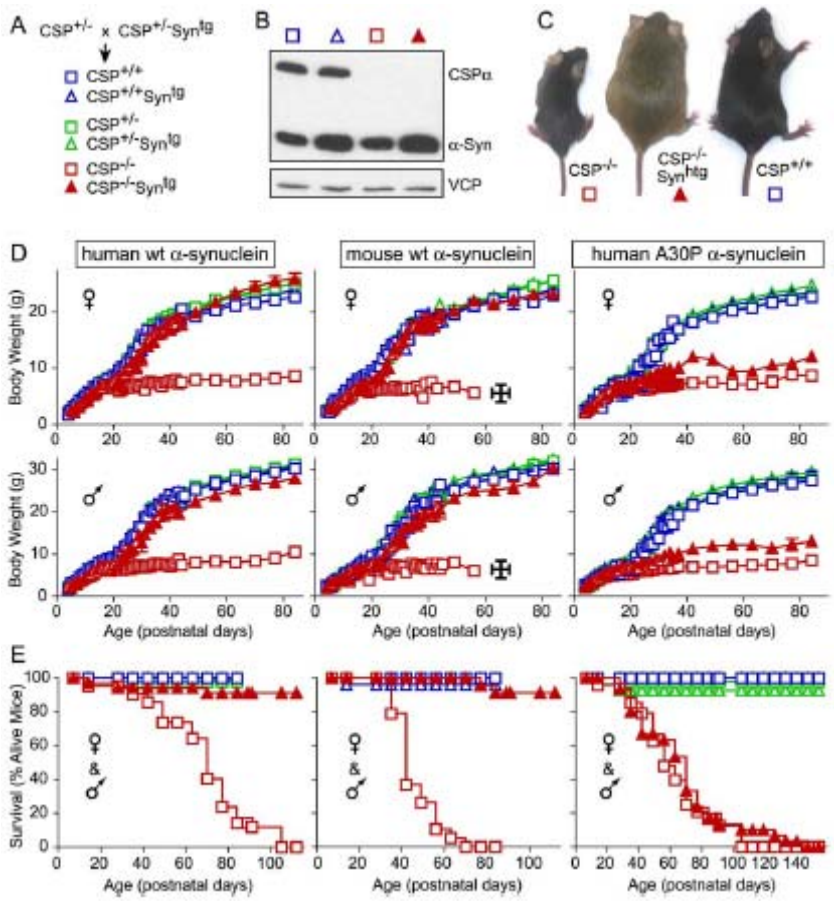

Figure 4.1 Effect of transgenic α-synuclein on the growth and survival CSPα KO mice. A. Breeding strategy. Heterozygous CSPα KO mice ($CSP^{+/-}$) were mated with heterozygous CSPα KO mice containing an α-synuclein transgene ($CSP^{+/-}Syn^{tg}$). **B.** Immunoblot analysis of brain proteins from wildtype and CSPα KO mice that express or lack transgenic human wildtype α-synuclein (20 µg protein/lane). Blots were probed with antibodies to CSPα, α-synuclein and VCP (as a loading control). **C.** Photograph of CSPα KO mice lacking ($CSP^{-/-}$) or containing the wildtype human α-synuclein transgene ($CSP^{-/-}Syn^{htg}$), and of a wildtype littermate control mouse ($CSP^{+/+}$) at 10 weeks. **D**. Body weights of littermate female (♀; top panels) and male mice (♂; bottom panels) as a function of age. **E.** Survival of female and male mice as a function of age (for the number of mice analyzed in panels D and E, see Suppl. Information). In D. and E., the left panels describe animals that express wildtype human α-synuclein, central panels wildtype mouse α-synuclein, and right panels A30P-mutant human α-synuclein. Results for

human A53T-mutant α-synuclein and an independent A30P mutant transgenic line are shown in Suppl. Figs. 3 and 4, respectively.

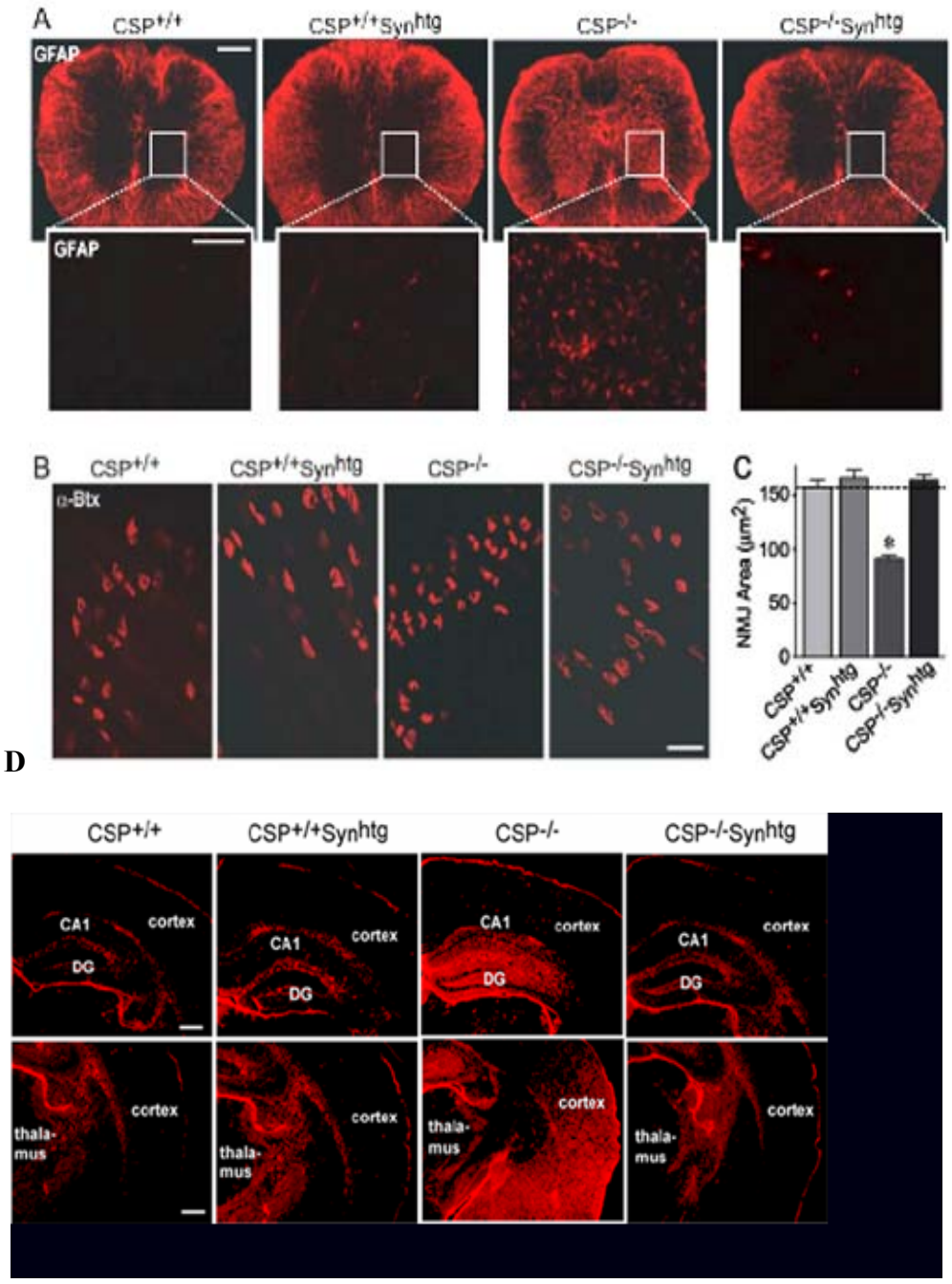

Figure 4.2 Transgenic α-synuclein reverses the neuropathology induced by deletion of CSPα. A. Spinal cord gliosis. Low- (top; calibration bar = 0.2 mm) and high-resolution micrographs (bottom; calibration bar = 0.1 mm) of transverse cervical spinal cord sections from mice with the indicated

genotypes. Sections were stained by immunofluorescence with GFAP antibodies. **B.** and **C.** Neuromuscular junction (NMJ) abnormalities. Panel B shows representative images of diaphragm NMJs labeled with fluorescent α-bungarotoxin (α-Btx; calibration bar = 50 μm). Panel C depicts quantitations of the NMJ size (n=3; asterisk indicates that the $CSP^{-/-}$ value is significantly different [$p<0.0001$] from all other three values, while these other values are not significantly different from each other). **D.** Images show immunofluorescence signals obtained by staining sagittal brain cryosections from littermate mice with antibodies to GFAP. The genotypes of the mice are indicated above the panels. The calibration bars (0.4 mm) shown on the left apply to all panels in the row. Abbreviations used: DG = dentate gyrus; CA1 = CA1 region of the hippocampus.

4.3.2 Transgenic α-synuclein rescues the motor impairment of CSPα-deficient mice.

CSPα KO mice exhibit a progressive loss of muscle strength and motor coordination (Fernandez-Chacon et al., 2004). To obtain a quantitative assessment of the motor behavior of CSPα KO mice, we employed a force actometer (Fowler et al., 2001). The actometer consists of a plate resting on four corner sensors that monitor the precise position and movement of a mouse on the plate. Non-transgenic and transgenic wildtype mice ($CSP^{+/+}$ and $CSP^{+/+}Syn^{htg}$) walk along the edges of the plate, exploring all four sides with even, smooth movements (Fig. 4.4A). In contrast, CSPα KO mice ($CSP^{-/-}$) usually stay in one sector of the force plate, and exhibit jagged and jerky movements that often appear to shoot out of the perimeter of the force plate. The jerky lines and phantom positions reflect ataxic behavior and sudden falls. Expression of wildtype α-synuclein completely reversed these locomotor deficits (Fig. 4.4A-B). Quantitation of the movement of mice on the force plate as the ataxia index (calculated as the area over which the mouse moves, divided by the net distance traveled; Fowler et al., 2001)

confirmed that CSPα KO mice are severely ataxic. Expression of wildtype human α-synuclein suppressed the ataxia of CSPα KO mice (Fig. 4.4D), whereas expression of A30P-mutant α-synuclein only partially improved the ataxia (Fig. 4.4C).

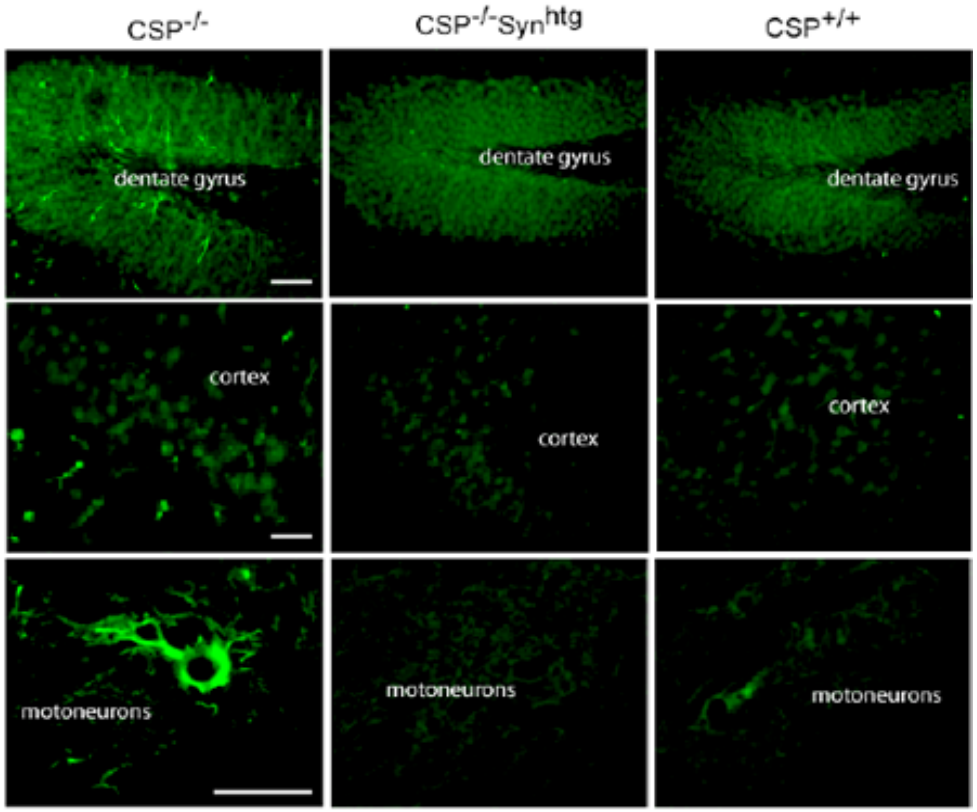

Figure 4.3 Transgenic α-synuclein prevents neurodegeneration enduced by deletion of CSPα

Staining of brain and spinal cord sections from CSPα KO mice lacking or containing the wildtype human α-synuclein transgene and from littermate wildtype control mice with Fluoro Jade B. Cryosections were stained as described previously. Calibration bars in the left panels (50 μm) apply to all panels in that particular row. Genotypes of the mice used for the sections are indicated on top (n=3; age of mice 40 days).

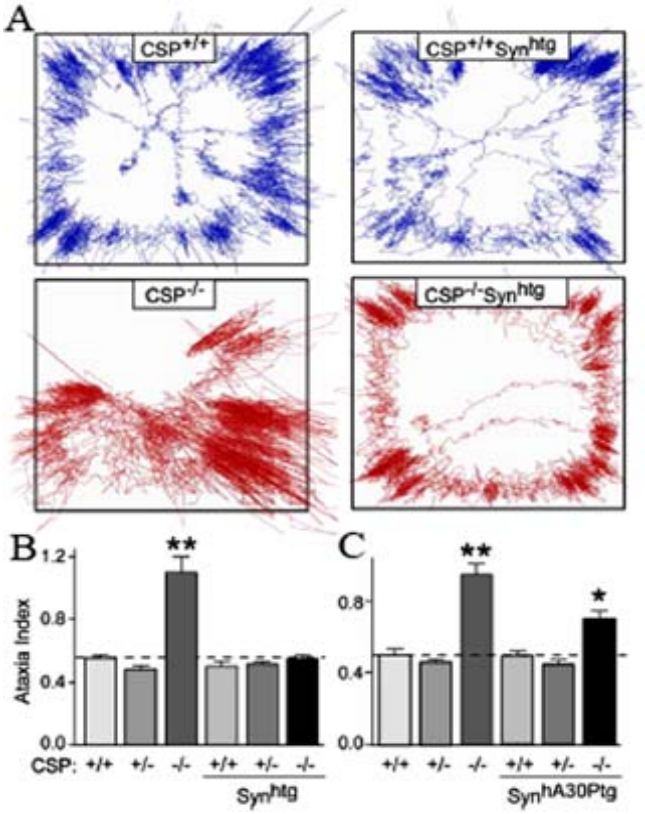

Figure 4.4 Rescue of the CSPα KO motor phenotype by transgenic wildtype but not A30P-mutant α-synuclein.

A. Locomotor traces of littermate wildtype ($CSP^{+/+}$; blue) and CSPα KO mice ($CSP^{-/-}$; red) without (left panels) or with the human wildtype α-synuclein transgene (right panels; Syn^{htg}). The traces display the position of the mouse as it explores the force plate (represented by the box) for a total of 6 min. Note that some of the traces outside the box in the CSPα KO mice were trimmed for the figure. **B** and **C,** ataxia indices of six week-old mice of the indicated genotypes expressing either wildtype (Syn^{htg} [D]) or A30P-mutant human α-synuclein ($Syn^{hA30Ptg}$ [E]). Ataxia indices were calculated from the force plate recordings as the area covered in 6 min divided by the net distance traveled (** = $p<0.001$ for all comparison except for the comparison of $CSP^{-/-}$ with $CSP^{-/-}Syn^{htgA30P}$ mice where $p<0.01$; * = $p<0.01$ for all comparisons).

4.3.3 Deletion of α- and β-synucleins exacerbates the CSPα KO phenotype.

The fact that overexpression of exogenous α-synuclein reverses the lethality of CSPα KO mice suggests that endogenous synucleins may normally dampen the progression of the CSPα KO phenotype. To test this hypothesis, we crossed CSPα KO mice with α/β-synuclein double KO mice that do not by themselves exhibit a major phenotype. We then measured the weight and survival of α/β-synuclein double KO mice with and without CSPα expression, and compared them to the weight and survival of the parental CSPα KO mice (Fig. 4.5).

Before P20, CSPα KO mice containing endogenous α- and β-synucleins were indistinguishable in weight from control mice (Fig. 4.1D). In contrast, at P8 the CSPα KO mice that additionally lack α- and β-synucleins already weighed less than their littermates, and at P20 were half the size of their CSPα wildtype littermates (Fig. 4.5A). Furthermore, triple KO mice lacking CSPα and α- and β-synucleins died precipitously within a few days after weaning (average life span = 23.8 ± 0.7 days; n=12), whereas CSPα KO mice expressing endogenous synucleins exhibited an extended mortality curve (average life span = 62.3 ± 2.2 days; n=102; Fig. 4.5B). Even when triple KO mice lacking CSPα and α- and β-synucleins were not weaned but left with their mothers, these mice did not survive for more than 30 days. Double KO mice that lacked both CSPα and α-synuclein but contained β-synuclein revealed an intermediate mortality curve (Fig. 4.5B), suggesting that α- and β-synuclein are redundant in this effect. The acceleration of the CSPα KO lethality by deletion of synucleins was accompanied by an accelerated neurodegeneration as evidenced by the absence of GFAP staining of gray matter in CSPα

KO mice containing endogenous synucleins at P10, but its presence in CSPα KO mice lacking synucleins (Fig.4.6).

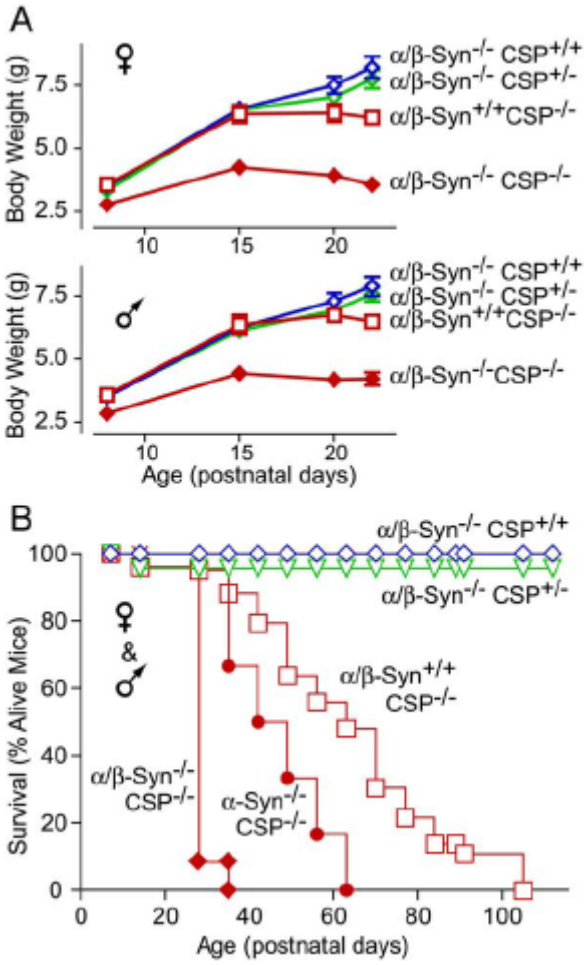

Figure 4.5 Deletion of endogenous α- and β-synucleins in mice exacerbates the CSPα KO phenotype. A. Body weights of pre-weaning female (♀) and male (♂) mice with various combinations of CSPα and α- and β-synuclein deletions as indicated (for precise breeding schemes and number of animals, see Suppl. Information). **B.** Postnatal survival of mice that lack α- and β-synucleins, and that additionally either contain or lack CSPα. The survival of the offspring from heterozygous matings of $CSP^{+/-}$α/β-$synuclein^{-/-}$ mice is plotted as a function of age; data for $CSP^{-/-}$ mice containing α- and β-

synucleins are pooled from the results of Fig. 2 (n = same as for panel A). In addition, survival analyses of α-synuclein KO mice lacking CSPα are shown (n=6).

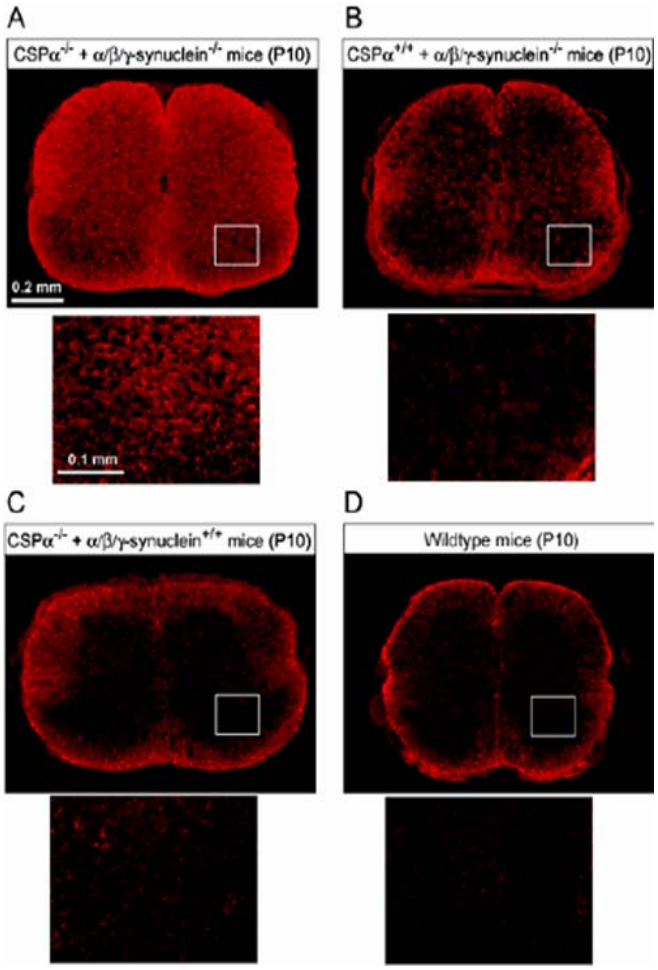

Figure 4.6 Deletion of synucleins accelerates neurodegeneration induced by CSPα-deficiency. Images depict transverse sections of spinal cord from mice of the indicated genotypes, analyzed at postnatal day P10 stained with an antibody to GFAP. The larger image depicts the spinal cord overview; a section from the gray matter (indicated by the white rectangle) is shown in the smaller image at higher magnification. The calibration bars in the lower left corners of the panels in A apply to all corresponding panels. Note that degeneration in CSP$^{-/-}$ α/β/γ-Syn$^{-/-}$ precedes onset of degeneration in CSP$^{-/-}$ α/β/γ-Syn$^{+/+}$ mice.

4.3.4 α-Synuclein and CSPα function independently but cell-autonomously.

Rescue of a lethal mouse mutant by a non-related transgene is highly unusual if not unique. This raises the question of whether transgenic α-synuclein acts cell-autonomously in collaboration with CSPα, or operates indirectly by a hormonal, developmental, or fortuitous action. In addressing this question, we noticed that retinal photoreceptor degeneration in CSPα KO mice is not rescued by the α-synuclein transgenes (Fig. 4.7). We then tested whether transgenic α-synuclein is expressed in photoreceptor cells, and found that it was only present in the inner plexiform synaptic layer, but not in photoreceptor synapses (Fig. 4.7).

The photoreceptor data indicate that α-synuclein only rescues neurodegeneration in cells that express the transgene, suggesting that α-synuclein may either function as a co-chaperone that cooperates with CSPα (and functionally replaces it in the KO mice), or act downstream of CSPα to prevent the deleterious consequences of the CSPα deficiency. To distinguish among these hypotheses, we first tested whether α-synuclein interacts with the chaperone Hsc70 and the co-chaperone SGT similar to CSPα (Tobaben et al., 2001). Using immunoprecipitation experiments, we found no interaction of synuclein with Hsc-70, SGT, or to CSPα (Fig 4.8). These results show that α-synuclein does not stably interact with the chaperones which constitute the enzymatically active CSPα-dependent chaperone complex. To test for a more transient interaction, we next explored the possibility that α-synuclein may replace CSPα in stimulating the Hsc70 ATPase activity, even if it does not stably interact with Hsc70. Although CSPα

powerfully stimulated Hsc70 ATPase activity, α-synuclein had no effect on the Hsc70 ATPase activity even in the presence of SGT, and furthermore did not alter the stimulation of Hsc70 ATPase by CSPα (Fig. 4.9). Finally, we explored the possiblity that α-synuclein may be a general chaperone that protects against all types of neurodegeneration by crossing transgenic α-synuclein mice with transgenic mice that express mutant superoxide dismutase (SOD), which causes a spinal cord neurodegeneration similar to that observed in CSPα KO mice (Gurney et al., 1994). However, we found that transgenic α-synuclein had no beneficial effect on this mutant (Fig 4.10). Together these data indicate that α-synuclein has no co-chaperone function, and rescues the CSPα-deficiency phenotype not by functionally replacing CSPα, but by preventing the deleterious consequences of its absence.

α-Synuclein is normally present in a random-coil conformation in solution, but avidly binds to phospholipid membranes in an α-helical conformation (Eliezer et al., 2001). Interestingly, A30P-mutant α-synuclein, but not the A53T mutant, is deficient in phospholipid binding (Lindquist, 2003), and does not fold properly on phospholipid surfaces as shown by circular dichroism spectroscopy and limited proteolysis (Fig. 4.11). Since transgenic A30P-mutant α-synuclein does not rescue the CSPα deficiency, this result indicates that α-synuclein needs to be in a fully phospholipid-bound conformation in order to function.

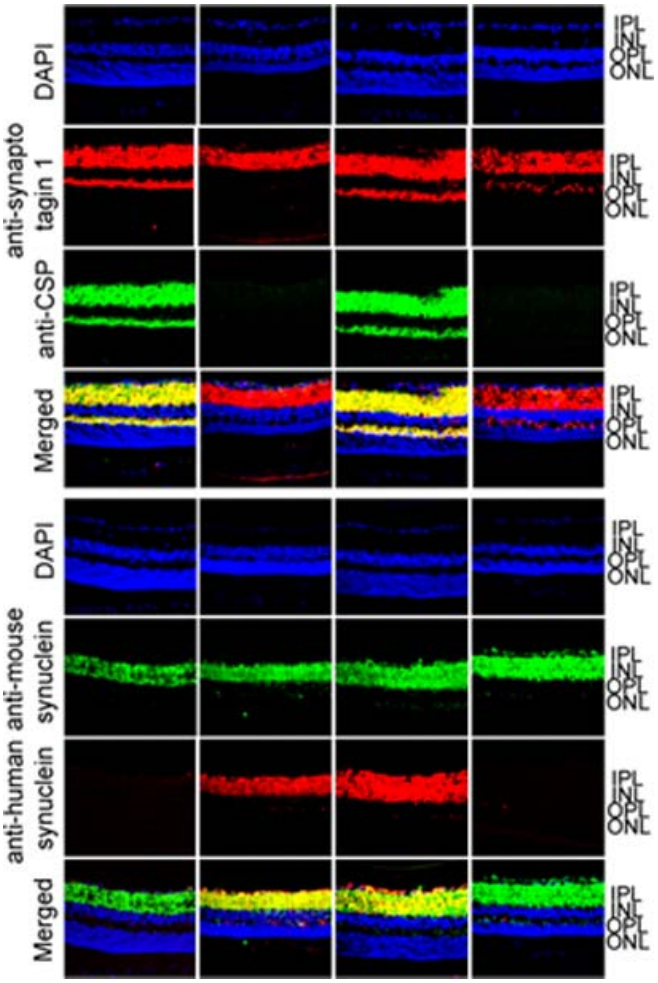

Figure 4.7 Analysis of retinal degeneration in CSPα-deficient mice containing or lacking transgenic expression of wildtype human α-synuclein. Immunofluorescence localization of CSPα and synaptotagmin 1 (top panels) and of transgenic and endogenous α-synuclein (bottom panels) in retina, compared to the location of nuclei visualized by DAPI staining. Different retina layers are indicated on the right (ONL = outer nuclear layer; OPL = outer plexiform synaptic layer; INL = inner nuclear layer; IPL = inner plexiform synaptic layer; asterisk labels the ONL that shrinks dramatically with photoreceptor degeneration). Note that the antibodies used for localization of human transgenic α-synuclein are human-specific, and that the OPL disintegrates in the CSPα KO as evidenced in the spotty synaptotagmin 1-labeling in the OPL whenever CSPα is deleted, independent of whether or not a human transgene is expressed in the IPL. Calibration bars in right lower panels (50 μm) applies to all panels.

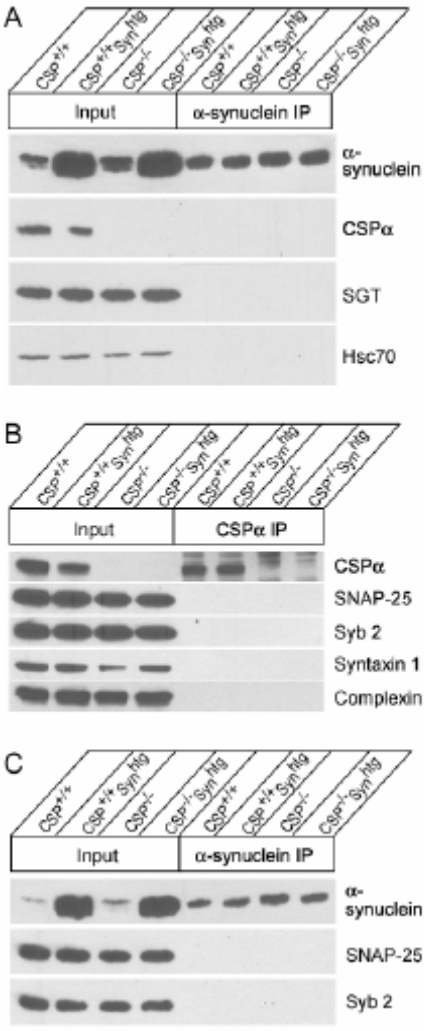

Figure 4.8 Immunoprecipitation analysis of α-synuclein and CSPα: lack of a direct interaction of α-synuclein with chaperone proteins, CSPα, or SNARE proteins. A. Proteins in brain homogenates from CSPα wildtype ($CSP^{+/+}$) and CSPα KO mice ($CSP^{-/-}$) lacking or containing transgenic expression of human wildtype α-synuclein (Syn^{htg}) were immunoprecipitated with an antibody to α-synuclein. Proteins in the input and immunoprecipitates (IP) were examined by immunoblotting with antibodies to α-synuclein, CSPα, SGT, and Hsc70. Positive bands were visualized by ECL. ***B***. Same as A., except that immunoprecipitations were performed with an antibody to CSPα, and immunoprecipitates were immunoblotted with antibodies to CSPα, SNAP-25, synaptobrevin/VAMP 2 (Syb 2), syntaxin 1, and complexins. ***C***. Same as A., except that immunoprecipitates were immunoblotted with antibodies to α-synuclein, SNAP-25, and synaptobrevin 2 (Syb 2).

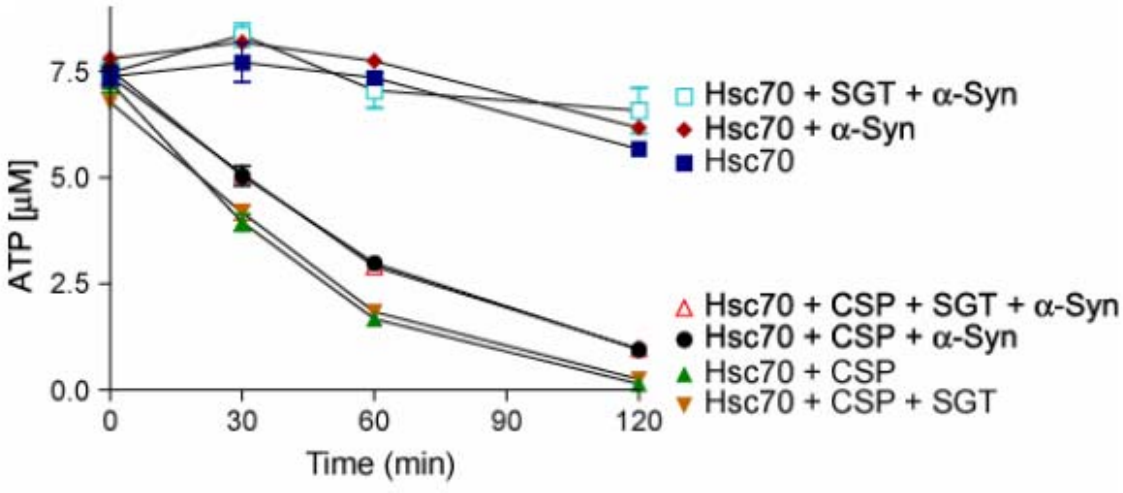

Figure 4.9 Analysis of enzymatic activity of α-synuclein: effect on the ATPase activity of the chaperone protein Hsc70. The ATPase activity of Hsc70 was measured using recombinant purified proteins. Hsc70 was incubated with the denoted proteins (0.5 μM Hsc70, CSP and SGT with 2 μM α-synuclein) at 37°C, and the reaction was stopped at the indicated times. ATP levels were measured using an ATP determination kit (Molecular Probes, Eugene OR). Figure shows data from a representative experiment that was repeated with similar results in 4 independent experiments. Note that α-synuclein neither stimulates Hsc70 ATPase activity, nor alters the stimulation of HSc70 ATPase activity by CSPα.

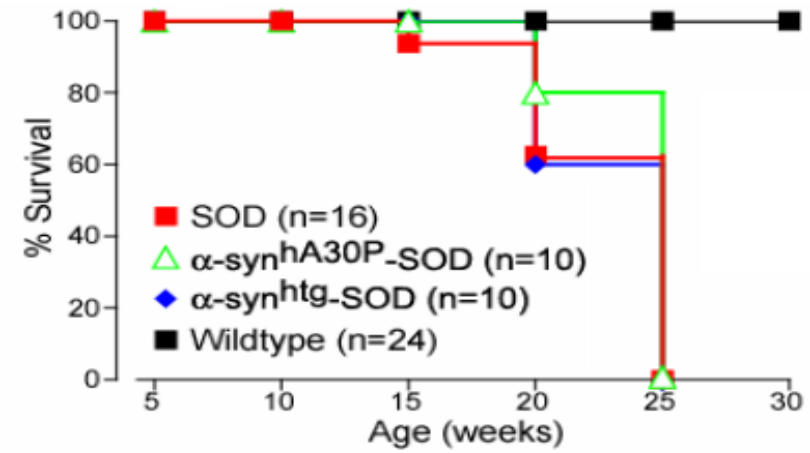

Figure 4.10 Effect of transgenic human α-synuclein transgene on the survival of mutant SOD transgenice mice. Heterozygous mutant SOD (superoxide dismutase) transgenic mice were purchased from Jackson Laboratories (Strain name: B6.Cg-Tg(SOD-G93A)1/Gur/J; JAX stock # 004435) and crossed to human wildtype and A30P-mutant α-synuclein transgenic mice. Survival of SOD transgenic mice containing or lacking the human wildtype and A30P-mutant α-synuclein transgene was analyzed. The percentage of mice that survive is plotted as a function of age. The numbers in the legend to the symbols on the right indicate the number of mice analyzed.

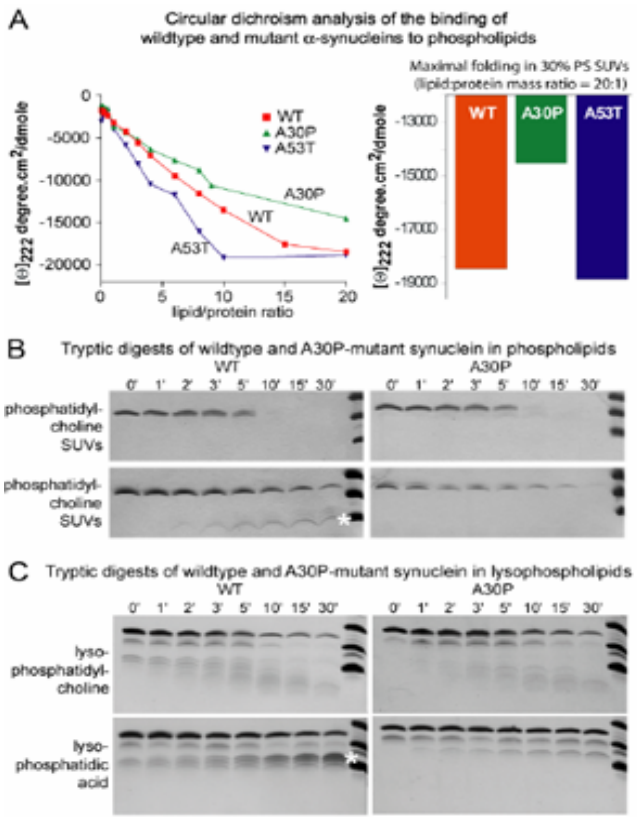

Figure 4.11 Analysis of the folding of wildtype (WT), A30P-mutant, and A53T-mutant α-synuclein induced by binding to liposomes. **A**. Recombinant synucleins were mixed with an increasing concentration of small unilamellar vesicles (SUVs) composed of 30% phosphatidylserine/70% phosphatidylcholine. In the left graph, the ellipticity of the protein was monitored at 222 nm as a function of the lipid/protein ratio. In the right bar diagram, the maximal ellipticity is shown at a saturating lipid/protein ratio (see Chandra, S., Chen, X., Rizo, J., Jahn, R., and Südhof. T.C. A broken α-helix in folded α-synuclein. *J. Biol. Chem.* **278**, 15313-15318 [2003]). ***B***, Digestion of human wildtype or A30P-mutant α -synuclein (40 µg) by trypsin (0.2%) in the presence of SUVs composed of only phosphatidylcholine which does not promote α-synuclein folding (*control*), or of 30% phosphatidylserine/70% phosphatidylcholine which does induce α-synuclein folding. Both vesicles were added at a lipid to protein ratio of 15:1. Digests were stopped at the indicated times, and proteins were analyzed on Tris-Tricine gels stained with Coomassie Blue. ***C,*** Digestion of human wildtype or A30P

mutant α-synuclein (40 μg) by trypsin (0.2%) in the presence of 500 μM lysophosphatidylcholine that does not bind α-synuclein (*control*) or lysophosphatidic acid that does bind α-synuclein. Digests were stopped at the indicated times, and proteins were analyzed on Tris-Tricine gels stained with Coomassie Blue. In panels B and C, the asterisks indicate N-terminal bands from wildtype but not A30P-mutant α-synuclein that are protected upon folding.

4.3.5 Impaired SNARE assembly in CSPα KO mice: effect of transgenic α-synuclein.

To search for potential CSPα target molecules that may be altered in CSPα KO mice, we performed quantitative immunoblotting experiments of brain proteins in CSPα KO and littermate control mice at P5, P10, and P40. Among more than 30 proteins examined, the levels of the plasma membrane SNARE protein SNAP-25 were selectively decreased by 30-40% at all ages examined (Figs. 4.12 and Tables 1 and 2). The levels of the related SNARE protein SNAP-23 were mildly decreased (~20%), but other synaptic SNARE proteins were not changed significantly. Hsc70, Hsp70 and α-synuclein also exhibited significant declines (~20-25%), consistent with a functional relation to CSPα. The decrease in SNAP-25 levels in CSPα KO mice was observed at the earliest time point examined (P5), prior to the onset of neurodegeneration (Fig. 4.12 Fig. 6). The decline in SNAP-25 is unique, as we have not observed a similar decline in more than 20 other lines of KO mice, including mice lacking the vesicular SNARE protein synaptobrevin/VAMP 2 that interacts with SNAP-25 (Schoch et al., 2001).

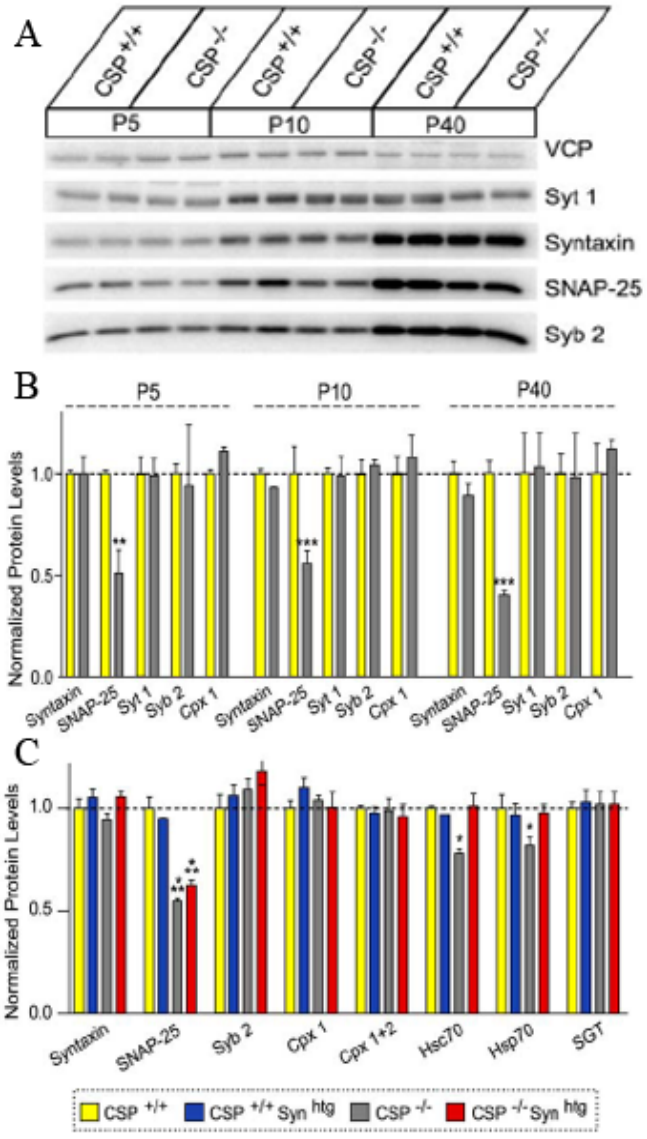

Figure 4.12 Effect of the CSPα deletion and of transgenic α-synuclein expression on SNARE protein levels. **A.** Representative immunoblots of selected brain proteins (abbreviations: Cpx, complexin; Syb 2 = synaptobrevin 2; Syp = synaptophysin; Syt 1 = synaptotagmin 1; VCP = vasolin-containing protein). **B.** Levels of selected brain proteins in wildtype (yellow bars) and CSPα KO mice (gray bars) at postnatal days P5, P10, and P40 (see Table 1 for a complete list of protein levels). C Levels of selected brain proteins in wildtype mice at P40 lacking (yellow bars) or containing transgenic human

wildtype α-synuclein (blue bars), or from CSPα KO mice lacking (gray bars) or containing transgenic human wildtype α-synuclein (*=P<0.05; **=P<0.01; ***=P<0.001 for panels B-C).

Transgenic overexpression of wildtype α-synuclein did not reverse the decline in SNAP-25 levels in CSPα KO mice (Fig. 4.12C and Table 2). Transgenic wildtype α-synucleins did, however, reverse the decline in Hsc70 and Hsp70 in the CSPα KO mice (Fig. 4.12C), although it did not cause a general upregulation of Hsc70, Hsp70, and other chaperones in wildtype mice (Table 2), consistent with the conclusion that α-synuclein is not a non-specific activator of chaperones (see above).

Specific decreases in SNAP25 protein levels suggest that α-synuclein and CSPα may alter SNARE function, but do not act via the same mechanism. This conclusion is supported by the finding that neither CSPα nor α-synuclein co-immunoprecipitated SNAP-25, synaptobrevin, or syntaxin (Figs. 4.8B and C). Although these results do not rule out a weak interaction between α-synuclein, CSPα, and SNARE proteins as yet, it does indicate that CSPα and α-synucleins are not stably bound to SNARE proteins.

During membrane fusion, SNAP-25 forms a complex with the plasma membrane SNARE protein syntaxin 1 and the synaptic vesicle SNARE protein synaptobrevin/VAMP (Söllner et al., 1993). To test whether, SNARE complex assembly is abnormal in CSPα KO mice, we immunoprecipitated SNARE complexes with antibodies to syntaxin 1 or synaptobrevin 2 and used ^{125}I-labeled secondary antibodies and phosphoimager detection to quantify the amount of SNARE proteins and of complexins which bind only to assembled SNARE complexes and thus report on the presence of SNARE complexes. We found a significant decrease in SNARE complexes

in CSPα KO mice; this decrease was reversed by transgenic wildtype α-synuclein but not by A30P-mutant α-synuclein (Figs. 4.13A-C). These results were confirmed with an independent assay in which SNARE complex assembly is measured on SDS-gels of non-boiled samples (Fig. 4.14). These data suggest that SNARE complex assembly is abnormal in the CSPα KO mice, but can be corrected by expression of wildtype human α-synuclein.

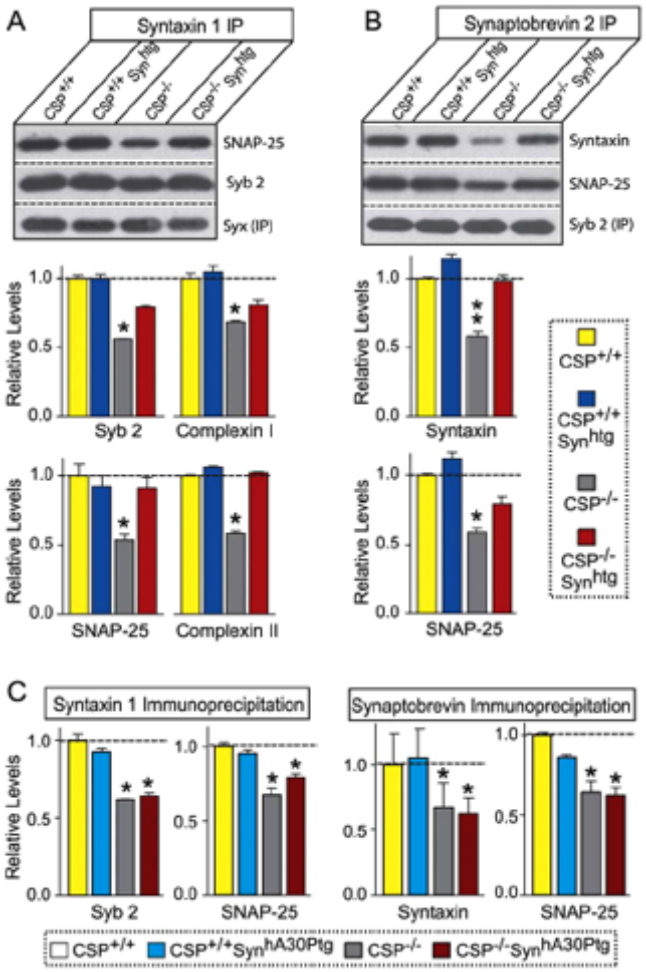

Figure 4.13 Effect of the deletion of CSPα and of transgenic α-synucleins on SNARE complex assembly

SNARE complexes were immunoprecipitated from brain homogenates from mice with the indicated genotypes, and analysed by quantitative immunoblotting. Immunoprecipitations were carried out with antibodies to syntaxin 1 (Syx; A and C) or synaptobrevin/VAMP (Syb; B and C). For A and B but not C, representative immunoblot are shown. Bar diagrams depict relative amounts of co-precipitated proteins (means ± SEMs; n=3). Asterisks indicate statistical significance: In A and B, * = $p<0.05$ and ** = $p<0.05$ for the $CSP^{-/-}$ sample compared all other samples (including the $CSP^{-/-}Syn^{htg}$ samples); in C, * = $p<0.05$ for the CSP-/- and the $CSP^{-/-}Syn^{hA30Ptg}$ samples compared to the $CSP^{+/+}$ and $CSP^{+/+}Syn^{htg}$ samples.

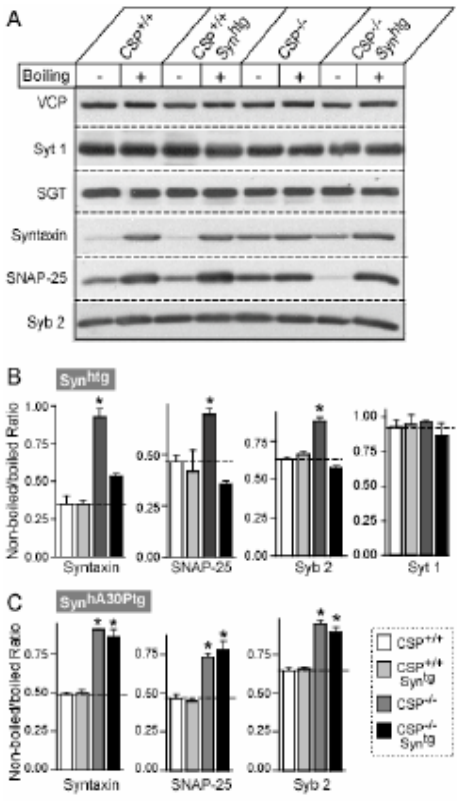

Figure 4.14 Effect of the CSPα deletion and of transgenic α-synuclein expression on SNARE complex assembly measured by comparison of monomeric SNARE proteins on SDS-polyacrylamide gels of boiled and non-boiled samples.

A. Representative immunoblots of brain proteins solubilized in Triton X-100 from mice with the indicated genotypes. Samples in SDS-sample buffer were subjected to SDS-PAGE and immunoblotting without or with boiling. Signals obtained with the antibodies indicated on the left (Syt 1 = synaptotagmin 1; SGT = small glutamine-rich tetratricopeptide repeat protein) were visualized by ECL. ***B.*** and ***C.*** Quantitation of the immunoblotting signals obtained for the monomers of the three synaptic SNARE proteins and of synaptotagmin 1 (Syt 1, used as a control). Signals were quantified with ^{125}I-labeled secondary antibodies and phosphorimager detection, and are depicted as the ratio of signal strength obtained without and with boiling of the samples prior to SDS-PAGE. The four genotypes described in panel A were analyzed for wildtype human α-synuclein transgenics (B), and the A30P-mutant (C). Data shown are means ± SEMs

(n=3 for B and C; asterisks indicate statistical significance at $p<0.01$ for the comparison of the $CSP^{-/-}$ sample lacking the transgene with the three other samples in B; and at $p<0.01$ for the two $CSP^{-/-}$ samples compared to the two $CSP^{+/+}$ samples in C.).

4.4 Discussion

In the present study, we demonstrate that transgenic α-synuclein overexpression prevents the lethality and neurodegeneration that is caused by deletion of CSPα (Figs. 4.1-4). Further, deletion of murine endogenous synucleins accelerated the neurodegeneration and death of CSPα-deficient mice (Fig. 4.5&4.6). In addition to transgenic human wild type synuclein, wildtype murine α-synuclein and A53T-mutant human α-synuclein were capable of rescuing the CSPα KO phenotype (Fig. 4.1). In contrast, the A30P-mutant human α-synuclein did not revert this lethal phenotype of CSPα deficiency. Deletion of CSPα led to a decrease in the SNARE protein SNAP-25 and to an impairment of SNARE complex assembly. Transgenic wildtype α-synuclein partially rescued the SNARE complex assembly defect, yet the A30P mutant failed to prevent this impairment of SNARE complexes (Fig. 4.13&4.14). Neither of the transgenes was capable of rescuing the decrease in SNAP-25 levels, suggesting that the SNAP25 decrease does not play a role in pathogenesis (Fig. 4.12). Together, these data suggest that α-synuclein acts to maintain the integrity of the presynaptic release machinery at nerve terminals following the introduction of insults such as CSPα deletion.

The differential activities of the A30P- and A53T-mutant α-synucleins in rescuing CSPα defieciency provide a clue to the mechanism of action of α-synuclein. Previous studies have illustrated that the A53T- mutation facilitates phospholipid binding in a

similar fashion to the wildtype by folding into an amiphipathic α-helical conformation, but the A30P-mutant α-synuclein has a reduced affinity for phospholipid binding and retains a random coil conformation. This suggests that α-synuclein functions are dependant on lipid binding and proper folding at the cytosol/membrane interface of synaptic vesicles. This observation is further supported by the fact that A30P transgenic α-synuclein is unable to rescue the lethal phenotype of CSPα KO mice, yet is capable of ameliorating their weight loss (Fig. 4.1).

This astonishing ability of α-synucleins to prevent the neurodegenerative lethal phenotype of CSP deficiency raises the concern of a genetic background effect. In addition, is this protective effect of α-synuclien a direct result of α-synuclein function or a secondary effect mediated by exogenous signal? Although, genetic background may favor the elimination of a lethal phenotype, it is highly unlikely to occur in multiple independent mouse lines, as were utilized in the present study. Observation of retinal degeneration in CSP KO mice helps to resolve the second question. CSP KO mice expressing transgenic α-synuclein displayed an absence of transgenic α-synuclein expression within photoreceptor neurons, and neurodegeneration was not prevented in these cells. This supports the hypothesis that α-synuclein prevention of neurodegeneration is cell autonomous in the context of CSP deficiency.

In addition, this study demonstrates that α-synuclein and CSPα functions are separate. *In vitro* experiments illustrate that α-synuclein is incapable of forming complexes with HSC70 and SGT (Fig.4.8). Moreover, transgenic α-synuclein was incapable of preventing neurodegeneration in a mouse model for Amyolaterial sclerosis commonly believed to be a misfolded protein disease (Fig.4.10). Although transgenic α-

synuclein is capable of reverting the decrease of HSC70 in CSPα KO mice, synuclein is incapable of rescuing the decrease in SNAP-25 levels (Fig.4.12). The decrease in SNAP-25 levels in CSPα KO mice appears not to contribute to pathogenesis, as this decline is present early in development before any detectable neurodegeneration (Fig. 4.6&4.12). This change in SNAP25 levels links CSP function in regulating SNAP-25 and associated proteins. This is further supported by evidence of SNARE protein complex impairment in CSP KO mice (Fig 4.13&4.14). Although the levels of SNAP-25 may not be related to the pathogenesis, SNARE complex impairment may result in the accumulation of misfolded SNARE proteins leading to inefficiency of synaptic transmission. This hypothesis is supported by the ability of synuclien to partially revert SNARE complex formations.

Synaptic transmission leads to synaptic vesicle exo- and endocytosis that is dependant on SNARE complex assembly and disassembly with each round producing disassembled unfolded SNARE proteins. Dedicating a presynaptic chaperone to prevent misfolding and to preserve SNARE protein functions may be essential for neuronal viability. We propose that deletion of CSPα causes the accumulation of toxic misfolded SNARE proteins that induce nerve terminal degeneration. α-synuclein serves to neutralize these toxic protein products in CSPα KO mice and avert their toxicity. The fact that α-synuclein needs to bind to membranes in order to prevent the lethal CSPα KO phenotype indicates that the toxic product of the CSPα KO harms neurons at the lipid/cytosol interface, again consistent with a role of SNARE proteins, which are thought to catalyze lipid fusion. In summary, our data suggest that decreases in CSPα and/or α-synuclein function may generally contribute to neurodegenerative diseases, and that

screening patients with neurodegenerative diseases for polymorphisms in the CSPα or synuclein genes may be rewarding.

Table 1

Total Protein Levels in Wild type and $CSP^{-/-}$ Brains

1A: Protein Levels at P5

Protein	$CSP^{+/+}$	$CSP^{-/-}$
SNAP-25 p913	**1.00 ± 0.212**	**0.60 ± 0.210**
SNAP25 71.1	**1.00 ± 0.015**	**0.51 ± 0.115**
Synaptotagmin 1	1.00 ± 0.083	0.99 ± 0.083
Syntaxin	1.00 ± 0.015	1.00 ± 0.084
Synaptobrevin 2	1.00 ± 0.047	0.94 ± 0.300
Munc18-1	1.00 ± 0.120	1.04 ± 0.056
α-SNAP	1.00 ± 0.016	0.95 ± 0.032
Complexin 1	1.00 ± 0.019	1.11 ± 0.020
Synapsin	1.00 ± 0.032	1.05 ± 0.042
Rab3a	1.00 ± 0.010	0.94 ± 0.031
NSF	1.00 ± 0.233	1.03 ± 0.038
Rab5a	1.00 ± 0.170	0.96 ± 0.010

1B: Protein Levels at P10

Protein	$CSP^{+/+}$	$CSP^{-/-}$
SNAP-25 p913	**1.00 ± 0.060**	**0.67 ± 0.125**
SNAP25 71.1	**1.00 ± 0.133**	**0.56 ± 0.061**
Synaptotagmin 1	1.00 ± 0.028	0.99 ± 0.095
Syntaxin	1.00 ± 0.022	0.93 ± 0.002
Synaptobrevin 2	1.00 ± 0.067	1.04 ± 0.027
Munc18-1	1.00 ± 0.280	0.90 ± 0.012
α SNAP	1.00 ± 0.007	0.95 ± 0.105
Complexin 1	1.00 ± 0.083	1.08 ± 0.015
Synapsin	1.00 ± 0.127	0.98 ± 0.120
Rab3a	1.00 ± 0.045	1.02 ± 0.008
NSF	1.00 ± 0.290	0.98 ± 0.181
Rab5a	1.00 ± 0.001	0.87 ± 0.120
SV2a	1.00 ± 0.163	1.11 ± 0.037
Velis	1.00 ± 0.115	0.93 ± 0.017
ERC	1.00 ± 0.104	1.05 ± 0.013
Intersectin	1.00 ± 0.075	0.92 ± 0.015
Synaptojanin 1	1.00 ± 0.083	0.96 ± 0.075
PSD95	1.00 ± 0.010	0.98 ± 0.208

1C: Protein Levels at P40

Protein	$CSP^{+/+}$	$CSP^{-/-}$
SNAP-25 p913	**1.00 ± 0.155**	**0.65 ± 0.035**
SNAP25 71.1	**1.00 ± 0.060**	**0.40 ± 0.025**
Synaptotagmin 1	1.00 ± 0.197	1.03 ± 0.170
Syntaxin	1.00 ± 0.057	0.89 ± 0.061
Synaptobrevin 2	1.00 ± 0.097	0.98 ± 0.216
Munc18-1	1.00 ± 0.012	0.98 ± 0.186
α-SNAP	1.00 ± 0.045	0.98 ± 0.080
Complexin 1	1.00 ± 0.144	1.12 ± 0.042
Synapsin	1.00 ± 0.018	1.07 ± 0.027
Rab3a	1.00 ± 0.035	0.92 ± 0.021
NSF	1.00 ± 0.207	0.90 ± 0.044
Rab5a	1.00 ± 0.021	0.93 ± 0.039
SV2a	1.00 ± 0.032	1.01 ± 0.142
Velis	1.00 ± 0.047	0.99 ± 0.023
ERC	1.00 ± 0.004	0.99 ± 0.003
Intersectin	1.00 ± 0.005	1.08 ± 0.014
Synaptojanin 1	1.00 ± 0.073	1.05 ± 0.019
PSD95	1.00 ± 0.035	1.15 ± 0.185
APP	1.00 ± 0.161	0.94 ± 0.113
Clathrin	1.00 ± 0.023	1.05 ± 0.066
Adaptin	1.00 ± 0.080	1.30 ± 0.081
Synaptogyrin 1	1.00 ± 0.086	0.84 ± 0.090
Dynamin	1.00 ± 0.132	0.86 ± 0.082
Hsc70	**1.00 ± 0.082**	**0.70 ± 0.028**
SV2b	1.00 ± 0.062	0.82 ± 0.030
Rabphilin	1.00 ± 0.043	0.87 ± 0.083

Table 2

Total Protein Levels in wildtype, $CSP^{-/-}$, and $CSP^{-/-}Syn^{htg}$ Brains

Proteins		$CSP^{+/+}$	$CSP^{+/+}$ Syn^{htg}	$CSP^{-/-}$	$CSP^{-/-}$ Syn^{htg}
Antibodies					
SNARES and associated proteins					
SNAP-25	***SMI81 1:10,000***	**1.00 ± 0.03**	**0.98 ± 0.14**	**0.66 ± 0.04**	**0.67 ± 0.17**
SNAP-25	***SMI81 1:20,000***	**1.00 ± 0.05**	**0.95 ± 0.00**	**0.56 ± 0.01**	**0.62 ± 0.03**
SNAP-25	***Cl 71.1***	**1.00 ± 0.10**	**0.99 ± 0.02**	**0.69 ± 0.09**	**0.63 ± 0.03**
SNAP-23	***P914***	**1.00 ± 0.07**	**0.90 ± 0.07**	**0.76 ± 0.02**	**0.81 ± 0.03**
Synaptobrevin2	*Cl 69.1*	1.00 ± 0.07	1.06 ± 0.05	1.09 ± 0.05	1.18 ± 0.05
Syntaxin	*U6251*	1.00 ± 0.05	1.05 ± 0.04	0.94 ± 0.03	1.06 ± 0.03
Syntaxin 1B	*4191*	1.00 ± 0.05	1.11 ± 0.07	1.05 ± 0.13	1.06 ± 0.07
Complexin I +II	*P942*	1.00 ± 0.01	0.98 ± 0.03	0.99 ± 0.06	0.96 ± 0.06
Complexin I	*L668*	1.00 ± 0.03	1.10 ± 0.05	1.04 ± 0.02	1.01 ± 0.07
NSF	*J372*	1.00 ± 0.01	1.06 ± 0.04	1.12 ± 0.02	1.14 ± 0.01
Munc18-1	*TL mono*	1.00 ± 0.05	1.05 ± 0.08	1.08 ± 0.05	1.06 ± 0.04
Munc18-1	*K329*	1.00 ± 0.04	1.08 ± 0.08	1.10 ± 0.05	1.09 ± 0.05
α–SNAP	*Cl 77.2*	1.00 ± 0.02	1.07 ± 0.03	1.01 ± 0.03	1.09 ± 0.05
Synaptotagmin 1	*Cl 41.1*	1.00 ± 0.00	1.06 ± 0.02	1.10 ± 0.02	1.10 ± 0.03
Synaptic proteins					
Rabphilin	*I734*	1.00 ± 0.09	1.11 ± 0.04	0.95 ± 0.04	1.02 ± 0.03
SCAMP	*R806*	1.00 ± 0.09	1.08 ± 0.02	0.96 ± 0.06	0.87 ± 0.03
Synapsin Ia+Ib	*E028*	1.00 ± 0.02	1.04 ± 0.08	1.13 ± 0.06	1.18 ± 0.09
Synapsin IIb	*E028*	1.00 ± 0.09	1.08 ± 0.04	1.09 ± 0.02	1.03 ± 0.04

Synaptogyrin I *P925*	1.00 ± 0.05	0.93 ± 0.01	0.94 ± 0.06	1.02 ± 0.02
Synaptojanin *T694*	1.00 ± 0.08	0.95 ± 0.13	1.02 ± 0.09	0.77 ± 0.08
Synaptophysin I *K831*	1.00 ± 0.07	1.20 ± 0.06	1.19 ± 0.00	1.13 ± 0.02
α-synuclein *T2270*	1.00 ± 0.07	5.98 ± 0.15	0.85 ± 0.06	4.91 ± 0.17
α-synuclein *Q698*	**1.00 ± 0.00**	**2.93 ± 0.11**	**0.79 ± 0.03**	**3.02 ± 0.24**
β-synuclein *Ab-1*	1.00 ± 0.03	0.73 ± 0.02	0.94 ± 0.04	0.76 ± 0.02
Tomosyn *U5403*	1.00 ± 0.04	1.04 ± 0.09	1.29 ± 0.05	1.33 ± 0.09
Veli *T813*	1.00 ± 0.04	1.03 ± 0.06	0.96 ± 0.02	1.16 ± 0.05
HSP's and chaperones				
Hsc70 *SPA816*	**1.00 ± 0.01**	**0.97 ± 0.00**	**0.78 ± 0.02**	**1.01 ± 0.06**
Hsp70 *SPA812*	**1.00 ± 0.07**	**0.98 ± 0.06**	**0.82 ± 0.04**	**0.98 ± 0.04**
SGT *Chat33*	1.00 ± 0.03	1.03 ± 0.06	1.02 ± 0.06	1.02 ± 0.06
Hsp40 *SPA400*	1.00 ± 0.07	1.13 ± 0.07	0.98 ± 0.11	0.85 ± 0.04
mito hsp *SPA801*	1.00 ± 0.05	1.01 ± 0.05	1.06 ± 0.04	1.20 ± 0.09
14-3-3 ε *TL mono*	1.00 ± 0.03	0.86 ± 0.08	0.83 ± 0.03	0.99 ± 0.02
Grp 78 *SPA826*	1.00 ± 0.14	1.06 ± 0.02	1.18 ± 0.23	0.89 ± 0.03

Chapter 5

Conclusion

Conclusion

Accumulating evidence for the pathogenesis of PD involves not only genetic mutations but several contributing factors that range from environmental effects, mitochondrial dysfunction, proteasomal dysfunction, and potentially other proteins (reviewed in introduction). Using transgenic mice that overexpress humans A30 and A53T mutant α-synuclein we illustrate that neurodegeneration resulting from the transgenic expression of α-synuclein not only involves α-synuclein but the UPS, elevated levels of ApoE, and insoluble Aβ peptides. In addition to the role of α-synuclein in the pathogenesis, we further reveal a potential function of α-synuclein in preventing neurodegeneration in CSPα deficient mice.

The identification of mutations in UCHL1, Parkin and α-synuclein, all support the hypothesis that misfolded proteins accumulate and impair proteasomal function leading to neuronal cell death (Dawson and Dawson, 2003; Giasson and Lee, 2003). In the asymptomatic α-synuclein transgenic mice, total ubiquitin levels are increased by 2 fold and the symptomatic transgenic mice reveal a 5 fold increase in total ubiquitin. This accumulation of ubiquitin may be indicative of proteasomal impairment in symptomatic transgenic mice. Studies based on the overexpression of α-synuclein in neuronal cultures have illustrated that specific UPS inhibitors bring about an accumulation of α-synuclein inclusions that results in neuronal cell death (Giasson and Lee, 2003; Nonaka et al., 2005). Transgenic expression of α-synuclein resulted in the accumulation of inclusions that are detectable with antibodies against α-synuclein, suggesting that the accumulation of misfolded α-synuclein may alter UPS function and contribute to PD pathogenesis. This accumulation of misfolded α-synuclein may result from its ability to form random

coiled conformations that have an increased propensity to form insoluble β-sheets. Symptomatic transgenic α-synuclein mice exhibited a decrease in α-synuclein solubility, consistent with the formation of misfolded and aggregated α-synuclein that may impair proteasome fuction.

Although ApoE has been strongly linked to Alzheimer's disease, its association with other neurodegenerative diseases including PD, amyotrophic lateral sclerosis, multiple sclerosis and Lewy body disorders has been reported recently (Mahley et al., 2006). The elevated levels of ApoE in α-synuclein transgenic mice is shown to be mechanistically important to the pathogenesis, since the deletion of ApoE delays α-synuclein neurodegeneration and increases overall survival. In SOD transgenic mice - a model for ALS, ApoE is also elevated and the ablation of ApoE resulted in an increase in survival (Haasdijk et al., 2002). The involvement of ApoE in α-synuclein-related neurodegeneration is the dramatically increased levels of ApoE in symptomatic mice, while the asymptomatic mice display a moderate increases in ApoE levels. The increase of ApoE in symptomatic transgenic mice correlates with an increase in insoluble Aβ peptides. *In vitro* and *in vivo* analyses suggest a general mechanism for ApoE in toxicity involves polymerization of Aβ peptide into amyloid filaments altering its clearance and deposition (Ma et al., 1994). Of the three major alleles of ApoE in humans, APOE4 (E4) has been shown to have the most severe toxic effects in neurodegenerative diseases. Much like E4, mouse ApoE has been shown to have similar pathobiological characteristics in transgenic animal models (Holtzman et al., 2000). Mutant APP transgenic mice displayed elevated levels of mouse ApoE that correlated with the increased Aβ levels, whereas the lack of mouse ApoE dramatically reduced Aβ peptide

deposition (Bales et al., 1997; Kuo et al., 2000). In patients with advance PD and AD, Aβ 42 levels are significantly decreased in CSF, consistent with impaired clearance and increased deposition (Mollenhauer et al., 2005; Mollenhauer et al., 2006; Sunderland et al., 2003). Involvement of Aβ in α-synuclein neurodegeneration has also been illustrated in double transgenic mice overexpressing mutant APP and α-synuclein that display an earlier onset for the α-synuclein degenerative disease with increased α-synuclein inclusions and oligomerization.

The link between ApoE, UPS and Aβ peptides in α-synuclein neurodegeneration suggest a common mechanistic pathway that may be shared in neurodegenerative diseases that include Alzheimer's disease, ALS, and Lewy body related diseases. The localization of extracellular Aβ and intracellular α-synuclein and ubiquitin implicate a signaling pathway that is mediated across the neuronal cell membrane. Perhaps the mediator of this signaling is ApoE that is localized both intracellular and extracellularly. This possibility raises several questions including, what receptors for ApoE are involved, does ApoE affect α-synuclein oligomerization directly as implicated in Aβ, or is ApoE elevated levels impairing proteasomal function?

The ability of α-synuclein to cause neurodegeneration is in direct contrast to its ability to prevent neurodegeneration in CSPα deficient mice. This remarkable finding has led to a potential function of α-synuclein that becomes apparent only under strenuous circumstances. CSPα is a co-chaperone protein that may aid in the refolding of SNARE and SNARE associated proteins and has proven to be essential for proper integrity of synaptic transmission and survival. CSPα deficient mice demonstrate a 50% decrease in SNAP25 protein levels prior to onset of neurodegeneration, suggesting decreased levels

of SNAP25 are not pathogenic. While the SNAP25 levels may not aid in the neurodegenerative process, they may suggest a potential function for CSPα in regulation of SNAP25 proteins levels. Transgenic expression of α-synuclein was incapable of rescuing the levels of SNAP25, separating the function of CSPα from α-synuclein.

Consistent with CSPα involvement in synaptic transmission, CSPα deficient mice exhibit impaired SNARE complex assembly that is partially rescued by human wildtype α-synuclein and human A53T mutant α-synuclein. In contrast, A30P mutant α-synuclein was incapable of rescuing the impairment of SNARE complexes and the lethality of CSPα deficiency. This discrepancy between the two mutations in their ability to prevent neurodegeneration may be due to the inability of the A30P α-synuclein to bind phospholipids. In the absence of phospholipids α-synuclein forms a random coil conformation, upon binding α-synuclein folds in amphiopathic α-helixes. This inability of the A30P α-synuclein to bind phospholipids properly suggests, α-synuclein function is dependent on phospholipids binding and proper folding.

Several studies have implicated a potential function for α-synuclein; in the present study we demonstrate α-synuclein function potentially targets SNARE complexes or SNARE associated proteins. Much like CSPα, α-synuclein may aid in the prevention of toxic misfolded SNARE complexes that impair synaptic function. The protective effect of α-synuclein is further illustrated to be specific for CSPα deficient and raises the possibility that a loss of function in α-synuclein and CSPα maybe important in neurodegenerative diseases.

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VITAE

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