The interplay between α-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies

Dissertation

In partial fulfillment of the requirements

for the award of the degree

"Doctor rerum naturalium" (Dr. rer. nat.)

Division of Mathematics and Natural Sciences

of the Georg-August-Universität Göttingen,

Faculty of Biology

within the doctoral program Molecular Physiology of the Brain

of the Georg-August University School of Science (GAUSS)

submitted by

Sibylle Elisabeth Eisbach

from Wickede (Ruhr), Germany

Göttingen 2015

Thesis Committee

Prof. Dr. Tiago Outeiro (1st Reviewer)

Neurodegeneration and Restorative Research, University Medical Center, Göttingen

Prof. Dr. Markus Zweckstetter (2nd Reviewer)

Max Planck Institute for Biophysical Chemistry, Göttingen

Dr. Sebastian Kügler

Neurology, AG Viral Vectors, University Medical Center, Göttingen

Extended Thesis Committee Members

Prof. Dr. Mikael Simons

Max Planck Institute for Experimental Medicine, Göttingen

Prof. Dr. Thomas Dresbach

Anatomy and Embryology, Göttingen University Medical School Center of Anatomy, Göttingen

Camin Dean, PhD

Trans-synaptic Signaling, European Neuroscience Institute, Göttingen

Day of oral examination: March 4, 2015

For everything there is a season,
and a time for every activity under heaven:
A time to be born and a time to die,
A time to plant and a time to uproot what was planted,
A time to kill and a time to heal,
A time to tear down and a time to build up,
A time to weep and a time to laugh,
A time to mourn and a time to dance,
A time to cast away stones and a time to gather stones together,
A time to embrace and a time to turn away,
A time to search and a time to give up searching,
A time to keep and a time to throw away,
A time to tear and a time to mend,
A time to be silent and a time to speak,
A time to love and a time to hate,
A time for war and a time for peace.

Ecclesiastes 3:1-8

Affidavit

I hereby declare that I have written this thesis entitled "The interplay between α-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies" independently and with no other sources and aids other than those quoted. This thesis has not been submitted elsewhere for any academic degree.

Sibylle Elisabeth Eisbach

Göttingen, January 2015

Disclaimer

Sections of Chapter 1 "Introduction" were published in an abbreviated version as a review article in the journal *Journal of Molecular Medicine entitled* "alpha-Synuclein and intracellular trafficking: impact on the spreading of Parkinson's disease pathology." by Sibylle E. Eisbach and Tiago F. Outeiro (Eisbach SE, Outeiro TF (2013) alpha-Synuclein and intracellular trafficking: impact on the spreading of Parkinson's disease pathology. J Mol Med (Berl) 91:693–703.) These sections include "Parkinson's Disease", "ASYN, a central player in PD", "ASYN and synaptic vesicles", "Rab GTPases", "ASYN and Rab GTPases" and "Spreading of ASYN pathology between cells" and are reproduced in this document in an altered version.

Figure 6 C and D as well as Figure 7 (altered) were previously published in the journal *Neurobiology of Disease* as part of a research article entitled "α-Synuclein interacts with the switch region of Rab8a in a Ser129 phosphorylation-dependent manner." by Guowei Yin, Tomas Lopes da Fonseca, Sibylle E. Eisbach, Ane Martín Anduaga, Carlo Breda, Maria L. Orcellet, Éva M. Szegő, Patricia Guerreiro, Diana F. Lázaro, Gerhard H. Braus, Claudio O. Fernandez, Christian Griesinger, Stefan Becker, Roger S. Goody, Aymelt Itzen, Flaviano Giorgini, Tiago D. Outeiro, Markus Zweckstetter (Yin G, Lopes da Fonseca T, Eisbach SE, Anduaga AM, Breda C, Orcellet ML, Szegő ÉM, Guerreiro P, Lázaro DF, Braus GH, Fernandez CO, Griesinger C, Becker S, Goody RS, Itzen A, Giorgini F, Outeiro TF, Zweckstetter M, Szegő EM (2014) α-Synuclein interacts with the switch region of Rab8a in a Ser129 phosphorylation-dependent manner. Neurobiol Dis 70C:149–161.)

Summary

Ageing related diseases become of ever more significance with the demographic shift currently prevalent in society. Dementia and movement disorders impact individuals and families alike, as the progressive decline in cognitive and physical abilities takes a toll on quality of life and an increasing strain on caretakers. Parkinson's disease (PD) is a neurodegenerative disorder that presents with movement-related symptoms due to degenerative processes in the midbrain, and that later can progress into changes in mood and behaviour, depression and dementia. PD primarily affects aged individuals, but genes of diverse cellular functions have been identified whose mutation can lead to an early—and even juvenile—onset of the disease. One major player in PD pathogenesis is α-Synuclein (ASYN), a small protein that has been identified as main component of PD-related protein depositions. The cellular function of ASYN is still unknown, but mutation and overexpression leads to toxic gain of function and a link to PD pathogenesis is therefore indisputable. Studies have shown that ASYN pathology interferes with vesicle trafficking and assembly of the transport machinery. Genetic screens aimed at identifying modulators of ASYN toxicity identified gene clusters involved in vesicular trafficking. Likewise, studies in yeast could show that toxic levels of ASYN disrupt several steps of the protein transport machinery, most notably the ER-to-Golgi trafficking step that is crucial for correct post translational modification of several proteins. Further, ASYN pathology drastically interferes with the homeostasis of Rab GTPases, a family of proteins involved with vesicle trafficking, and it has been demonstrated that members of this family can alleviate ASYN toxicity. Here we show in a comprehensive screen of Rab GTPases with a mammalian cell model of ASYN inclusion formation that ASYN pathology leads to perturbation of Rab-related trafficking steps on a large scale. We identify two different endosomal pathways dysregulated upon appearance of intracellular protein depositions: the endosomal-lysosomal pathway which includes the early endosome, and the trans-Golgi network (TGN) recycling pathway. The small Rab GTPases Rab5A, Rab7 and Rab8A have fundamental impact on ASYN inclusion formation, secretion and toxicity. We demonstrate that Rab8A modulates ASYN inclusion formation and acts protective from ASYN mediated toxicity in our cellular model. Early endosomal Rab5A mislocalises upon ASYN inclusion formation, while lysosomal Rab7 increases ASYN inclusion formation, but fails to colocalize with them. We further use size exclusion chromatography (SEC) and enzyme linked immunosorbent assay (ELISA) to show that depending on their activity state, Rab5A and Rab7 increase

ASYN particulate size and modulate secretion. Finally evaluating an animal model overexpressing human ASYN in a pan-neuronal manner we demonstrate upregulation of the lysosomal Rab7 and the protease Cathepsin D (CatD) in brain regions responsible for movement, motivation and memory formation. Our work both in mammalian cell culture as well as transgenic animals suggest that ASYN pathology impacts endosomal trafficking pathways, but also demonstrate the ability of proteins associated with the endosomal transport system to modulate ASYN associated toxicity. Thereby, anomalies in the trafficking machinery associated with endosomes caused by ASYN dysregulation might be contributors to PD pathogenesis.

Zusammenfassung

Mit fortschreitendem Durchschnittsalter der Bevölkerung gewinnen altersbedingte Krankheiten immer mehr an Signifikanz. Demenz und Einschränkungen der Beweglichkeit wirken sich auf Individuen sowie auf Familien aus, da die progressive Abnahme kognitiver und physischer Fähigkeiten ihren Tribut von der Lebensqualität Betroffener sowie den Pflegenden fordert. Morbus Parkinson (PD) ist eine neurodegenerative Erkrankung, welche sich durch Symptome des Bewegungsapparates äußert, bedingt durch degenerative Prozesse im Mittelhirn, und welche mit Veränderungen des Gemütszustandes, Verhaltens sowie Depression und Demenz fortschreiten können. PD betrifft in der Regel ältere Personen, jedoch wurden Gene verschiedener zellulärer Funktionen identifiziert, deren Mutation zu einer frühen oder gar juvenilen Ausprägung der Krankheit führen kann. Ein Hauptakteur in PD ist α-Synuclein (ASYN), ein kleines Protein welches in PD-typischen Proteinablagerungen gefunden wurde. Die zelluläre Funktion von ASYN ist immernoch unbekannt, Mutation oder Überexpression jedoch können zu einem hypermorphen Phänotyp führen und die Verbindung zu PD ist daher unumstritten. Studien haben gezeigt, dass ASYN mit Proteintransportwegen und der Aufstellung der Transportmaschinerie interferiert. Genetische Rasterstudien identifizierten Modulatoren von ASYN-Toxizität in Genclustern des Vesikeltransports. Ebenso konnten Studien in Hefe zeigen, dass Überexpression von ASYN diverse Transportwege stört, besonders zu beachten ist hier der ER-zu-Golgi Transportweg, welcher kritisch für Posttranslationale Modifikationen verschiedener Proteine ist. Des Weiteren greift ASYN-Pathologie störend in die Homöostase von Rab GTPasen ein, eine Proteinfamilie involviert in Vesikeltransport, manche deren Mitglieder ASYN-Toxizität reduzieren können. In dieser Studie zeigen wir in einer Rasteruntersuchung mit Rab GTPasen in einem Säugerzellmodell von ASYN-Proteinanreichungen, dass die ASYN-Pathologe zu einer weitreichenden Störung von Rab GTPase assoziierten Transportwegen führt. Wir identifizieren zwei unterschiedliche endosomale Stoffwechselwege welche beim Auftreten von ASYN-Proteinablagerungen fehlreguliert werden: der endosomale-lysosomale-Proteintransportweg welcher das frühe Endosom beinhaltet, sowie den trans-Golgi Netzwerk (TGN) Transportweg. Die kleinen Rab GTPasen Rab5A, Rab7 und Rab8A haben fundamentale Auswirkungen auf die Formation von ASYN-Proteinansammlungen, Sekretion und Toxizität. Wir zeigen dass Rab8A in der Lage ist ASYN-Proteinansammlungen zu modulieren und agiert protektiv in Bezug auf zelluläre Toxizitätslevel in unserem Modell. Rab5A, ein Protein des frühen

Endosoms, fehllokalisiert mit Formation der Ablagerungen, während das lysosomale Rab7 die Anzahl der Ablagerungen erhöht, aber nicht ihnen kolokalisiert. Des Weiteren benutzen wir Größenexklusionschromatographie (SEC) und Enzyme Linked Immunosorbent Assay (ELISA) um zu zeigen, dass Rab5A und Rab7 in Abhängigkeit ihres Aktivitätszustandes die Partikelgröße von ASYN ändert und die Sekretion moduliert. Die abschließende Bewertung eines Tiermodells welches humanes ASYN pan-neuronal überexpremiert zeigte, dass lysosomales Rab7 und die Protease Cathepsin D (CatD) in Hirnregionen verantwortlich für Bewegung, Motivation und Gedächtnis herausreguliert sind. Unsere Arbeit sowohl in Säugerzellkultur sowie in transgenen Tieren deutet darauf hin, dass die ASYN-Pathologie Auswirkungen auf das endosomale Transportsystem hat, aber zeigt auch die Fähigkeit von Proteinen, welche mit diesem Transportsystem assoziiert sind, die Toxizität von ASYN zu modulieren. Daher schließen wir, dass Anomalien in der Transportmaschinerie von Endosomen, welche durch Fehlregulation ASYN verursacht wurden, zur Entstehung der PD Pathologie beitragen.

Content

1. Introduction

An aging society faces unique challenges. Some of these are to be found on the socio-economic scale, but even more important are the ones in the health sector. Aging-associated diseases range from cardiovascular conditions to cancer, hypertension and neurodegenerative diseases, to only name a few. Neurodegenerative conditions occur as several autonomous disorders, often characterized by subcellular protein deposits that can be located either inside or outside the cell.

Examples of these disorders are amyloidoses and tauopathies, such as Alzheimer's disease (AD) which shows the accumulation of extracellular plaques and intracellular neurofibrillary tangles. Another example of neurodegenerative diseases is the family of synucleinopathies, which are characterized by the accumulation of intracellular inclusions primarily composed of the protein α-Synuclein (ASYN). The main types of synucleinopathies are PD, dementia with Lewy Bodies (DLB) and multiple system atrophy (MSA).

1.1 Parkinson's Disease

PD was first described by James Parkinson in 1817 (Parkinson, 1817). Currently, PD is estimated to affect up to 4% of the general population over 80 years of age (de Lau and Breteler, 2006). The major motor symptoms associated with PD are resting tremor, postural instability, rigidity and bradykinesia starting in the fine motor skills (Jankovic, 2008). On a neuropsychiatric level, the symptoms include decreased cognitive speed and corresponding cognitive dysfunction, progressing dementia with accompanying alterations of mood and behavior have also been observed (Braak et al., 2002a, 2002b, 2004). In other independent studies, non-classical symptoms were recently linked to PD; these include sleep disturbance, constipation, impotence and anosmia that often set in decades before the onset of the cardinal motor PD symptoms described above (Barone et al., 2009; Ferrer, 2011). PD is an idiopathic disorder and the causes are still unclear. Several factors that have been proposed to contribute to disease, including genetic, epigenetic and environmental circumstances, such as trauma and toxin exposure. While some toxins could be shown to cause symptoms concurrent with parkinsonism, with metals and neurotoxins being most prominent (Dauer and Przedborski, 2003), these

along with trauma and dementia pugilistica (Mendez, 1995; Erlanger et al., 1999), can be ruled out in most cases.

Investigations of the molecular pathology of the disorder have revealed a progressive loss of dopaminergic neurons located in the substantia nigra (Davie, 2008). These neurons project into the striatum, the region of the midbrain that is responsible for motor control and coordination (Hodge and Butcher, 1980; Davie, 2008). Frederic Lewy described protein depositions in surviving neurons in *post mortem* brain tissue of PD sufferers (Rodrigues e Silva et al., 2010). These depositions are now called Lewy bodies (LBs) and are considered the major pathological hallmark of PD. They consist largely of ASYN, but also stain positive for ubiquitin (Spillantini et al., 1997; Goedert, 2001; Dauer and Przedborski, 2003; Engelender, 2008).

At the beginning of the century, Heiko Braak proposed a staging of the disease according to LB pathology (Braak et al., 2004). The pattern of LB formation is ascending from the medulla towards the neocortex, in concordance to the progression of the symptoms. LB pathology confined to the brain stem either does not lead to symptoms or individuals thus affected only show non-classical symptoms. Only when LBs can be found in the substantia nigra, and about 50% of the dopaminergic neurons have died, the motor symptoms set in (Braak et al., 2002a, 2002b, 2004; Angot et al., 2010). When pathology progresses into the neocortex, neuropsychiatric symptoms are observed (Braak et al., 2002a, 2002b, 2004). As neurons are postmitotic and terminally differentiated cells they are especially vulnerable to stress and injury and rely especially on an intact protein transport machinery to maintain their integrity and basic function. However, the reason for the special vulnerability of dopaminergic neurons is still not clear. Dopamine is a catecholamine and as such sensitive to reactive oxygen species, so one possibility lies in disruption of redox homeostasis (Dexter et al., 1987; Fariello, 1988; Sofic et al., 1988, 1992; Spina and Cohen, 1989; Sian et al., 1994; Hashimoto et al., 1999; Nakaso et al., 2013). This hypothesis is supported by the decreased glutathione and increased iron concentrations in the brains of affected individuals (Dexter et al., 1987; Sofic et al., 1988, 1992; Spina and Cohen, 1989; Sian et al., 1994). Oxidation of dopamine caused the oxidative insults of these two conditions might lead to cytotoxicity.

By now, several genes have been associated with PD (Table 1). Over the past decades, one of the strongest associations with the disease has turned out to be with ASYN (Figure 1), a small protein found both in the nucleus and at presynaptic termini

(Maroteaux et al., 1988). Many research efforts have been concentrated on this protein, whose cellular function is still not fully understood. We will further try to shed some light on what is known about ASYN and how it relates to the molecular events of PD pathology.

Table 1: Genetics of PD

Locus	**Gene**	**Description**	**Reference**
PARK 1/4	*SNCA*	Presynaptic/nuclear protein	(Polymeropoulos et al., 1996)
PARK2	*Parkin*	Ubiquitin ligase	(Kitada et al., 1998)
PARK3	SPR(?)		(Gasser et al., 1998)
PARK5	*UCH-L1*	Ubiquitin protease	(Liu et al., 2002)
PARK6	*PINK1*	Mitochondrial protein kinase	(Hatano et al., 2004)
PARK7	*DJ-1*	Multifunctional protein	(Bonifati et al., 2003)
PARK8	LRRK2	Leucine-rich repeat kinase	(Gasser, 2009)
PARK9	ATP13A2	Lysosomal ATPase	(Ramirez et al., 2006)
PARK10	(?)		(Li et al., 2002)
PARK11	GIGYF2		(Lautier et al., 2008)
PARK12	(?)		(Pankratz et al., 2002)
PARK13	Omi/HTRA2	Serine Protease	(Strauss et al., 2005)
PARK14	PLA2G6	Phospholipase	(Paisan-Ruiz et al., 2009)
PARK15	FBXO7	F-box protein	(Shojaee et al., 2008)
PARK16	(?)		(Satake et al., 2009)
PARK17	VPS35	Retromer Complex	(Wider et al., 2008)
PARK18	EIF4G1	Translation Initiation Factor	(Chartier-Harlin et al., 2011)
PARK19	DNAJC6	DNAJ/HSP40 homolog	(Edvardson et al., 2012)
PARK20	SYNJ1	Synaptojanin 1	(Krebs et al., 2013; Quadri et al., 2013)

1.2 ASYN, a central player in PD

ASYN is the main component of LBs (Spillantini et al., 1997) and it has long been defined as a natively unfolded protein able to assume α-helical secondary structure (Chandra et al., 2003) (Figure 1B), form homodimers, and fibrillize into detergent insoluble aggregates (Conway et al., 1998). Several studies also discussed a potential tetrameric structure under physiological conditions (Bartels et al., 2011; Wang et al., 2011). The α-helically folded form of the protein is the one thought to preferentially interact with membranes. In any case, the existence of tetrameric ASYN remains controversial (Binolfi et al., 2012; Fauvet et al., 2012a, 2012b). This can also be attributed to a follow up study that found differently modified, native and recombinant ASYN to have similar electrophoretic mobility, mass, and circular dichroism spectra concurrent with results for monomers (Fauvet et al., 2012b). Due to the exhaustive nature of the study employing transgenic mice expressing human ASYN, rat brains expressing human ASYN after AAV infection, human erythrocytes and brain post mortem samples as well as recombinant protein (Fauvet et al., 2012b), it seems more likely that ASYN exists predominantly as a natively unfolded monomer.

While LBs are the pathological hallmark of PD, the deposits are found in surviving neurons, which might present an effort of the cells to protect themselves by sequestering exogenous protein into LBs. Due to this hypothesis, it might be that lower molecular weight ASYN oligomers constitute the toxic species (Winner et al., 2011). Lesions from autopsied PD brains show a marked increase in S129 hyperphosphorylated ASYN (Fujiwara et al., 2002). Still, the exact determinants that lead to ASYN misfolding and fibrillization remain unclear. Nonetheless, multiple factors might be involved in this process.

Given that ASYN interacts with membranes, it is likely that fibril formation might interfere with normal membrane dynamics (Zhu et al., 2003; Fortin et al., 2004; Jo et al., 2004), and that the physiological function of ASYN might be related to membrane integrity or protein trafficking pathways. Several studies support the interaction of ASYN with the trafficking machinery: ASYN regulates the size of the presynaptic vesicle pool in primary hippocampal neurons (Murphy et al., 2000), might have a chaperone activity to other presynaptic membrane proteins (Chandra et al., 2005) and plays a role in SNARE complex assembly (Burré et al., 2010; Thayanidhi et al., 2010), can interact with Rab

GTPases (Sung et al., 2001; Dalfó et al., 2004a; Cooper et al., 2006; Gitler et al., 2008; Soper et al., 2008, 2011; Liu et al., 2009; Sancenon et al., 2012; Rendón et al., 2013; Breda et al., 2014; Chutna et al., 2014; Yin et al., 2014), and is involved in vesicle recycling (Ben Gedalya et al., 2009; Nemani et al., 2010; Scott and Roy, 2012). Additional evidence for the association of ASYN with membranes can be derived from biochemical and biophysical studies: acetylation at the N-terminus of ASYN leads to an increased membrane binding affinity (Bartels et al., 2011; Fauvet et al., 2012a; Kang et al., 2012; Maltsev et al., 2012), while nitration of C-terminally-located tyrosines leads to a partial abolishment of membrane binding (Sevcsik et al., 2011).

Missense mutations and multiplications of the gene encoding for ASYN cause autosomal dominant forms of PD (Golbe et al., 1990; Polymeropoulos et al., 1996, 1997; Polymeropoulos, 1997; Krüger et al., 1998; Singleton et al., 2003; Chartier-Harlin et al., 2004; Zarranz et al., 2004; Kiely et al., 2013; Proukakis et al., 2013; Pasanen et al., 2014) (Figure 1A). So far, six familial missense mutations of ASYN have been found: A30P (Krüger et al., 1998), E46K (Zarranz et al., 2004), H50Q (Proukakis et al., 2013), G51Q (Kiely et al., 2013) A53E (Pasanen et al., 2014) and A53T (Golbe et al., 1990; Polymeropoulos et al., 1996; Polymeropoulos, 1997). Additionally, duplication (Chartier-Harlin et al., 2004) and triplication (Singleton et al., 2003) of the SNCA locus also lead to disease onset. All of these studies provide a strong link between ASYN and PD. Exogenous amounts of wild type (wt) ASYN and disease-associated mutations caused by overexpression has been shown to induce toxicity in a variety of cellular and animal models, such as yeast, worms, flies, or mice (Masliah et al., 2000; van der Putten et al., 2000; Giasson et al., 2002; Outeiro and Lindquist, 2003; Singleton et al., 2003; Chartier-Harlin et al., 2004).

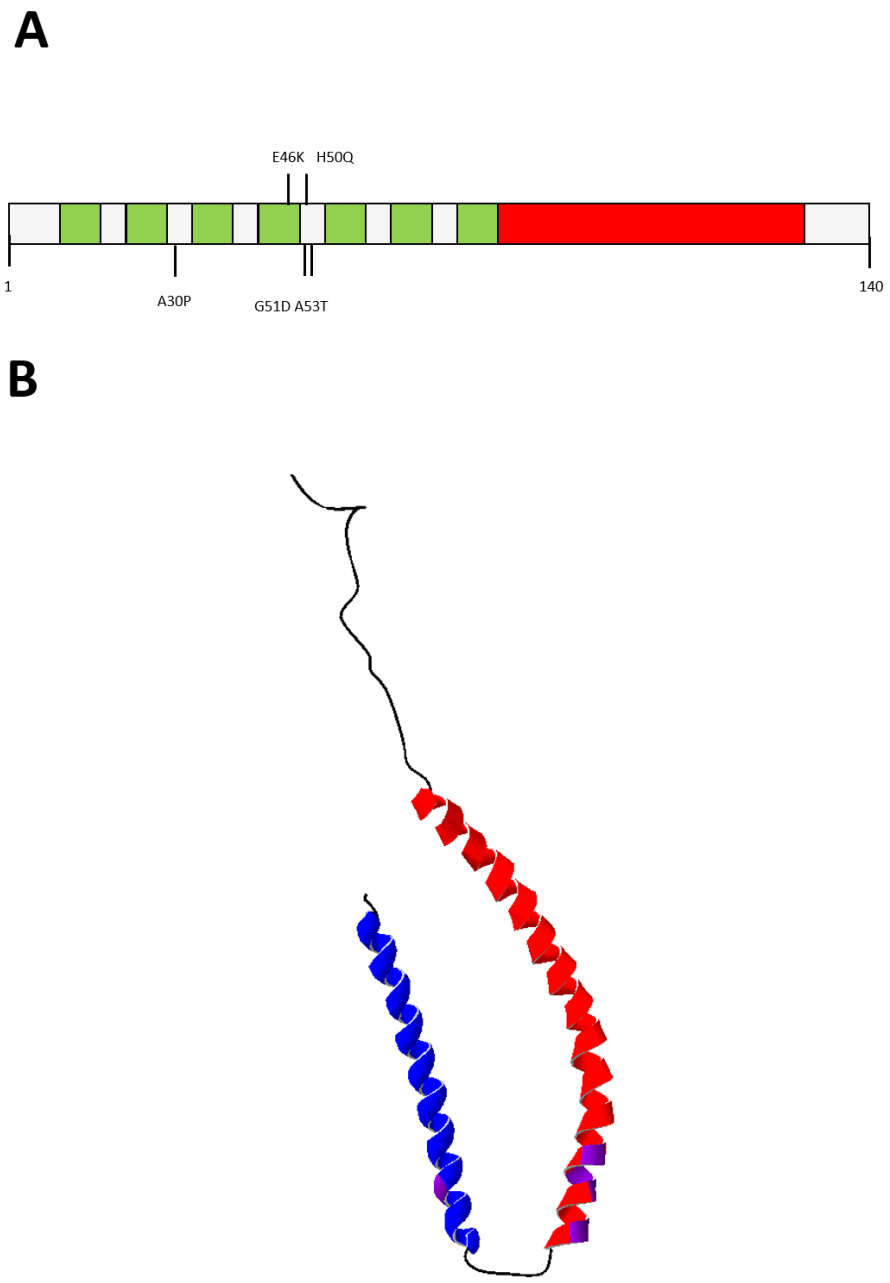


Figure 1: ASYN mutations and secondary structure. (A) The N-terminus with KTKEGV repeats (green) is able to assume α-helical structure (Jao et al., 2004) upon binding to membranes as displayed in (B). The non-amyloid component (NAC) domain is displayed in red. The C-terminus is highly unstable and modifications can aid aggregate formation. Amino acid positions A30, E46, H50, G51 and A53 have been associated with familial PD. (B) Structure of membrane bound ASYN colored in secondary structure succession by Swiss Pdb-Viewer (Guex and Peitsch, 1997). Sites of familial mutations are marked in purple. PDB entry 1XQ8 (Ulmer et al., 2005).

Overexpression of human wt and mutant ASYN in mice leads to lesions in the spinal cord, brainstem, hippocampus and neocortex as well as motor phenotypes (Masliah et al., 2000; van der Putten et al., 2000; Giasson et al., 2002; Rockenstein et al., 2002;

Fleming et al., 2004). Interestingly, expression of the ASYN transgene in a cysteine string protein α (CSPα) mouse knockout model is able to rescue neurodegeneration caused by the null mutation (Chandra et al., 2005). ASYN also seems to play a role in SNARE complex assembly (Burré et al., 2010; Thayanidhi et al., 2010), which is further validated by the fact that ASYN co-immunoprecipitates with SNAP-25, and directly interacts with VAMP2 *in vitro* (Burré et al., 2010).

Further evidence for a role of ASYN in the cellular transport machinery stems from studies in yeast. While yeast does not have a native homologue for ASYN, nevertheless, it has proven to be an invaluable model organism. In genome wide screens in yeast, multiple genes were identified to enhance or suppress ASYN toxicity (Outeiro and Lindquist, 2003; Willingham et al., 2003; van Ham et al., 2008). It is striking that many of the thus identified gene clusters are related to either lipid metabolism or vesicular transport. A high percentage of these genes have human orthologs. It was first proven in yeast that Ypt1, the yeast homologue of mammalian Rab1, can alleviate a ER-to-Golgi vesicle trafficking block caused by ASYN (Cooper et al., 2006; Gitler et al., 2008). The results of these studies could be repeated in other model systems (Cooper et al., 2006).

In an RNAi screen in *Caenorhabditis elegans*, several genes associated with trafficking were identified to act neuroprotective on ASYN-induced toxicity (Hamamichi et al., 2008; Kuwahara et al., 2008; van Ham et al., 2008).

1.3 ASYN and synaptic vesicles

ASYN is present at presynaptic termini (Maroteaux et al., 1988). This, along with its membrane binding capability and association with synaptic vesicles suggests a role in endo- or exocytosis, which are essential processes for the function and survival of neuronal cells. Under physiological conditions synaptic vesicles dock to and fuse with the plasma membrane, which releases neurotransmitters into the synaptic cleft. They are then replenished via recycling, as well as from the distal vesicle pool. This vesicle reserve pool is diminished upon ASYN knockdown in primary hippocampal neurons, and this suggests that ASYN might be involved in the regulation of vesicle pools (Murphy et al., 2000; Cabin et al., 2002).

Electrophysiological studies support this observation, as they show no alteration in basal transmission, but depletion of the docked vesicles and distal reserve pool upon prolonged stimulation (Cabin et al., 2002). Supporting this is the observation that aged ASYN null mice show reduced dopamine content in the striatum, while no such progressive loss could be observed in the substantia nigra *pars compacta* (Al-Wandi et al., 2010). The normal function of ASYN therefore might be involved in the dopamine cycle, however this does not explain the toxic gain of function observed upon increased gene dose or mutation. If ASYN should be a deciding factor in exocytosis, oxidative insult might lead to a diminishment of membrane binding of ASYN and, therefore, to dysfunction in neurotransmitter release (Sevcsik et al., 2011). Increased expression of ASYN in chromaffin PC12 cells concurs with an inhibition of dopamine release, but it increases the number of vesicles docked to the plasma membrane (Larsen et al., 2006). These results are somewhat controversial, but might be cell line specific. A reduction in released vesicles was also observed in mouse primary hippocampal cultures (Nemani et al., 2010). While subsequent *in vivo* studies with wt and A30P ASYN revealed no alteration in amount of docked vesicles in primary chromaffin cells, a significant reduction of neurotransmitter release was shown (Larsen et al., 2006), as well as an inhibition of synaptic transmission in acute hippocampal slices, electrochemical caudate putamen measurements and primary midbrain dopaminergic neurons (Nemani et al., 2010; Scott et al., 2010; Lundblad et al., 2012; Scott and Roy, 2012). Due to these findings, it is possible that ASYN pathology is related to secretion and vesicle recycling. Vesicle clustering and reclustering is non-uniform in synapses of ASYN overexpressing animals and this goes in hand with unusual, non-uniform vesicle sizes (Nemani et al., 2010; Scott et al., 2010). Overall changes to the ultrastructure of the presynapse seem plausible due to these changes in the vesicle pools and synaptic transmission. Whether this directly related to abnormal ASYN interactions with synaptic vesicles or components of the underlying trafficking machinery remains unknown.

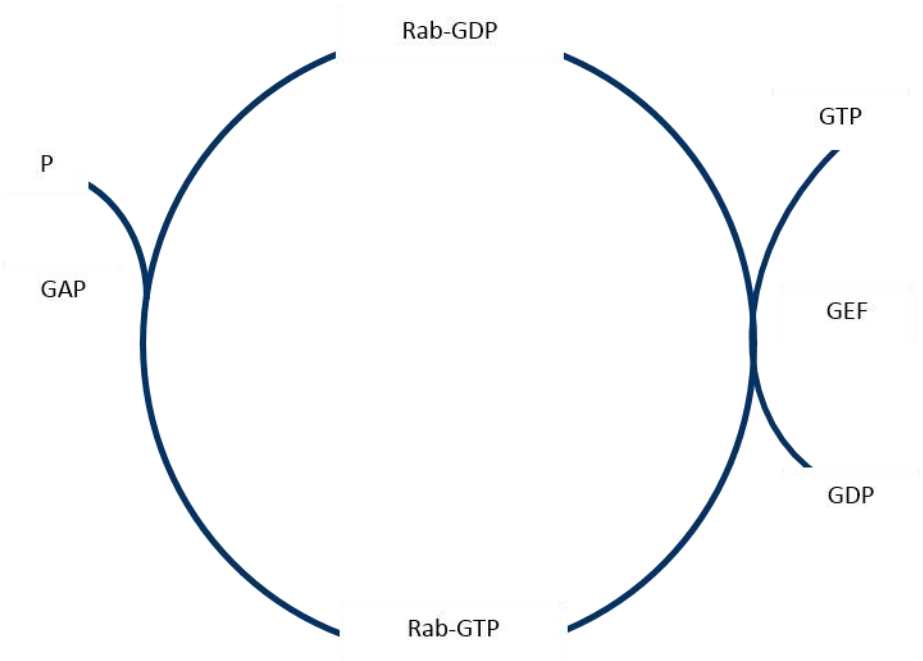
1.4 Rab GTPases

The family of Rab GTPases is a class of peripheral membrane proteins mainly involved in trafficking. They are molecular switches and present key regulatory factors in a multitude of trafficking and vesicle movement steps between organelles.

More than 60 members of the Rab family have so far been identified in mammals, many of them with up to four isoforms, with 11 homologues in yeast. Rabs are highly conserved (Pereira-Leal and Seabra, 2001; Chan et al., 2011; Diekmann et al., 2011) with a GTPase fold that can be found in all members of the Ras superfamily identified so far and which is responsible for the activation state of the protein. The C-terminus is highly variable and one of the defining structural characteristics between different members of the Rab GTPase family (Lee et al., 2009).

Rab GTPases are present in a GTP-bound—active—and GDP-bound—inactive—conformation (Figure 2A) and cycle between the target membrane and the cytosol. They can be considered molecular switches, as they cover a significant role in the formation, budding, fusion, molecular transport as well as tethering of a vesicle. GDP is exchanged for GTP by guanosine exchange factors (GEFs) and GTP is cleaved by interaction of Rabs with a GTPase activating protein (GAP) (Fischer von Mollard et al., 1994a; Lee et al., 2009) (Figure 2A). Rabs can themselves interact with other proteins, which are then called effectors and carry out diverse functions, depending on their localization and specific function in the membrane trafficking pathway (Pfeffer, 2001; Lee et al., 2009; Hutagalung and Novick, 2011) (Figure 2B). Thus, the specific Rab is one factor that contributes to membrane and thereby organelle identity (Chavrier et al., 1990b; Pfeffer, 2001) (Figure 2B, Figure 3, Figure 4A). Rab GTPases mutants have been described that are impaired in intrinsic GTP hydrolysis, termed constitutive active (Bucci et al., 1992, 2000; Vitelli et al., 1997), or that have a greatly reduced affinity to GTP, termed dominant negative (Bucci et al., 1992, 2000; Vitelli et al., 1997).

A


Rab-GDP
GTP
P
GAP
GEF
GDP
Rab-GTP

B

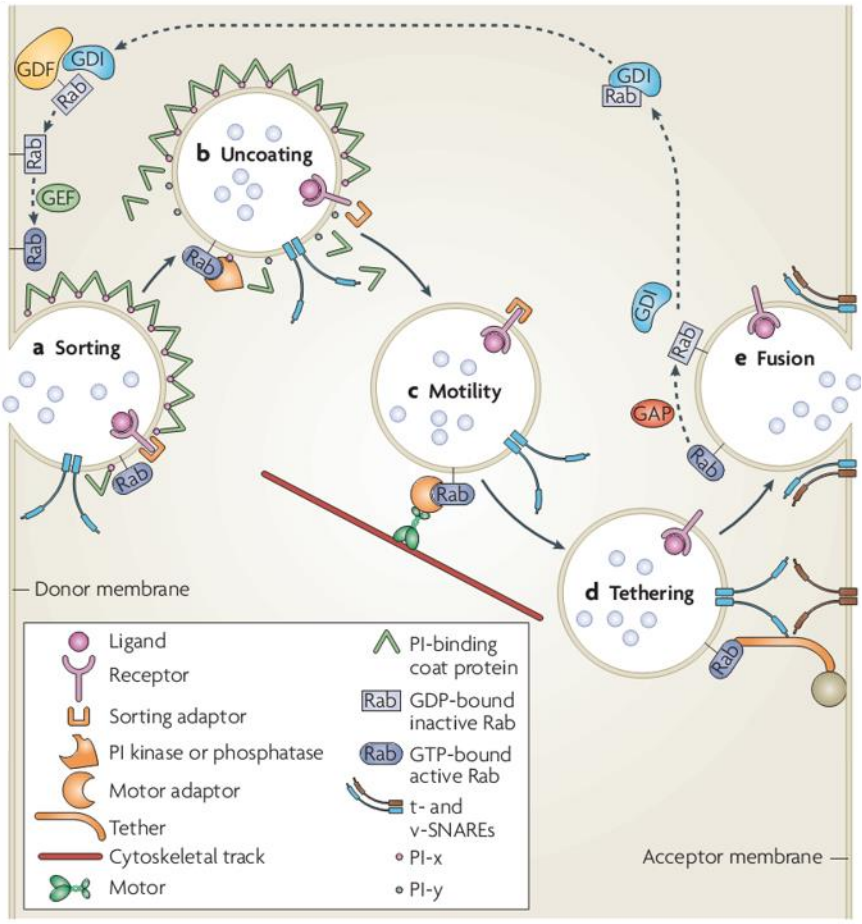

GDF
GDI
Rab
GEF
b Uncoating
a Sorting
c Motility
e Fusion
GAP
d Tethering
Donor membrane
Acceptor membrane
Ligand
Receptor
Sorting adaptor
PI kinase or phosphatase
Motor adaptor
Tether
Cytoskeletal track
Motor
PI-binding coat protein
GDP-bound inactive Rab
GTP-bound active Rab
t- and v-SNAREs
PI-x
PI-y

Figure 2: Rab GTPases are molecular switches that act to coordinate membrane trafficking. (A) Rabs are present in active GTP-bound and an inactive GDP-bound state. Conversion from the inactive to the active state is facilitated by GEFs and causes a conformational change that activates the protein, the active state is recognized by effector proteins. These GAPs stimulate the inherent GTPase activity and an inorganic phosphate molecule is released, rendering the Rab inactive. (B) Rabs functions in vesicle trafficking. Distinct membrane trafficking steps that can be controlled by a Rab GTPase and its effectors (indicated in orange). (a) An active GTP-bound Rab can activate a sorting adaptor to sort a receptor into a budding vesicle. (b) Through recruitment of phosphoinositide (PI) kinases or phosphatases, the PI composition of a transport vesicle might be altered (the conversion of PI-x into PI-y) and thereby cause uncoating through the dissociation of PI-binding coat proteins. (c) Rab GTPases can mediate vesicle transport along actin filaments or microtubules (collectively referred to as cytoskeletal tracts) by recruiting motor adaptors or by binding directly to motors (not shown). (d) Rab GTPases can mediate vesicle tethering by recruiting rod-shaped tethering factors that interact with molecules in the acceptor membrane. Such factors might interact with SNAREs and their regulators to activate SNARE complex formation, which results in membrane fusion. (e) Following membrane fusion and exocytosis, the Rab GTPase is converted to its inactive GDP-bound form through hydrolysis of GTP, which is stimulated by a GAP. Targeting of the Rab-GDP dissociation inhibitor (GDI) complex back to the donor membrane is mediated by interaction with a membrane-bound GDI displacement factor (GDF). Conversion of the GDP-bound Rab into the GTP-bound form is catalysed by a GEF. (Figure 2B and accompanying description originally by Harald Stenmark (Stenmark, 2009).)

The exchange of one Rab replacing another on a membrane, e.g. from early endosome to late endosome, is called Rab conversion (Rink et al., 2005; Poteryaev et al., 2010). In this process, the loss of one Rab is coordinated with the acquisition of another, each binding to its own domain on the target membrane (Sönnichsen et al., 2000; Rink et al., 2005). On the molecular level, this conversion is facilitated by Rab effector molecules (Rink et al., 2005; Poteryaev et al., 2010).

Rabs are integral membrane proteins and due to their previously mentioned involvement in ASYN pathology a prime target for further investigation. For this study, we selected three Rab GTPases to investigate further in the context of ASYN pathology, namely Rab5A, Rab7 and Rab8A which we will highlight further.

1.4.1 Rab5A

Rab5A is a regulator of clathrin-mediated endocytosis as well as early endosome biogenesis and fusion (Chavrier et al., 1990a; Bucci et al., 1992; Fischer von Mollard et al., 1994b; Stenmark et al., 1994; Zeigerer et al., 2012) (Figure 3). Overexpression of wt

and constitutively active Rab5 leads to accelerated endocytosis and abnormally large endosomes (Bucci et al., 1992), while dominant negative Rab5 can inhibit endocytosis and prevent the fusion of early endosomal membranes (Stenmark et al., 1994). Rab5 is involved in endocytic sorting in axonal trafficking (Deinhardt et al., 2006) and has recently been found to be necessary for the formation of the autophagosome (Ao et al., 2014). No disease associated mutations have been identified as of yet, which highlights its general importance in trafficking events. On the other hand, Rab5 is able to aid in facilitating pathogenic entrance into cells (Gimenez et al., 2015). Rab5 interacts with mutant ASYN A30P *in vivo* (Dalfó et al., 2004b) and is upregulated in human cases of mild cognitive impairment and AD (Ginsberg et al., 2010, 2011).

1.4.2 Rab7

Rab7 has been dubbed "a key to lysosomal biogenesis" (Bucci et al., 2000) and is an important regulator of the endo-lysosomal system as well as autophagosome formation (Chavrier et al., 1990a; Mukhopadhyay, 1997; Bucci et al., 2000; Gutierrez et al., 2004; Deinhardt et al., 2006; Silverman et al., 2011; Ng et al., 2012; Aloisi and Bucci, 2013; Ao et al., 2014). The late endosome and lysosome only achieve their identity after Rab conversion where Rab5 is exchanged for Rab7 at their discreet domains on the endosomal membrane (Rink et al., 2005) (Figure 3). Overabundance of wt or expression of constitutively active Rab7 lead to enlarged late endosomes and lysosomes, localized mainly in the perinuclear region, while expression of dominant negative Rab7 leads to reduced acidity and dispersal of the lysosome (Bucci et al., 2000). Several studies have associated other mutations of Rab7 with the inherited motor and neurological disorder Charcot-Marie-Tooth disease type 2B (Spinosa et al., 2008; McCray et al., 2010; Bucci and De Luca, 2012). Besides these, one study found Rab7 to be upregulated in human cases of mild cognitive impairment and AD (Ginsberg et al., 2010, 2011). Interestingly, Rab7 interacts with leucine-rich-repeat-kinase 2 (LRRK2), another gene associated with familial forms of PD (Gasser, 2009; Dodson et al., 2012; Gómez-Suaga et al., 2014). In *D. melanogaster*, mutant LRRK2 homologue interferes with Rab7 mediated lysosomal positioning (Dodson et al., 2012). In HeLa and HEK293 cells, expression of mutant LRRK2 leads to impaired receptor degradation, possibly by downregulating Rab7 activity and the associated pathway (Gómez-Suaga et al., 2014). Expression of constitutive active

Rab7 rescues this delay and restores the late endocytic trafficking pathway (Gómez-Suaga et al., 2014).

Both Rab5 and Rab7 are key factors ensuring normal progression through the endocytic pathway (Figure 3).

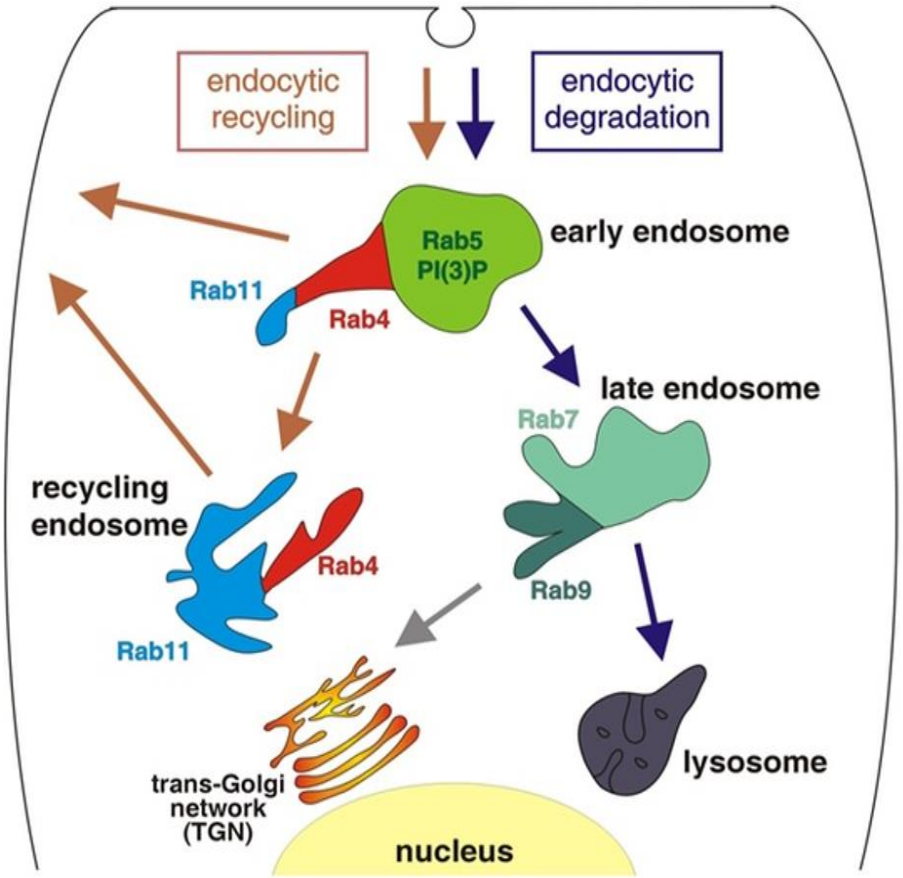


Figure 3: Model, describing endocytic organelles as a mosaic of membrane domains. Each Rab domain fulfills a different cellular function. Cargo enters the cells via the Rab5-mediated endocytic pathway and first enters Rab5 positive structures. Rapid sorting into Rab4 and Rab11 positive recycling endosomes occurs via sorting mechanisms. In the endocytic degradation pathway cargo is shifted from sorting endosomes into late endosomes positive for Rab7 and Rab9. From here material is either recycled via the trans-Golgi network or degraded in the lysosome. (Modified from Marino Zerial, Max-Planck-Institute of Molecular Cell Biology and Genetics, Dresden (Zerial and Sönnichsen, n.d.; Sönnichsen et al., 2000).)

1.4.3 Rab8A

Rab8A is located in the TGN where it regulates apical transport events that pass through recycling endosomes (Hattula et al., 2006; Henry and Sheff, 2008), placing it in the secretory pathway. Compartments positive for Rab8A can be tubular in shape (Hattula et al., 2006), and constant activation leads to disturbances in protein sorting events (Henry and Sheff, 2008), while expression of the dominant negative version of the protein has an effect of cell shape and adhesion (Hattula et al., 2006). As Rab8A is also involved in ciliogenesis, disturbances in Rab8A homeostasis are implicated in microvillus inclusion disease and Bardet-Biedl syndrome (Nachury et al., 2007; Erickson et al., 2008).

1.5 ASYN and Rab GTPases

Several studies have linked Rab GTPases to PD and ASYN pathology thus far. In brain tissue from DLB and MSA patients as well as in transgenic mouse lines, co-immunoprecipitation studies showed interaction between ASYN and Rab3A, Rab5 and Rab8 (Dalfó et al., 2004a, 2004b; Dalfó and Ferrer, 2005) (Figure 4B). Rab3A is the neuronal isoform of Rab3 and present in almost all synapses in the brain. Knockout mutants are viable and fertile due to redundancy (Fischer von Mollard et al., 1991; Geppert et al., 1994). One of its effectors is rabphilin (Geppert et al., 1994), and the same study also showed an interaction with ASYN (Dalfó et al., 2004a). Interaction of high molecular weight species of ASYN and Rab3A, Rab5, Rab8 and rabphilin could only be found in the detergent insoluble fraction of patients' brains, while slight interaction with the monomer was observed in the soluble fraction of control brains (Dalfó et al., 2004b; Dalfó and Ferrer, 2005). Rab3 and Rab8 are involved in exocytosis, while Rab5 is in the endocytic pathway and due to the aforementioned results and similar findings in A30P transgenic mice, it has been hypothesized that these two crucial cellular functions are dysregulated in synucleinopathies (Dalfó et al., 2004b) (Figure 4B). Overexpression of inactive Rab5a can reduce cell death induced by exogenous addition of ASYN (Sung et al., 2001; Potokar et al., 2012). Further, in an ASYN transgenic mouse model, as well as brains from DLB and AD patients, Rab5-positive endosomal structures are enlarged and Rab5 is dysregulated (Sancenon et al., 2012) (Figure 4B). In a cell model with ASYN A30P overexpression, co-expression of Rab1A, Rab7 and Rab8A yielded a two-fold higher secretion of ASYN into the medium, while Rab7 and Rab8A also decreased toxicity

(Ejlerskov et al., 2013). In the same study, modulation of Rab27A activity could show a modulation of ASYN secretion and mortality. While extracellular ASYN is endocytosed in a Rab regulated manner, recycling and exocytosis also involve Rab GTPases. Rab11a, which characterizes recycling endosomes, interacts with endocytosed ASYN in cellular models, where it modulates secretion, ASYN aggregation and toxicity (Liu et al., 2009; Chutna et al., 2014). Heat shock protein 90 (HSP90) interacts with Rab11a, but is also involved in the recycling pathway of endocytosed ASYN (Liu et al., 2009). Whether these results could be exploited for clearance of intracellular ASYN or ASYN aggregates remains unknown, although ASYN and Rab11A have been shown to interact *in vivo* in mice (Chutna et al., 2014) and could alleviate ASYN mediated toxicity in *Drosophila melanogaster* model (Breda et al., 2014), possibly by restoring homeostasis to the synaptic vesicle pool (Breda et al., 2014).

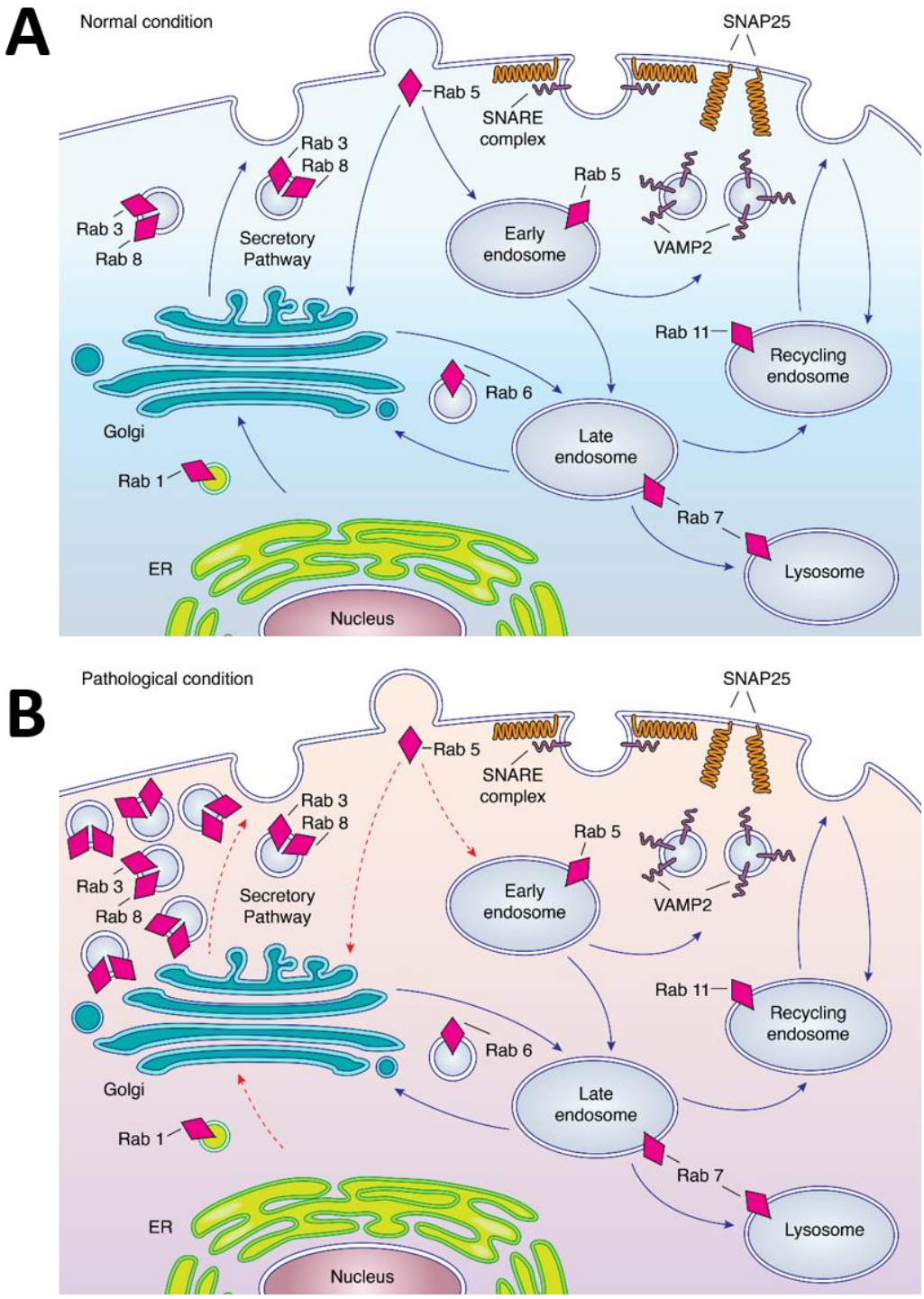


Figure 4: Hypothetical model for vesicular trafficking pathways with selected regulatory factors associated with ASYN pathology. (A) Under normal homeostatic conditions cargo vesicles bud off the ER and are shuttled to the Golgi apparatus with the help of Rab1. After processing in the Golgi, vesicles are processed by the TGN and either enter the secretory pathway where they are associated with Rab8 and Rab3 or are transported to one endosomal compartments. Rab5 mediated endocytosis directs vesicles either to the Golgi apparatus or to an endosome. Anterograde as well as retrograde traffic between the endosome and the Golgi is facilitated by Rab6. As the endosome matures, Rab5 is replaced by Rab7 at its membrane, signifying a slow acidification and eventual lysosomal function. Rab11 at recycling endosomes facilitates a

steady exchange with the extracellular space. Fusion of vesicles with the plasma membrane for exocytosis is facilitated by zippering of SNARE proteins; t-SNAREs, here SNAP25, and v-SNAREs, here VAMP2, zipper together to enable fusion of the two membranes. (B) Hypothetical model of how several regulatory factors could be affected by ASYN toxicity. Several discrete trafficking steps are associated with ASYN pathology. ASYN oligomers associate with the ER membrane, causing stress and leading to cellular toxicity. Aggregation of ASYN leads to a trafficking block between the ER and the Golgi apparatus, which can be alleviated by overexpression of Rab1. The TGN and secretory pathway are inhibited, and vesicular clustering can be observed. Rab5 mediated endocytosis is inhibited and the formation of SNARE complexes is disturbed. *Interrupted arrows* signify disturbances in vesicular trafficking pathways. ASYN is not displayed due to unknown nature of aggregation state, the condition is instead symbolized by *shading* of the cytoplasm. (Modified from (Eisbach and Outeiro, 2013).)

Interactions between ASYN and the family of Rab GTPases have been most extensively studied in yeast, where several vesicle trafficking steps were identified to be affected by ASYN overexpression (Figure 4). An ASYN mediated ER to Golgi trafficking (Cooper et al., 2006) which could be repeated in cell free systems, where transport is reduced by 50% (Gitler et al., 2008). Overexpression of the Rab1 homologue Ypt1 alleviates the trafficking block and rescues cytotoxicity. Rab1 could afterwards reduce toxicity in flies, nematodes primary neuronal cultures (Cooper et al., 2006). In yeast, ASYN overexpression leads to protein foci attached to the plasma membrane (Gitler et al., 2008; Soper et al., 2008), but strong overexpression of Ypt1 was able to reduce the size of the foci and ASYN toxicity (Gitler et al., 2008). The Rab homologues Ypt31, Sec4, Ypt6, Vps21, Ypt52 and Ypt7 all colocalize with ASYN accumulations in yeast, but none of these genes suppresses ASYN toxicity. Human Rab1, Rab3a and Rab8a were able to significantly rescue toxicity (Gitler et al., 2008) in *C. elegans*. It is worth highlighting these three Rabs are associated with Golgi or TGN trafficking respectively. Subsequently, Yptp6p, Yptp7p, Ypt10p, Ypt31p, Ypt32p, Ypt51p and Ypt53p were found to colocalize with ASYN in an with only Ypt10p partially localizing to its normal target membrane (Soper et al., 2011). In knockout mutants of Rab homologues that express ASYN, inactivation of Ypt6p led to an increase in vesicle clustering. In yeast expressing ASYN and Ypt6p reduced vesicle clustering was observed this yeast homologue of Rab6 to be a regulator of ASYN toxicity. In the same study, deletion of Ypt7p, yeast homologue of Rab7, and Ypt51p, homologue of Rab5, also led to increases in vesicle clustering, but to a lesser degree than Ypt6p (Soper et al., 2011). These proteins are all involved neuronal endocytosis, the TGN and the endosomal pathway (Fischer von Mollard et al., 1994b; Sung et al., 2001; Rink et al., 2005; Utskarpen et al., 2006; Potokar et al., 2012), which

suggests that ASYN pathology could interfere with Golgi or endosome trafficking pathways. Further evidence of this has been found in one of our own studies, in which we could prove ASYN interaction with Rab8A *in vitro* as well as *in vivo* and found Rab8A interact with the C-terminus of ASYN and modulates inclusion formation and toxicity both in a cellular and a *D. melanogaster* model (Yin et al., 2014).

In ASYN transgenic *C. elegans*, pan-neuronal knockdown of Rab7 caused severe motor and growth abnormalities (Kuwahara et al., 2008). In primary neurons, axonal transport of Rab7 positive compartments is impaired by immobilized ASYN inclusions (Volpicelli-Daley et al., 2014).

Another Rab-related ASYN interactor is prenylated Rab acceptor protein 1 (PRA1) (Lee et al., 2011). PRA1 binds to Rab1, Rab3a, Rab5 and Rab6, placing it into the Golgi and endosomal trafficking pathways (Figueroa et al., 2001). In cells co-expressing PRA1 and ASYN, vesicles were translocated towards the cell periphery (Lee et al., 2011).These studies suggest that overexpression of ASYN seems to interfere with a variety of trafficking steps; from transport vesicles of newly translated proteins between ER and Golgi to endo- and exocytosis and retrograde protein transport. Interestingly, numerous members of the Rab GTPase family appear to be dysregulated by ASYN, whether this is causative to the trafficking defects or a consequence of them remains unknown, as well as how exactly ASYN interferes with vesicular transport events.

1.6 Spreading of ASYN pathology between cells

PD is a progressive disease and when we consider the different stages (Braak et al., 2004) that suggest LB pathology to be ascending from the medulla into the neocortex concurrent with physical and cognitive symptoms (Braak et al., 2002a, 2002b, 2004), we have to take the presence of an infectious agent into account. In an effort to alleviate PD motor symptoms, patients received embryonic nigral transplants into their own midbrains (Freed et al., 2001). In post mortem investigations, researchers discovered the presence of LB-like pathology in these transplants (Kordower et al., 2008a, 2008b; Li et al., 2008). Studies with stem cells transplants in mice have yielded comparable results (Desplats et al., 2009). This is surprising because PD is a disease that mostly occurs sporadically in aged individuals and, therefore, LBs—the pathological hallmark of the disease—should not be present in transplanted embryonic tissue. While

microenvironmental factors in diseased brains could be at fault and cause inclusion formation, the connection to Braak staging is obvious. The high concentration of ASYN in LBs make it an attractive candidate and indeed, several studies have shown that ASYN can be secreted (El-Agnaf et al., 2003; Liu et al., 2009; Jang et al., 2010) and that it can enter cells from the extracellular space via Rab mediated endocytosis (Sung et al., 2001) and even a clathrin-dependent pathway (Liu et al., 2007; Ben Gedalya et al., 2009). The question remains of how such a transmission would be possible. One attractive explanation is that of a prion-like mechanism of transmission, especially since studies have proposed a similar mechanism for amyloid-β and tau in AD (Angot et al., 2010; Steiner et al., 2011). While secretion and uptake of ASYN probably present natural cellular mechanisms, ASYN inclusion formation would likely require a seed. In spread of prion disease, a single misfolded PrP molecule is sufficient to act as a nucleus leading to further events of misfolding (Laurén et al., 2009). Accordingly, one pathologic event in ASYN folding or oligomerization would be enough to infect the rest of the cell and lead to similar events in neighboring cells, if secretion and uptake occur (Angot et al., 2010; Steiner et al., 2011). This theory was already successfully tested using neuroblastoma cells (Hansen et al., 2011). In another study, inoculation of mice brains with brain extracts from sick mice led to an earlier onset of symptoms and immuoreactivity to disease-associated ASYN throughout the brain (Bétemps et al., 2014). Another *in vivo* study where the authors injected recombinant ASYN fibrils into the substantia nigra of wt mice reported slow neurodegeneration due to neuronal dysfunction due to ASYN spreading in a prion-like manner (Masuda-Suzukake et al., 2013). Progressive loss of dopaminergic neurons with motor deficits could be shown after seeding of recombinant ASYN fibrils into the striatum, indicating cell-to-cell spread of pathogenic ASYN in anatomically connected brain regions (Luk et al., 2012).

Interestingly, oligomers in the instance of ASYN present the smallest unit with the ability to seed aggregation, as it has been shown that fibrils from recombinant ASYN are also able to seed aggregation in primary neurons (Volpicelli-Daley et al., 2011, 2014) and then be transported to the soma in an axon dependent manner (Volpicelli-Daley et al., 2011, 2014; Freundt et al., 2012). How large oligomer fibrils and aggregates would be able to be secreted is still under debate, however we should consider the possibility that these might originate from dead cells releasing their content into the extracellular space (Steiner et al., 2011). It has been shown that ASYN can be released into the extracellular space via exosomes (Emmanouilidou et al., 2010; Danzer et al., 2012), small

membranous vesicles derived from late endosomes (Bucci et al., 2000). Both monomeric and oligomeric ASYN is packaged into exosomes and secreted (Emmanouilidou et al., 2010; Danzer et al., 2012), and can be toxic for several cell types when exosome containing medium is added to them (Emmanouilidou et al., 2010; Danzer et al., 2012).

Ultimately, cell-to-cell spread of ASYN would explain correlation between symptom severity and ascending pathology as described by Braak staging and presents a feasible event in PD progression.

1.7 Protein degradation pathways and ASYN pathology

The ubiquitin-proteasome system (UPS) consists of ubiquitin chains attached to cytoplasmatic proteins that mark them for degradation, and proteasomes that consist of 20S and 19S subunits. This 26S proteasome is barrel-shaped and breaks proteins into short peptides that are recycled by peptidases (Wong and Cuervo, 2010). Protein degradation is an ATP-dependent process and the pathway is well-defined in eukaryotes: Target proteins destined to be degraded are first covalently bound to ubiquitin via E1 enzymes in energy consuming reaction. More ubiquitin molecules follow with the help of ubiquitin-carriers, E2 enzymes, and ubiquitin ligases, E3 enzymes. Eventually these reactions lead to a ubiquitin chain that is recognized by the regulatory particle of the 19S proteasome subunit. Here the protein is unfolded prior to degradation by the 20S proteasome subunit and its components are hydrolyzed to amino acids and re-introduced to the cellular metabolism (Haas and Siepmann, 1997; Glickman and Ciechanover, 2002). The UPS is an essential cellular pathway whose deregulation can lead to accumulation of harmful—misfolded peptide chains, excess amounts or protein, and lead to pathological conditions including neurodegeneration (Bedford et al., 2008).

Following the discovery of Lewy bodies staining positive for ubiquitin (Kuzuhara et al., 1988), evidence of proteasomal dysfunction in PD was uncovered (McNaught and Jenner, 2001). Supporting this evidence are is a rat model of PD which is induced by proteasome inhibitors (McNaught and Olanow, 2006) that shows the core pathological features including motor deficits and degeneration of dopaminergic neurons. Proteasome activity is also reduced in toxin-induced models of PD, namely following

exposure of cells and animals to rotenone and MPTP (Fornai et al., 2005; Betarbet et al., 2006; Chou et al., 2010).

The autophagy-lysosomal pathway encompasses all degradation processes whose ultimate target is the lysosome or lysosomal membrane. Lysosomes are membrane-enclosed vesicles that contain a variety of cellular proteases, hydrolases and maintain an acidic pH (Cuervo and Dice, 1998). Autophagy is a process intimately linked to the functioning of the lysosome, as autophagic vesicles will fuse with the lysosomal membrane in order for the hydrolases to digest the content of the autophagosome. It is the process by which especially cytosolic proteins are degraded and can be roughly structured into macroautophagy, microautophagy, and chaperone mediated autophagy (CMA) (Mizushima et al., 2002). During macroautophagy, a double membrane forms around proteins or organelles, and the resulting vesicle is called the autophagosome (Marzella et al., 1981; Mizushima et al., 2002). It is induced by starvation or injury and acts in conjunction with autophagy-related-genes (Atgs), which regulate the different pathways. The autophagosome will eventually fuse with a lysosomal membrane and release its content into the lysosome (Cuervo and Dice, 1998; Wong and Cuervo, 2010). The small GTPase Rab7 is a key regulatory factor in this process (Jäger et al., 2004). Meanwhile, microautophagy describes the direct absorption of cytoplasmatic proteins into the lysosome via invagination of the membrane (Marzella et al., 1981). CMA on the other hand presents a selective and specific pathway that involves heat shock complex (hsc) 70. Hsc70 is a chaperone, that will transport a protein to the lysosome if it encounters an hsc70 –recognition site on the protein in question (Cuervo and Dice, 1998; Bandyopadhyay et al., 2008). The whole complex is then transported to and admitted into the lysosome via receptor binding, where the protein is unfolded and degraded (Cuervo and Dice, 1998).

ASYN degradation seems to follow both pathways, although degradation of the protein is not fully understood in pathological conditions. However, mounting evidence suggests that both the UPS as well as the autophagic pathway are involved (Kuzuhara et al., 1988; Webb et al., 2003; Cuervo et al., 2004; Lee et al., 2004; Cookson, 2005; Qiao et al., 2008; Sevlever et al., 2008; Klucken et al., 2012; Petroi et al., 2012; Crabtree et al., 2014).

LBs are ubiquinated, indicating that they might be subject of degradation by UPS (Kuzuhara et al., 1988; Cookson, 2005). Furthermore, Parkin, an E3 ligase, is a gene

associated with early onset of PD upon mutation and is also localized in LBs (Kitada et al., 1998; Schlossmacher et al., 2002)

ASYN degradation by the lysosomal pathway may either present a parallel or an alternative mode of clearance. Autophagy-induction by the drug rapamycin leads to increased degradation of ASYN (Webb et al., 2003), while ASYN mutation impairs CMA (Cuervo et al., 2004). After translocation to the lysosome, the lysosomal protease CatD was shown to degrade wt ASYN (Qiao et al., 2008; Sevlever et al., 2008; Crabtree et al., 2014). Previous studies further showed oligomeric ASYN in cell culture (Lee et al., 2004) and aggregates in yeast, a cell model, as well as patient brains (Klucken et al., 2012; Petroi et al., 2012) to be degraded by the lysosome and vacuole respectively. CatD is proteinase located in the lysosome requiring pH ranges from 3.5-5, which is synthesized as an inactive precursor protein called Procathepsin D (Hasilik et al., 1982; Fusek and Vetvicka, 2005). Only upon association with the lysosome and maturing to its mature form by cleavage is CatD functional (Hasilik et al., 1982; Fusek and Vetvicka, 2005; Guha and Padh, 2008). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well-known substrate of the lysosome (Aniento et al., 1993) and can be used, alongside CatD, to assess lysosomal function. However, due to its surface cysteines, GAPDH is redox sensitive and prone to form aggregates (Nakajima et al., 2007). The same study found GAPDH aggregation to be promoted by β-amyloid.

A failing or overwhelmed degradation system may not be causative if PD, but can thus be considered a contributing factor.

2. Aim of this study

The objective of this study was to investigate the mutual effects between ASYN and intracellular trafficking pathways. For this purpose we screened a library of EGFP tagged Rab GTPases in a cellular model that displays the formation of intracytoplasmatic ASYN inclusions. We identified pathways impacted by ASYN inclusion formation and compared these findings with the expression of non-inclusion prone ASYN in the same cells. We further investigated whether Rab GTPases of the selected pathway had an influence on ASYN inclusion formation or cellular ASYN distribution. This study examined ASYN secretion and toxicity in the presence and absence Rab GTPase overexpression. Afterwards we compared these findings in both our employed models. Finally, we analysed the identified pathways in a mouse model of ASYN pathology.

3. Materials and Methods

Unless indicated otherwise, materials were purchased at Carl Roth GmbH (Karlsruhe, Germany) or Sigma Aldrich (St. Louis, MO, USA).

3.1 Mice

Animal care was conducted in house, according to institutional guidelines with a 12 h light / 12 h dark cycle and ad libitum access to food and water. Genotypes of Thy1-aSyn were determined prior to start of the experiment via polymerase chain reaction (PCR) from DNA isolated from tail tissue. Only male mice were used for experiments as the ASYN transgene was inserted on the X-chromosome and random X-inactivation might otherwise interfere with transgene expression (Rockenstein et al., 2002; Chesselet et al., 2012; Magen et al., 2012). Thy1-ASYN of 13 weeks, 26 weeks and 39 weeks were obtained and sacrificed by cervical dislocation. Wt littermate controls were used for all experiments. Cortex (CTX), Hippocampus (HC), Midbrain (MB) and Striatum (STR) were isolated for analysis of protein expression.

3.2 Molecular Biology Methods

3.2.1 Transformation of *Escherichia coli* DH5α

Competent *E. coli* cells were thawed on ice. 0.5 µg/µl of plasmid were added to the competent cells and incubated on ice for 30 minutes. Each sample was heat-shocked at 42° C for 30 seconds and incubated on ice for 2 minutes. 250 µl of SOC-Medium was added to each sample and incubated for 1 hour at 37° C while horizontally shaking at 600 rpm (Thermomixer comfort, Eppendorf, Hamburg, Germany). For high yield expression, 150 ml LB medium were inoculated, supplemented with 100 mg/ml Kanamycin or 200 mg/ml Ampicillin. For low yield expression, 5 ml LB medium were inoculated, supplemented with 100 mg/ml Kanamycin or 200 mg/ml Ampicillin.

3.2.2 Plasmid Isolation

3.2.2.1 Small Scale Plasmid Isolation

Plasmids were purified with the Invisorb Spin Plasmid Mini Two (STRATEC Biomedical AG, Birkenfeld, Germany) kit according to the manufacturer's instructions.

3.2.2.2 Large Scale Plasmid Isolation

Plasmids were purified with the NucleoBond Xtra Midi (Macherey & Nagel, Düren, Germany) kit according to the manufacturer's instructions.

3.2.3.3 Agarose Gel Electrophorersis

All samples were brought on a 1% agarose gel for plasmids or 2% agarose gel for PCR products from tail tissue and were run at 120 V. GeneRuler 1 kb DNA Ladder (Fermentas, Thermo Scientific, Waltham, MA, USA) was used as standard.

3.2.4 Polymerase Chain Reaction (PCR)

PCR was conducted for genotyping of animals. Tail tissue was digested by overnight incubation in 2.5 mg/ml Proteinase K. DNA was extracted via isopropyl ethanol precipitation. PCR sample preparation utilized 10x sample buffer (Genecraft, Cologne, Germany), dNTPs, $MgCl_2$, Taq Polymerase (Genecraft, Cologne, Germany), with primers Thy1 209F (CTGGAAGATATGCCTGTGGA) and Thy1 211R (GAGGAAGGACCTCGAGGAAT).

Reactions were run with the Eppendorf Master Gradient Cycler with 2-step PCR yielding a 110 bp product.

3.3 Cell Culture

Human neuroglioma H4 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; PAN-Biotech GmbH, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS; PAN-Biotech GmbH, Aidenbach, Germany) and 1% Penicillin/Streptamycin (PAN-Biotech GmbH, Aidenbach, Germany) at 37° C and 5% CO_2.

For *in vitro* experiments, cells were routinely plated 24 h prior to use at a density of $5*10^4$ cells/ml. Cell count was determined using a Neubauer counting chamber.

3.4 H4 Cell Transfection

3.4.1 Metafectene

H4 cells plated 24 h prior to transfection a density of $5*10^4$ cells/ml. Transfection was conducted according to manufacturer's instructions at a ratio of DNA:Metafectene (Biontex Laboratories GmbH, Planegg, Germany) of 1:2.

3.4.2 Calcium-Phosphate Transfection

H4 cells plated 24 h prior to transfection a density of $5*10^4$ cells/ml. Several hours before transfection, fresh medium was supplied. Transfection was conducted in 6-well multiwell plates (Corning Life Science, Corning, NY, USA), 12-well multiwall plates (Corning Life Science, Corning, NY, USA) on coverslips coated with 0.1% gelatine (VWR, Radnor, PA, USA) or 10 cm cell culture dishes (Corning Life Science, Corning, NY, USA). Table 2 shows the amounts of solutions for different well sizes. To transfect, 1xHBS (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mM NaCl, 5 mM KCl, 0.75 mM $Na_2HPO4*2H_2O$, 6 mM Dextrose, pH 7.1) was mixed with appropriate amounts of DNA. Right after, 2.5 M $CaCl_2$ was added and mixed again immediately. The mixture was incubated for 20 min before being added dropwise to the cells. The plate or dishes was rocked gently to mix transfection reaction with medium. After overnight incubation, medium was changed for full medium. Samples for further experiments were taken 48 h after transfection.

Table 2: Overview of $CaPO_4$ transfection reaction for different culture sizes

Format	Culture Media	Transfection Media	1x HBS	2.5 M $CaCl_2$
12 well	1 ml	600 µl	36 µl	2.1 µl
6 well	2 ml	1.5 ml	86 µl	5.1 µl
10 cm	10 ml	8 ml	500 µl	30 µl

3.5 Protein Chemistry

3.5.1 Immunocytochemistry

A list of primary and secondary antibodies can be found in Table 3.

H4 cells were fixed 48 h after transfection with 4% PFA in PBS for 20 min at RT. After washing once with 1x PBS, cells were permeabilized for 20 min with 0.1%-0.5% Triton X-100 at RT. After blocking for 1 h with 1.5% bovine serum albumin (BSA; NZytech, Lisbon, Portugal), samples were incubated with primary antibody diluted 1:500-1:2000 in 1.5% BSA, either 3 h at RT or over night at 4° C. Secondary antibody diluted 1:2000 in 1.5% BSA was added after washing three times 5 min with PBS for 2 h at RT. Samples were washed three times 5 min with PBS prior to incubation with Hoechst 33258 (Molecular Probes, Eugene, OR, USA) 1:5000 in PBS for 5 min. If immunocytochemistry was conducted on cover slips, these were subsequently mounted with Mowiol 4-88 (Calbiochem, Merck Millipore, Darmstadt, Germany). Samples were visualized with Leica DMI 6000B (Leica Camera, Solms, Germany).

Table 3: Primary and secondary antibodies used in immunocytochemistry

Antibody	Manufacturer	Order No	Dilution
Primary Antibodies			
Purified Mouse Anti-α-Synuclein	BD Bioscience	610787	1:2000
Rab 7 Antibody (H-50)	Santa Cruz Biotechnology	sc-10767	1:500
Secondary Antibodies			
Alexa Fluor 488 Donkey anti-mouse IgG	Molecular Probes	A21202	1:2000
Alexa Fluor 555 donkey anti-mouse IgG	Molecular Probes	A31570	1:2000
Alexa Fluor 488 donkey anti-rabbit IgG	Molecular Probes	A21206	1:2000
Alexa Fluor 555 donkey anti-rabbit IgG	Molecular Probes	A31572	1:2000

3.5.3 Solubilisation of Proteins and Protein Quantification

Following transfection, cells were solubilised 48 h later (unless indicated otherwise) with radioimmuniprecipitation assay (RIPA) buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Sodium-Dodecyl-Sulphate (SDS), 1% Nonidet P40, 0.5% Sodium-Deoxycholate, α-complete (La Roche, Basel, Switzerland)). Cell lysates were centrifuged 15 min at 10,000 g (Sigma 1-15 K, Sigma Aldrich Co. LLC, St. Louis, MO, USA) and supernatant was transferred into a new 1.5 ml reaction tube.

Brains were lysed in animals lysis buffer (250 mM Sucrose, 50 mM Tris pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$, 5 mM KCl, 12 mM Sodium-Deoxycholate, 1% Triton X-100, α-complete protease inhibitor cocktail (La Roche, Basel, Switzerland)) using Percellys 24 homogenisator (bertin technologies, Montigny-le-Bretonneux, France). Afterwards lysates were centrifuged 10 min at 4° C and 10,000 g. The supernatants were transferred into a fresh tube and proteins content was quantified.

Protein quantification was done on the basis of Bradford's assay for rapid protein quantification (Bradford, 1976). In short, 1 µl of cell lysate was added to 49 µl of sterile water and 150 µl of Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Inc.,

Hercules, CA, USA). Absorbance at 595 nm over 450 nm was measured using the Infinite M200 PRO (Tecan Ltd., Maennedorf, Switzerland) plate reader. All samples were measured in triplicate.

3.5.4 Western Blot Analysis

Detection of proteins on immunoblots was performed to detect ASYN, Rab GTPases fused to EGFP, untagged Rab7, CatD, β-actin and GAPDH.

3.5.4.1 SDS-PAGE

Cellular lysates or brain samples were separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 12%-15% separating gels with 7.5% stacking gels (Acrylamide/Bis-acrylamide ratio 37.5:1 (Carl Roth GmbH, Karlsruhe, Germany), 0.01 % SDS in ddH_2O, 0.1 % tetramethylethylenediamine (TEMED), and 1 % ammonumpersulfat (APS)). 40 µg of sample for cell lysates or 20 µg of sample for brain lysates were prepared with five time Laemmli buffer (250 mM Tris pH 6.8, 10% SDS, 1.25% Bromphenol Blue, 5% β-Mercaptoethanol, 50% Glycerol) and incubated 5 min at 96° C. Samples were loaded onto gel together with 5 µl PageRuler Plus Prestained Protein Ladder (Perbio Science Deutschland, Bonn, Germany). Electrophoresis was performed in one time SDS-Running Buffer (125 mM Tris, 960 mM Glycine) for 90 min at 120 V in Bio-Rad Mini-Protean 3 Mini Vertical Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3.5.4.2 Transfer of Proteins to Nitrocellulose Membrane

Subsequent to SDS-PAGE, sandwich tank Western Blot was performed by transferring proteins from SDS matrix to Protean nitrocellulose transfer membrane (Schleicher & Schuell Bioscience GmbH, Dassel Germany) using Bio-Rad Mini-Protean 3 Mini Trans-Blot module (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sandwich was assembled with blotting paper (Schleicher & Schuell Bioscience GmbH, Dassel Germany) and membrane pre-soaked in one time transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol). Transfer was conducted at 300 mA for 90 min.

3.5.4.3 Immunodetection

A list of primary and secondary antibodies used can be found in Table 4.

After protein transfer, nitrocellulose membranes were blocked with 5% milk or 5% BSA in tris-buffered saline (TBS) to remove unspecific signal for 1 h at RT. Afterwards, membrane was in primary antibody (Table 4) diluted in 5% BSA in TBS 1:1000-1:5000 supplemented with sodium acid for preservation purposes, overnight a 4° C. Membranes were washed three times for 5 min in TBS supplemented with 0.1% Tween (TBST). Afterwards, membranes were incubated for 30-45 min in secondary antibody conjugated with HRP (Table 4) diluted 1:20,000 in TBS. Membranes were washed three times for 5 min in TBST. Afterwards, membranes were visualized using AlphaImager (Alpha Innotech, San Leandro, CA, USA) or Fusion Fx (Vilber Lourmat, Marne-la-Vallée, France) with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA). Membranes were stripped or dried at RT and stored for potential future use. Densitometric quantification was conducted using Image J 1.49m, distributed by the National Institute of Health, USA.

Table 4: Primary and secondary antibodies used in Western blot analyses

Antibody	Manufacturer	Order No	Dilution
Primary Antibodies			
Purified Mouse Anti-α-Synuclein	BD Bioscience	610787	1:2000
Cathepsin D Antibody (C-20)	Santa Cruz Biotechnology	sc-6486	1:5000
GAPDH Antibody	Thermo Scientific	PA1-988	1:2000
Rab 7 Antibody (H-50)	Santa Cruz Biotechnology	sc-10767	1:1000
Monoclonal Anti-ß-Actin antibody	Sigma-Aldrich	A5441	1:10,000
Secondary Antibodies			
ECL Mouse IgG, HRP-Linked	GE Healthcare	NXA931	1:20,000
ECL anti rabbit IgG, HRP-Linked	GE Healthcare	NA934V	1:20,000
HRP-conjugated donkey anti-goat	Jackson Immunoresearch	705-035-003	1:20,000

3.5.4.4 Membrane Stripping

To remove primary and secondary antibody for detection of more proteins, membranes were stripped by incubating them in stripping buffer (200 mM Glycine, 500 mM NaCl, pH 2.8) for 15-20 min. Subsequently, membranes were re-blocked and antibody incubation could proceed.

3.5.5 Immunodetection of ASYN with Sandwich ELISA

For a list of antibodies employed see Table 5.

In order to detect ASYN released by cells into the extracellular space, we performed direct enzyme-linked immunosorbent assay (ELISA), so called sandwich ELISA (compare Figure 5). One day prior to experiment, a 96 well plate, High Bind, polystyrene, flat bottom (Corning, Corning, NY, USA) was coated with 1 µg/ml capture antibody against ASYN diluted in PBS (PAN-Biotech GmbH, Aidenbach, Germany). The plate was incubated overnight at 4°C covered with laboratory film. The next day the plate was washed five times using the Multidrop Combi (Thermo Scientific, Waltham, MA, USA) with ELISA washing buffer (0.0325 mM $NaH_2PO_4*H_2O$, 24.5 mM $Na_2HPO_4*2H_2O$, 150 mM NaCl, 0.1% Tween-20, pH 7.5). The plate was blocked for at least 2 h at RT with ELISA blocking buffer (PBS, 0.1% BSA) shaking at 900 rpm on the OS-500 plate shaker (VWR, Radnor, PA, USA). Sample standards were prepared with recombinant monomeric ASYN (with thanks to Dr. Anna Villar-Piqué) in ELISA incubation buffer (PBS, 0.01% BSA, 0.1% Tween-20) in duplicate or triplicate at concentrations of 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 2.5 nM, 5 nM, 10 nM, 25 nM, 50 nM, 75 nM and 100 nM. After blocking, plate was washed 5 times with ELISA washing buffer. Sample standards and 200 µl sample were loaded onto the plate, samples were loaded either in technical duplicates or triplicates. The plate was incubated for 2 h at RT shaking at 900 rpm on the OS-500 plate shaker (VWR, Radnor, PA, USA) covered in laboratory film. Afterwards, plate was washed 5 times times with ELISA washing buffer. An α/β/γ-synuclein antibody was used as primary detection antibody and it was prepared at 1 µg/ml in ELISA incubation buffer. The plate was incubated 1 h at RT shaking at 900 rpm covered in laboratory film. Afterwards, plate was washed 5 times times. HRP-conjugated anti-rabbit antibody was used for secondary detection at a concentration of 1:10,000 diluted in ELISA incubation buffer. The plate was incubated for 45-60 min at RT shaking at 900 rpm covered in laboratory film. Subsequently, the plate was washed 5 times with ELISA washing buffer. Room tempered

TMB Chromogen Solution (Life Technologies, Carlsbad, CA, USA) was used as HRP substrate and 100 µl/well were incubated with the samples. The reaction was stopped after 1-30 min, when the blue color had sufficiently developed but before signal saturation, with 1 M sulfuric acid. The absorbance at 450 nm was measured using the Infinite M200 PRO (Tecan Ltd., Maennedorf, Switzerland). Calculations were conducted by nonlinear regression curve fit with sigmoidal dose-response equation using Prism 4.03.

Table 5: Antibodies used in sandwich ELISA

Purpose	Antibody	Manufacturer	Order No	Dilution
Capture	Human Synuclein-alpha MAb (Clone 189105)	R&D Systems	MAB1338	1 µg/ml
Primary Detection	α/β/γ-synuclein Antibody (FL-140)	Santa Cruz Biotechnology	sc-10717	1 µ/ml
Secondary Detection	ECL anti rabbit IgG, HRP-Linked	GE Healthcare	NA934V	1:10,000

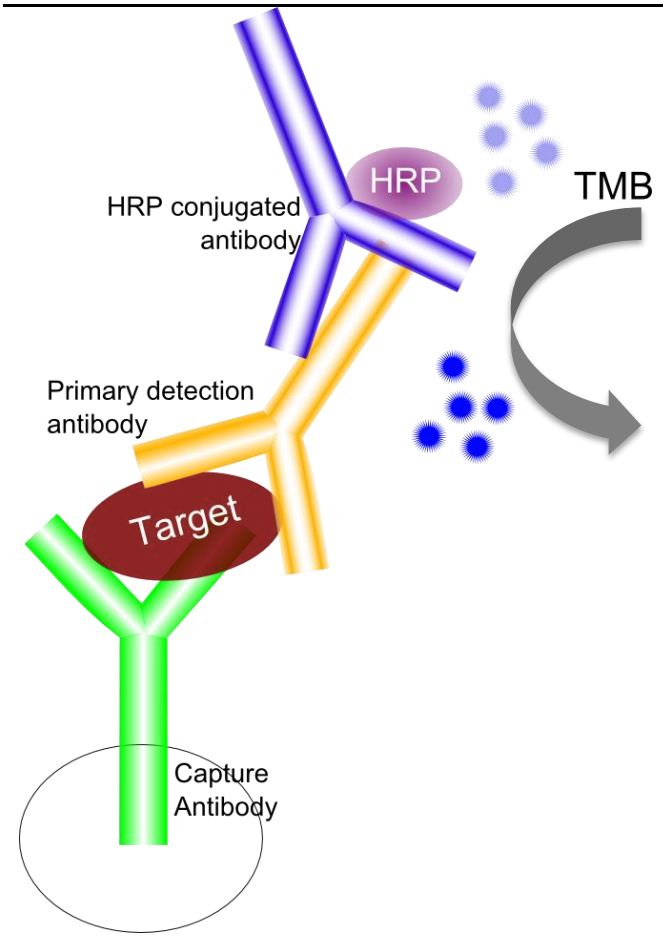


Figure 5: Schematic of sandwich ELISA. A microtiter plate is coated with capture antibody targeted against epitope of protein of interest. The target protein binds to the capture antibody upon incubation. A second antibody targeted against another epitope is employed as primary detection antibody. A third antibody targeted against the primary detection antibody and conjugated with horse radish peroxidase (HRP) is used for detection. The HRP catalyzes the TMB substrate into a blue precipitate.

3.5.6 Size Exclusion Chromatography

To detect a shift in particulate size of ASYN, SEC was performed using high performance liquid

chromatography (HPLC). H4 cells were grown in 10 cm cell culture dishes dishes and transfected with a total of 25 µg ASYN, GFP, Rab5 and Rab7 constructs. 48 h after transfection, cells were collected in phosphate lysis buffer (PBS, 0.5% Triton X-100), freshly supplemented with protease inhibitor α-complete (La Roche, Basel, Switzerland) and centrifuged for 15 min at 10,000 g to remove cellular debris. After total amount of protein was determined, 2-3 mg total protein in a volume of 500 µl was filtered through a 0.45 µm Spin-X centrifuge filter and then loaded onto a Superose 6 (Superose 6 10/300GL, GE Healthcare Life Science, Sweden) column with subsequent performance of HPLC (Äkta Purifier 10, GE Healthcare Life Science, Sweden) in 50mM ammonium acetate pH 7.4 buffer with a flow rate of 0.5 ml/min (Äkta Purifier operation was conducted by Tomás Lopes da Fonseca). Fractions of 500 µl were collected and stored at 4° C up until 24 h after collection.

3.5.6.1 Dot Blot

All HPLC samples were boiled for 10 min at 95° C at 650 rpm and then spun down briefly at 10,000 g and 4° C. Samples were loaded fully into two rows of a 96 well Dot Blot machine constructed in house. A vacuum pump was used to suck sample through a 0.2 µm pore size Protean nitrocellulose membrane (Schleicher & Schuell Bioscience GmbH, Dassel, Germany). The membrane was subsequently blocked with 5% skim milk in TBS to prevent unspecific staining for 1 h. Membranes were incubated with primary antibody diluted in 1% skim milk in TBS or 5% BSA in TBS to 1:2000 overnight at 4° C. Membranes were washed three times with TBST. Subsequently, membranes were incubated with secondary antibody diluted 1:10,000 in TBS. Afterwards, membranes were visualized using Fusion Fx (Vilber Lourmat, Marne-la-Vallée, France) with Immobilon Western Chemiluminescent HRP Substrate (Merck Millipore, Billerica, MA, USA).

3.6 Cell Viability

48 h after transfection, culture supernatant from 6-well multiwell plates was collected and cytotoxicity was determined via release of LDH into the culture medium with the Cytotoxicity Detection Kit (LDH) (La Roche Ltd, Basel, Switzerland). Basic LDH release was measured from vehicle-treated controls for background correction. Total LDH release was determined by incubating cells in culture medium supplemented with 2% Triton X-100. Absorbance of 96 well plate with samples was measured at 490 nm with the Infinite

M200 PRO (Tecan Ltd., Maennedorf, Switzerland). Experimental values were calculated in percentages relative to total lysis and normalized to mock transfected cells.

4. Results

4.1 Screen of Rab GTPase library with model of ASYN inclusion formation identifies putative targets

The family of Rab GTPases are integral parts of the intracellular vesicle trafficking machinery with a variety of tasks and up to four isoforms for proteins. Although some members of this family could already be identified to be involved in PD and be dysregulated with ASYN pathology, a comprehensive screen with a single model was missing as of the conception of this study. In order to address this, we screened a Rab GTPase library containing almost all human Rab GTPases (a kind gift from Prof. Mikael Simons) and their isoforms in the human H4 neuroglioma cell line. These we cotransfected with a previously described variant of ASYN that is modified at the C-terminus (SynT), rendering it prone to inclusion formation (Figure 7B). This model has the advantages of forming LB-like inclusions in a cellular context (McLean et al., 2001; Klucken et al., 2012; Yin et al., 2014). All Rabs are EGFP tagged and SynT was visualized via immunocytochemistry (Figure 7B). All experiments were evaluated using epifluorescence microscopy and were analyzed for changes either in the presentation of the Rab protein or the SynT. Each experiment was accompanied by a control that expressed an empty vector alongside the target protein, Rab GTPase or SynT. Table 6 summarizes the findings, which include both changes to the intracellular distribution of various Rab proteins as well as to the presentation of the SynT inclusions. All experiments were conducted a total of three times to exclude handling errors, but evaluation initially was of qualitative, rather than quantitative nature. All observed effects were consistent in all experiments. Each Rab protein has a distinct pattern of localization. Rabs located to the membrane of the same compartment have a similar distribution pattern, but neither exactly the same. This was also true for isoforms. The pattern of distribution and the localization of the Rabs changed dramatically upon coexpression of SynT. Generally, many Rab GTPases colocalized at least partially with ASYN positive inclusions and lost their distinct distribution pattern, instead they took on a diffuse presentation throughout the cytoplasm. Rab7 was an intriguing exception here, as it showed no colocalization with inclusions in any of the experiments. Contrary to observations made of the Rabs, only few effects on SynT could be determined:

coexpression with Rab7 yielded an apparent increase in inclusion formation and no localization with the inclusions, while coexpression of Rab8A and SynT resulted in fewer condensed nuclei, hinting at a decrease in toxic effect and Rab35 seemed to shift SynT localization to the nucleus.

Table 6: Rab GTPase library screen reveals effects of ASYN inclusion formation on intracellular trafficking proteins

Rab GTPase	Organelle Localization / Target Membrane (Hutagalung and Novick, 2011)	Effect on Rab distribution with SynT coexpression	Effect on SynT inclusions with Rab coexpression
Rab1A	ER, Golgi	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab1B	ER, Golgi	Diffuse presentation in the cytoplasm, colocalization SynT	No effect
Rab2A	ER, ER-Golgi intermediate department, Golgi	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab2B	ER, ER-Golgi intermediate department, Golgi	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab3A	Secretory vesicle, plasma membrane	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab3B	Secretory vesicle, plasma membrane	Maintenance of localization, colocalization SynT	No effect
Rab3C	Secretory vesicle, plasma membrane	Maintenance of localization, colocalization SynT	No effect
Rab3D	Secretory vesicle, plasma membrane	Maintenance of localization, colocalization SynT	No effect

Rab4A	Early endosome	Partial maintenance of localization, colocalization with SynT inclusions	No effect
Rab4B	Early endosome	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab5A	Early endosome	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab5B	Early endosome	Partial maintenance of localization, colocalization with SynT inclusions	No effect
Rab5C	Early endosome	Partial maintenance of localization, colocalization with SynT inclusions	No effect
Rab6A	Golgi	Partial maintenance of localization, colocalization with SynT inclusions	No effect
Rab6B	Golgi	Partial maintenance of localization	No effect
Rab7	Late endosome,lysosome, phagosomes	Maintenance of localization	Increase of inclusion formation
Rab8A	Secretory vesicle, plasma membrane	Colocalization with SynT and SynT inclusions	Fewer condensed nuclei
Rab8B	Secretory vesicle, plasma membrane	Partial maintenance of localization	No effect

Rab11A Q70L	Golgi, recycling endosome, early endosome	Maintenance of localization, partial colocalization SynT inclusions	No effect
Rab11A S25N	Golgi, recycling endosome, early endosome	Maintenance of localization	No effect
Rab12	Golgi, secretory vesicle	Diffusion through the cytoplasm	No effect
Rab13	Cell/tight junctions, TGN, recycling endosome	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab15	Early/sorting endosome	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect
Rab17	Recycling endosome	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab18	Golgi, lipid droplets	Partial maintenance of localization	No effect
Rab20	Golgi, endosome	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect
Rab22A	Early endosome	Diffusion through the cytoplasm	No effect
Rab23	Plasma membrane, endosome	Maintenance of localization	No effect
Rab25	Recycling endosome	Partial maintenance of localization, partial colocalization with	No effect

		SynT inclusions	
Rab26	Secretory granules	Partial maintenance of localization	No effect
Rab27A	Secretory vesicles	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab27B	Secretory vesicles	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab28	Unknown	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab30	ER, Golgi	Partial maintenance of localization	No effect
Rab31	TGN, endosome	Partial maintenance of localization, partial colocalization with SynT inclusions	No effect
Rab32	Mitochondria	Maintenance of localization	No effect
Rab33A	Golgi, dense-core vesicles	Partial maintenance of localization, partial colocalization with SynT inclusions	No effect
Rab33B	Autophagoome	Partial maintenance of localization, partial colocalization with SynT inclusions	No effect

Rab35	PM, endosome	Maintenance of localization	Observed decrease in inclusion formation, relocalization of SynT to the nucleus/nuclear envelope
Rab36	Golgi	Maintenance of localization	No effect
Rab37	Secretory granules	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect
Rab38	Melanosomes	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect
Rab40A	Golgi, ER	Diffuse presentation in the cytoplasm, colocalization with SynT inclusions	No effect
Rab40B	Golgi, ER	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect
Rab40C	Golgi, ER	Relocalization to the vicinity of the nucleus	No effect
Rab43	ER, Golgi	Diffuse presentation in the cytoplasm, partial colocalization with SynT inclusions	No effect

4.2 Rab GTPases colocalize with ASYN inclusions and modulate their formation

Cell survival is a crucial parameter in the investigation of neurodegenerative diseases and Rab8A was shown in the past to have a beneficial effect in regards to ASYN mediated toxicity (Gitler et al., 2008). Rab8A is located in the TGN and at tubular recycling endosomes (Figure 6A), regulating exocytosis and other sorting events. We could show that Rab8A colocalized with SynT mediated inclusions (Figure 6B) and overexpression of Rab8A resulted in an increase of cells with inclusions by 30% (Figure 6C). At the same time, the S129 phosphomimetic mutant of SynT, S129D, increased the number of inclusions per cell (Figure 6D) and a block of phosphorylation with the S129A mutation resulted in more cells without aggregates (Figure 6D). (Assessment of inclusion formation and statistical analysis were performed by Tomás Lopes da Fonseca.)

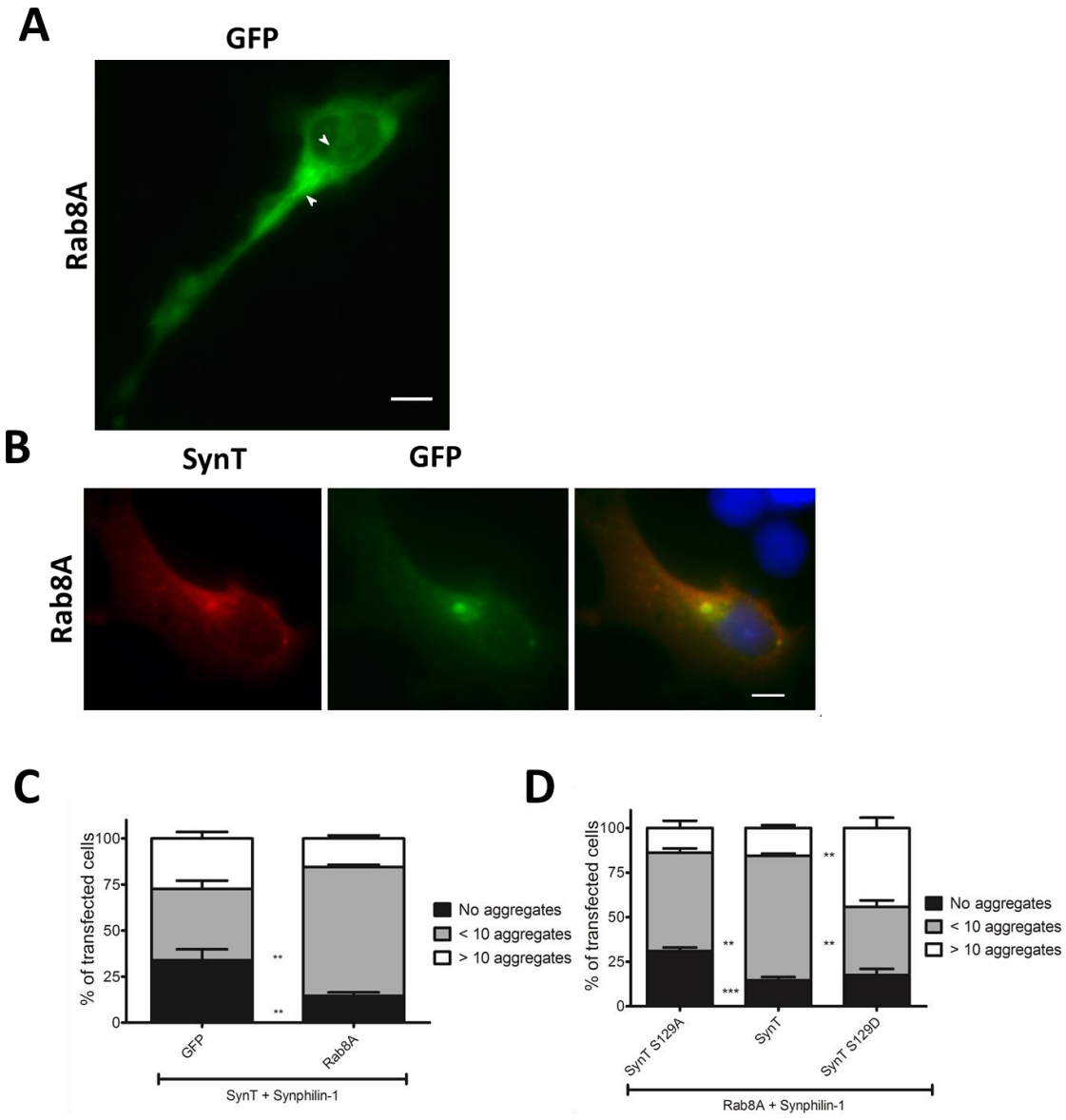


Figure 6: Rab8A modulates ASYN inclusion formation cells. (A) Representative live cell image of H4 cells expressing Rab8A. The arrowhead marks typical structure of tubular endosomal compartments. (B) Rab8A loses its localization and instead colocalizes with SynT mediated inclusions. (C) The presence of the phosphomimetic variant S129D of SynT increases the number of inclusions per cell but not the total percentage of cells with aggregates. On the other hand the S129A SynT mutant showed a reduction in the percentage of cells with inclusions. ** $p \leq 0.01$, *** $p \leq 0.001$ Two-way ANOVA. Scale bar: 20 µm. (Statistics originally published in 2014 (Yin et al., 2014).)

We selected Rab5A and Rab7 because they occupy the same target membrane at different maturation stages of the vesicle, the endosome, albeit at different domains. Both are important regulators of trafficking steps in the endocytic-lysosomal pathway and are key proteins for the progression through the degradation pathway. Both have a

similar, yet distinctly different distribution pattern in the cell (Figure 7A). Rab5A is located at early endosomes, also called sorting endosomes, where it mediates fusion of endocytic vesicles with the early endosome as well as helps sorting cargo into different endosomal compartments. The decision about the fate of internalized cargo is made at the sorting endosome and can enter either the endocytic recycling or endocytic degradation pathway. Overexpression of Rab5A leads to enlarged endosomes. Rab7 is an identifier of late endosomes and lysosomes—vesicles that maintain a mild to strong acidic environment—and mediates progression through the autophagic pathway. Overexpression of Rab7 leads to more and enlarged lysosomes that are often localized in the perinuclear region.

When coexpressed with SynT, the subcellular localization of EGFP-Rab5A, but not EGFP-Rab7, changed in the presence of ASYN positive inclusions (Figure 7C). EGFP-Rab5A colocalized with ASYN inclusions in a in a significantly stronger manner than EGFP-Rab7 (Figure 7D). Next we determined the percentage of cells with ASYN inclusion formation in control conditions and with co-expression of EGFP-Rab5A or EGFP-Rab7. In comparison we found no significant difference between control conditions and co-expression with EGFP-Rab5A, although a trend could be established towards an increase in inclusion formation (Figure 7E). With EGFP-Rab7 co-expression, significantly more cells showed inclusion formation than in control conditions (Figure 7E).

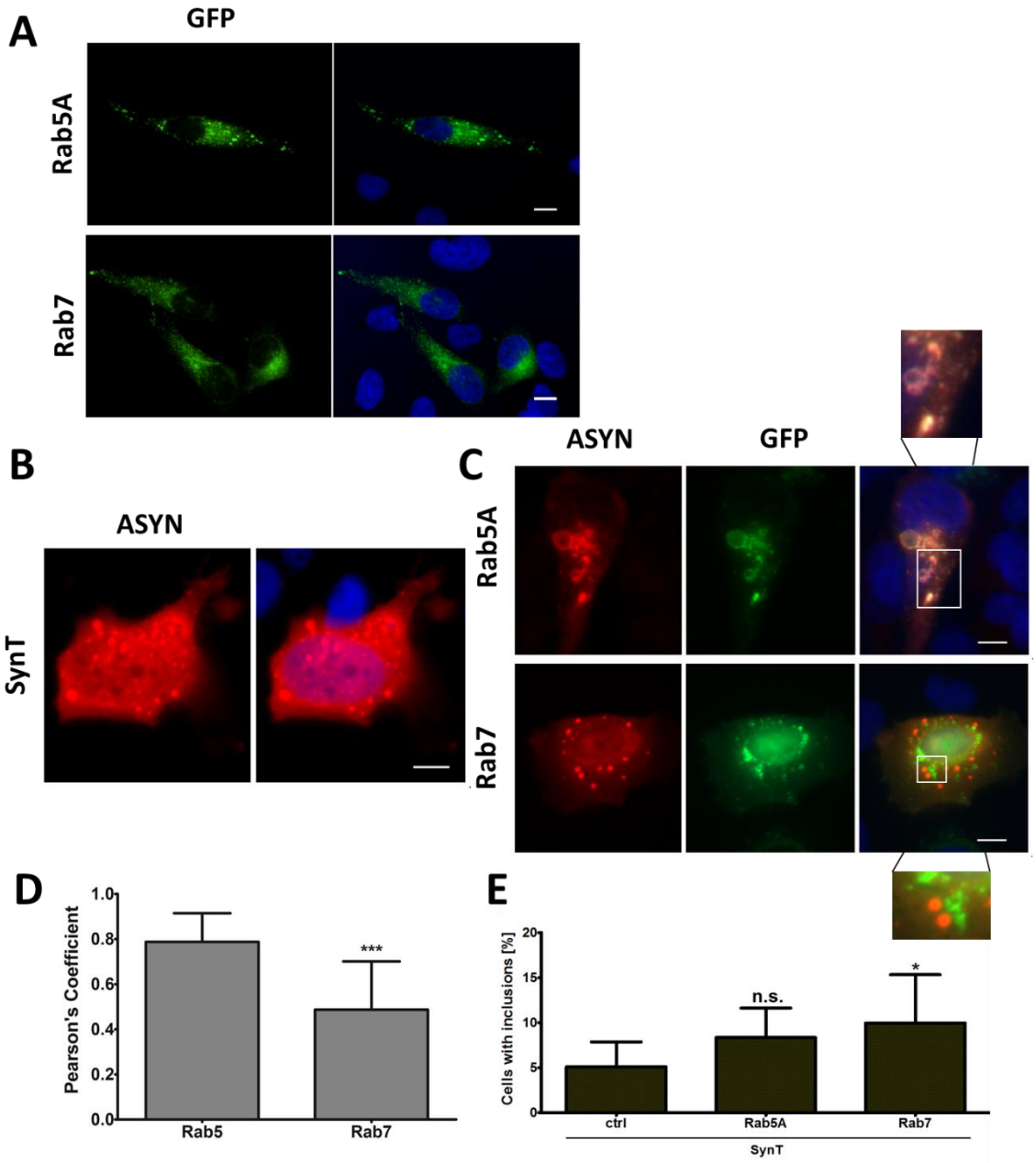


Figure 7: ASYN inclusions lead to mislocalization of trafficking markers and Rab7 modulates inclusion formation. (A) Rab5A and Rab7 distribution in the cell. Both Rab proteins are located at their target membranes with vesicular distribution. (B) ASYN inclusion formation due to a modified C-terminus, termed SynT. (C) Expression of SynT with Rab GTPases. Rab5A is localized away from its target membrane and localizes with ASYN inclusions. Rab7 does not colocalize with ASYN inclusions. Insets show blow up images of regions of interest (ROI) framed in white. (D) Rab5A shows higher colocalization with ASYN inclusions than Rab7. (D) Rab7 increases the percentage of cells showing inclusions. All statistical analysis are from n=3-4 independent experiments and represent mean ± S.D.; * $p \leq 0.05$ One-tailed paired t-test, *** $p \leq 0.001$ Two-tailed paired t-test, Scale bar: 10 µm.

Expression of wt ASYN lacks inclusion formation in the way we observed with SynT (Figure 7B, Figure 8A). Instead, ASYN is distributed throughout the cytosol with little to no obvious structural features (Figure 9A). We also see no formation of inclusions anywhere in our investigated cells when we co-express Rab GTPases (Figure 8B). However upon investigation of subcellular localization under these conditions, we could determine a strong trend towards a far more structured presentation of ASYN than in our previous observations (Figure 8B). These structures look not unlike vesicles. At the same time, investigations into the subcellular localization showed a strong colocalization with both EGFP-Rab5A and EGFP-Rab7 (Figure 8B, C). We could further show that while ASYN colocalized with EGFP-Rab5A, the effect with EGFP-Rab7 co-expression was significantly stronger (Figure 8C).

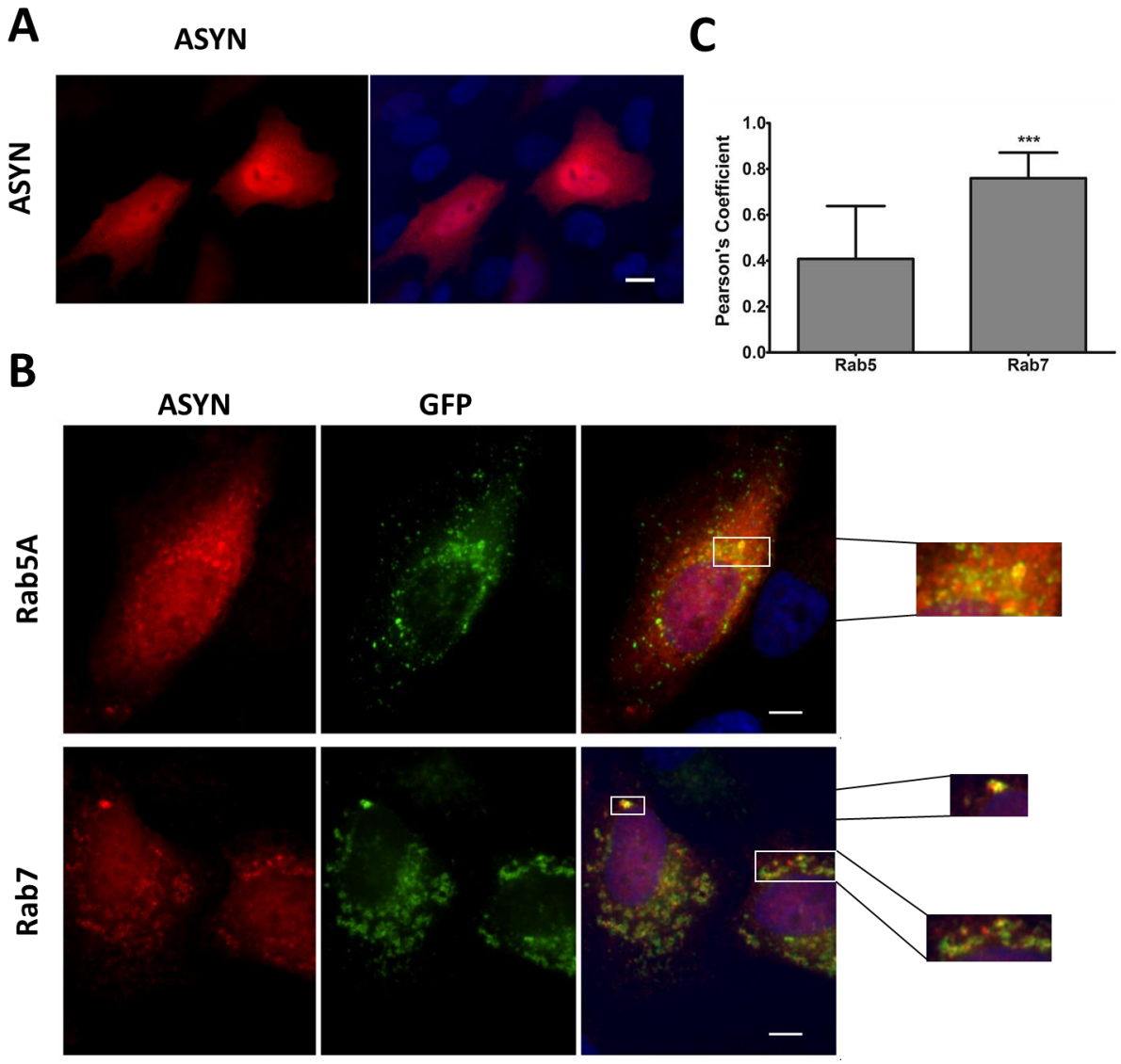


Figure 8: Wild type ASYN vesiculates with the overexpression of Rab GTPases. (A) Wt ASYN is distributed in the cytosol with few structural features. (B) Wild type ASYN presents in vesicular structures that colocalize with Rab5A and Rab7 when either is overexpressed. Insets show blow up images of ROI framed in white. (C) Native ASYN colocalizes stronger with Rab7 positive structures than Rab5A positive structures. All statistical analysis are from n=3-4 independent experiments and represent mean ± S.D.; *** $p \leq 0.001$ Two-tailed paired t-test, Scale bar: 10 µm

4.3 ASYN particulate size is modulated by endosomal Rab GTPases

The vesiculation of wt ASYN in the presence of endosomal Rab GTPases is an unexpected side effect we observed during the expression of Rab5A and Rab7 alongside ASYN (Figure 8B). We were curious whether this meant a change in ASYN conformation. Generally, ASYN is a natively unfolded monomer with membrane binding capabilities, although it has been argued that ASYN presents as a tetramer in an intracellular context (Bartels et al., 2011; Wang et al., 2011). However, different genetic contexts might be able to alter the organization state of proteins.

To investigate the particulate size of ASYN, we employed SEC via a high performance liquid chromatography (HPLC) machine and a dot blot apparatus made in house. A schematic can be seen in Figure 9A. Blotting the A1-D11 fractions revealed a size shift of ASYN from the C3-C6 fractions, corresponding to 44 kDa to 17 kDa (Figure 9B), which is concurrent with the monomeric fraction in our hands, to fractions spanning B1 to C7 fractions in the presence of EGFP-Rab5A (Figure 9C). These correspond to high molecular weight oligomers of up to 670 kDa or above. In the presence of EGFP-Rab7, we could observe a similar shift in size, although these concentrated in the upper B and C fractions (Figure 9D). Figure 9E shows a graphical representation of the Dot Blots, and a clear shift is visible from the lower molecular weight fractions when ASYN is expressed only alongside EGFP to higher molecular weight fractions when endosomal Rab GTPases are present.

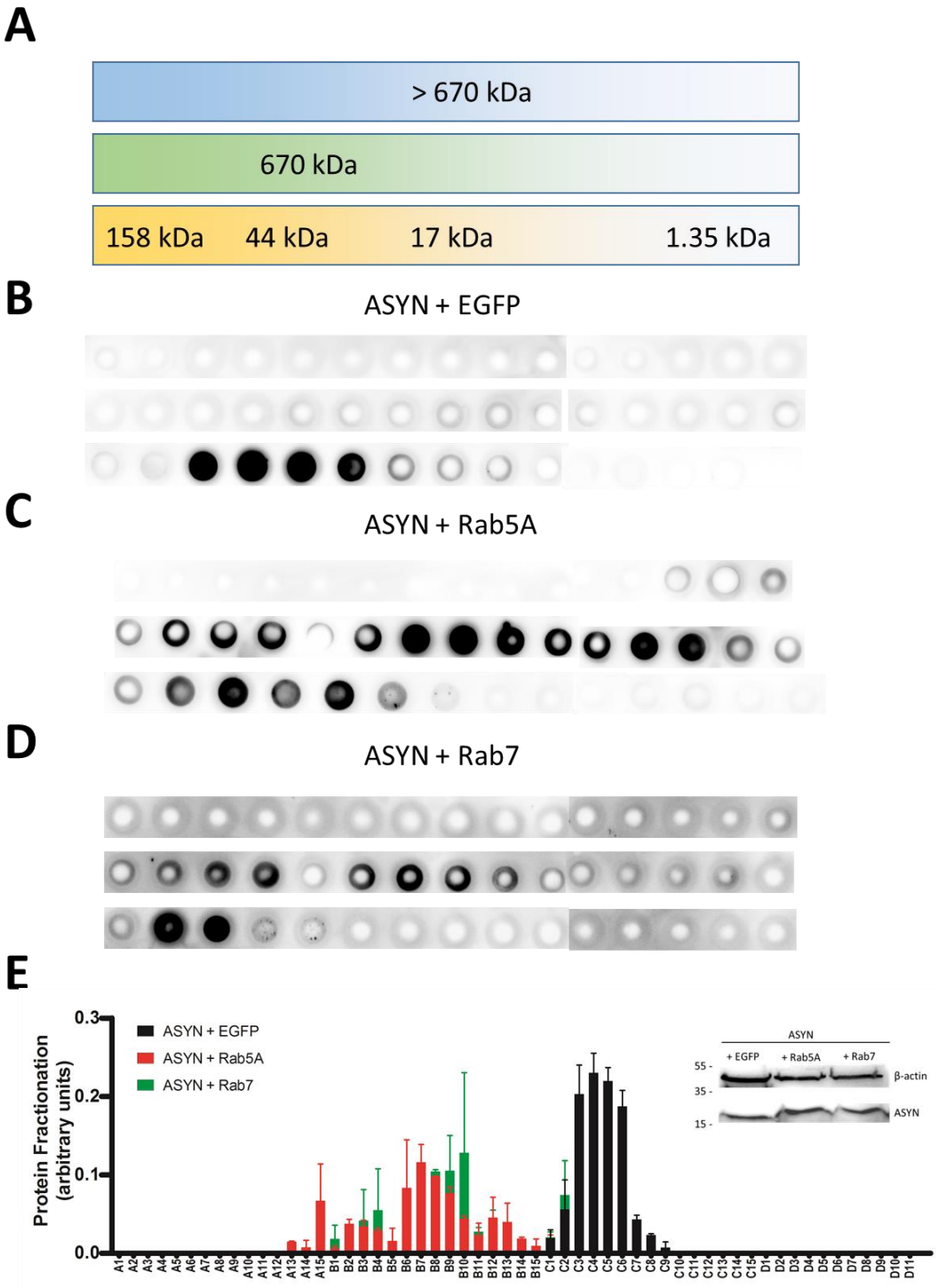


Figure 9: Rab GTPases change the particulate size of ASYN. (A) Size schematic of the HPLC fractions, blue: Fraction A1-A15, void volume; green: Fraction B1-B15, 670 kDa; yellow: Fraction C1-C15, 150 kDa – 1.35 kDa. (B) Dot Blot of control condition, ASYN and EGFP, only monomeric ASYN can be found in the C fraction. (C) ASYN and Rab5A coexpression, a shift to higher molecular sizes from 670 kDa to monomeric sizes. (D) ASYN and Rab7 coexpression lead to a shift to 670 kDa to 158 kDa. (E) Graphical representation of ASYN fractioning with different genetic contexts: co-expression with GFP (black), Rab5A (red) and Rab7 (green). Inset shows ASYN protein levels remain the same; β-actin was employed as loading control. All experiments are representative of n=3 independent experiments.

4.4 Endosomal Rab GTPases modulate ASYN secretion and toxicity, depending on their cellular function

While Rab5A is not directly involved in the secretory pathway, it is an important mediator of the modular system of endosomes. This system includes not only the early and late endosome, but also the recycling endosome. Each of these compartments is the domain of different Rab GTPases with different functions, although all of them are connected to the sorting endosome. Hence, manipulating early endosomal markers in ASYN overexpressing cells might lead to an effect on secretion.

For this purpose, human H4 neuroglioma cells were cotransfected with either SynT or wt ASYN, as well as EGFP-Rab5A or EGFP-Rab7. To investigate whether any observed effects were a direct results of the overexpression of the Rab protein, we also transfected the constitutive active, GTPase activity deficient mutants EGFP-Rab5A Q79L and EGFP-Rab7 Q67L as well as the dominant native, GDP-bound mutants EGFP-Rab5A N133I and EGFP-Rab7 T22N (all mutants generous gifts of G. Schiavo and C. Bucci) in parallel. The experiments were performed utilizing a Sandwich ELISA, the principal idea of which is displayed in Figure 5. Briefly, a high binding microtiter plate is coated with a capture antibody, which is highly specific for an epitope on ASYN. The sample is incubated with the plate before a second antibody is used for primary detection. Lastly, a secondary detection antibody targeted against the primary detection antibody coupled with horse radish peroxidase is used for detection. An enzymatic substrate reveals protein levels in the sample in a colorimetric manner. The resulting standard curve can be seen in Figure 10A.

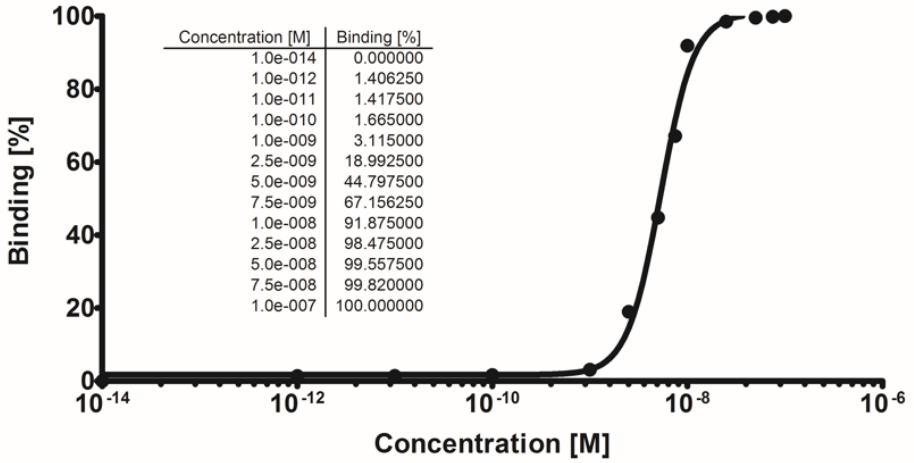
Co-expression of EGFP-Rab5A Q79L and wt ASYN leads to an increase in secretion (Figure 10B), indicating accelerated sorting activity from the early endosome into the recycling pathway. This is confirmed by a sharp drop in secretion with the co-expression of the dominant negative mutant, although secretion is not entirely abolished (Figure 10B), hinting at a secondary release pathway independent of the early endosome. Expression of all three EGFP-Rab7 mutants along wt ASYN leads to a decrease in ASYN release indicative or higher protein turnover (Figure 10B) despite equal protein amounts (Figure 10F, G).

Employing the inclusion model yields a sharp decrease in ASYN release with the co-expression of the constitutive active mutant of EGFP-Rab5A, while release is almost

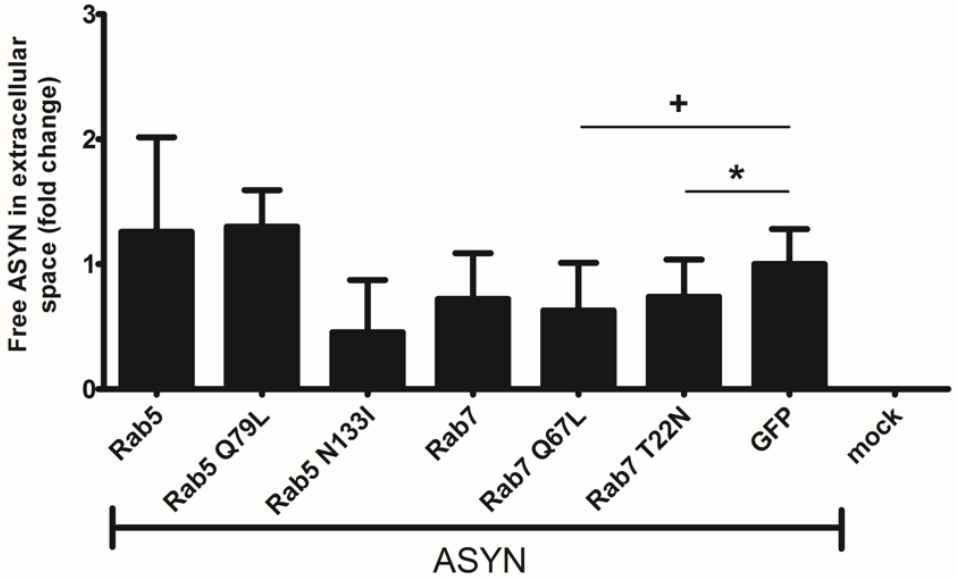
unchanged upon expression with the dominant negative mutant (Figure 10C). For EGFP-Rab7 we once more see a decrease in ASYN release in all three mutants (Figure 10C), indicative of higher protein turnover or a blocked release pathway, despite equal amounts of protein (Figure 10F, H).

To ensure these effects, and especially the high variances in the experiments with EGFP-Rab5A wt, were not due to increased release of the material of dead cells into the medium, we performed an LDH assay. This enzymatic assay measures the concentration in the culture medium in a colorimetric manner. No significant differences could be determined between experiments and control conditions (Figure 10D, E). In our previous study (Yin et al., 2014) we found SynT mediated cellular toxicity decreasing significantly with Rab8A co-expression when compared to SynT expression alone (Figure 11A). This rescue was not due to altered protein levels (Figure 11B), and we could repeat this effect with the phospho-mutants of SynT (Figure 11). Whether this effect is related to altered aggregation behavior Figure 6 remains unknown (Yin et al., 2014).

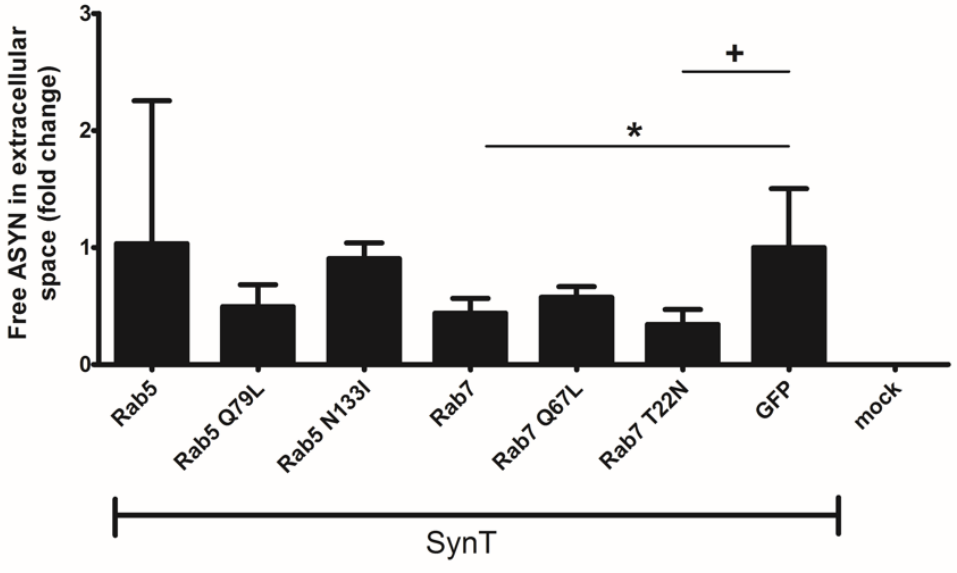
A


Concentration [M] | Binding [%]
1.0e-014 | 0.000000
1.0e-012 | 1.406250
1.0e-011 | 1.417500
1.0e-010 | 1.665000
1.0e-009 | 3.115000
2.5e-009 | 18.992500
5.0e-009 | 44.797500
7.5e-009 | 67.156250
1.0e-008 | 91.875000
2.5e-008 | 98.475000
5.0e-008 | 99.557500
7.5e-008 | 99.820000
1.0e-007 | 100.000000
Binding [%]
100
80
60
40
20
0
10-14
10-12
10-10
10-8
10-6
Concentration [M]

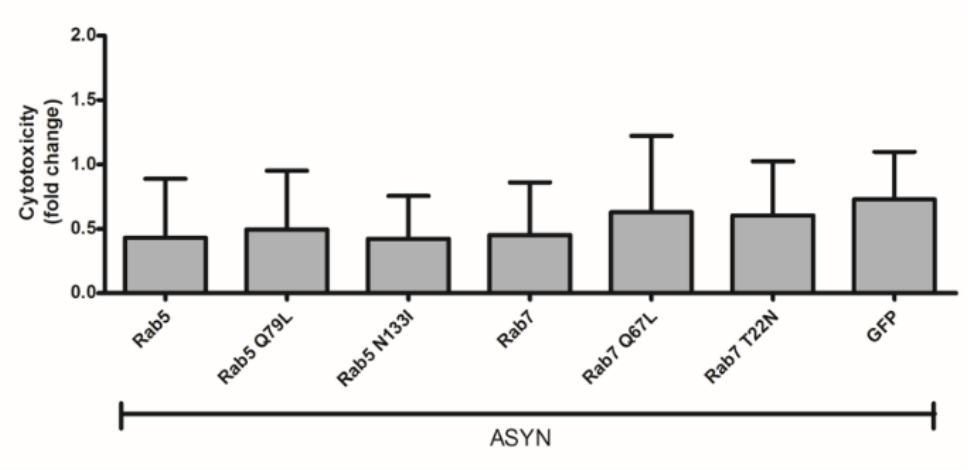
B


Free ASYN in extracellular space (fold change)
3
2
1
0
+
*
Rab5
Rab5 Q79L
Rab5 N133I
Rab7
Rab7 Q67L
Rab7 T22N
GFP
mock
ASYN

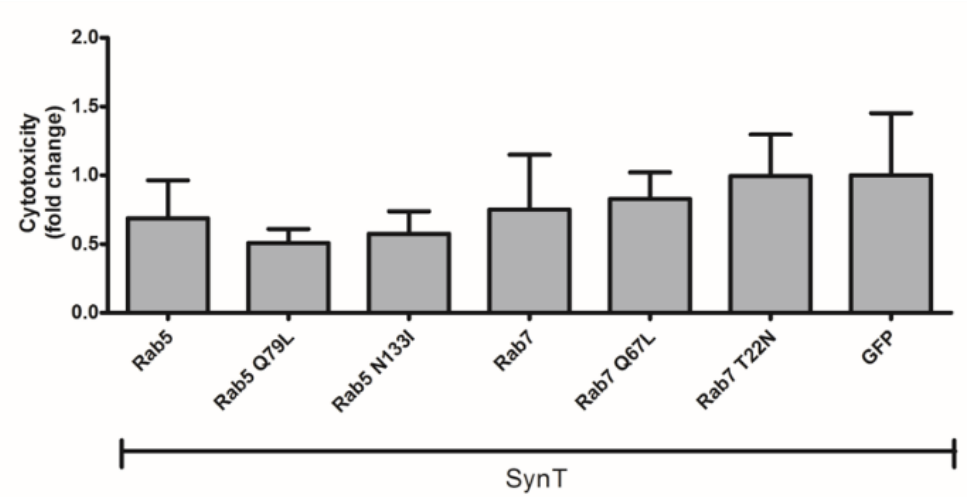
C


Free ASYN in extracellular space (fold change)
3
2
1
0
+
*
Rab5
Rab5 Q79L
Rab5 N133I
Rab7
Rab7 Q67L
Rab7 T22N
GFP
mock
SynT

D


Cytotoxicity (fold change)
2.0
1.5
1.0
0.5
0.0
Rab5
Rab5 Q79L
Rab5 N133I
Rab7
Rab7 Q67L
Rab7 T22N
GFP
ASYN

E


Cytotoxicity (fold change)
2.0
1.5
1.0
0.5
0.0
Rab5
Rab5 Q79L
Rab5 N133I
Rab7
Rab7 Q67L
Rab7 T22N
GFP
SynT

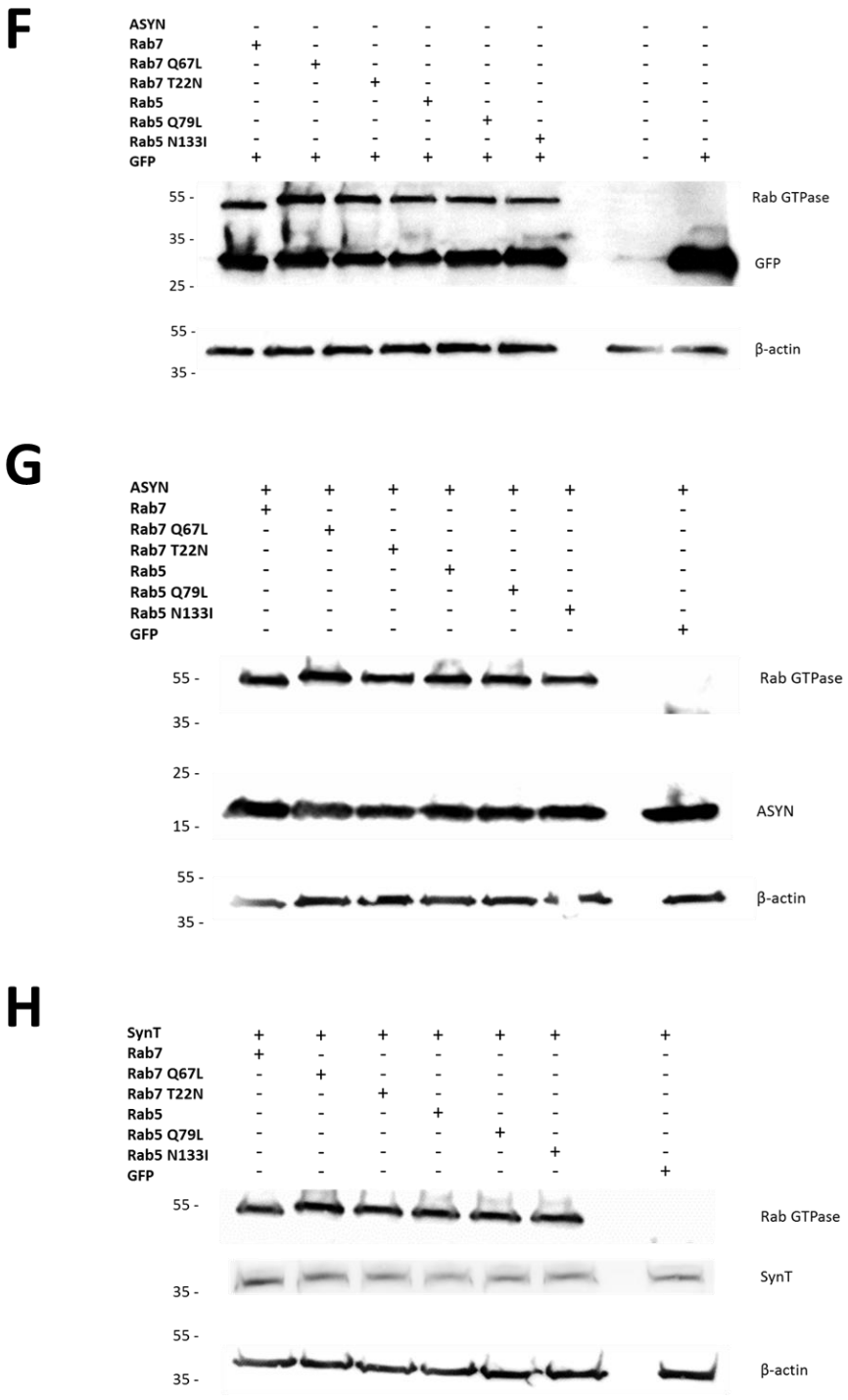


Figure 10: Endosomal Rab GTPases modulate secretion of ASYN but do not influence toxicity. (A) Standard curve for calculation of secreted protein. Table inset shows molarities and percentage of binding. (B) Fold change of secretion compared between native ASYN with GFP for control and expression alongside Rab5A and Rab7 wt and activity mutants. (C) Fold change of secretion compared between native ASYN with GFP for control and expression alongside Rab5A and Rab7 wt and activity mutants. (D, E) Expression of Rab GTPases and activity mutants with ASYN or SynT do not interfere with cell survival. (F, G, H) Expression of ASYN or SynT with Rab5A and Rab7 wt or constitutive active or dominant negative mutants do not change protein

expression. All analyses represent n=4 independent experiments and statistical data is displayed as mean ± S.D.; + 0.1 < p < 0.05, * p ≤ 0.05 One-tailed paired t-test.

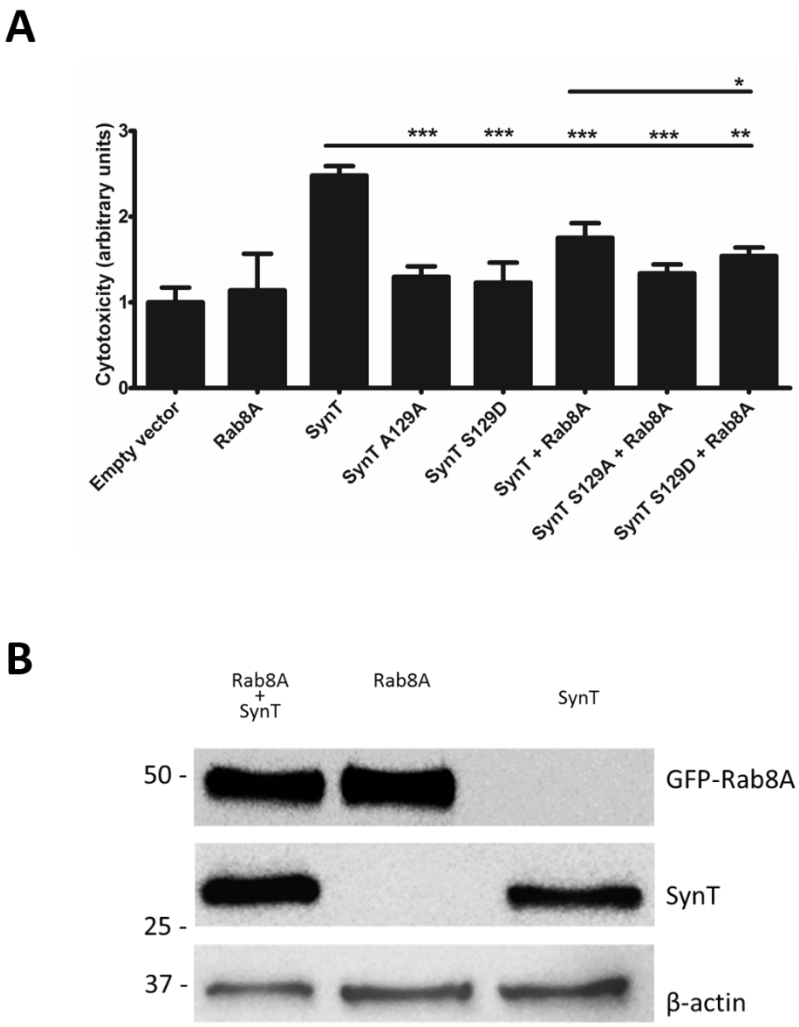


Figure 11: Rab8a rescues SynT-induced cellular toxicity. (A) Expression of SynT increases toxicity by more than two-fold and is rescued by co-expression of Rab8A. All data are presented relative to total cell death and were normalized to empty vector control. Data is presented as mean ± S.D. * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001 Two-tailed paired t-test two-tailed paired t-test. (B) Protein expression levels remain unchanged in experimental conditions. β-actin as employed as loading control. Experiments represent n=3-5 independent experiments. (Modified, original version published 2014 (Yin et al., 2014).)

4.5 Lysosomal and degradation markers are dysregulated with constitutive ASYN expression *in vivo*

Since especially Rab7 is involved in the progression through the degradation pathway and taking the previous findings into account, we finally investigated several markers of the degradation pathway *in vivo*. For this study, we utilized a mouse model overexpressing full length human wt ASYN under the murine Thy1 promoter on a C57Bl6 background that has been developed in the laboratory of Elizier Masliah (Rockenstein et al., 2002). Only males were used for this study, since the ASYN transgene is inserted on the X-chromosome and expression of the transgene in females in all cell types is not guaranteed due to random X-inactivation. We initially investigated animals at 26 weeks of age and then selected two more timepoints at 13 and 39 weeks to represent young adult age and aged individuals. The mouse model overexpresses ASYN in a moderate to strong manner, develops proteinase K resistant ASYN inclusions and shows striatal dopamine loss at 14 months of age (Lam et al., 2011; Chesselet et al., 2012). To investigate protein expression, mice were sacrificed at the indicated age, brains were harvested and dissected to isolate cortex (CTX), hippocampus (HC), midbrain (MB) and striatum (STR). These areas were selected due to their importance to movement, coordination, and memory. Brain areas were lysed, immunoblotted and blots were stained for ASYN, Rab7, CatD, GAPDH as well as β-actin as a loading control. All data was analyzed for changes due to genotype (Figure 12) and afterwards also for changes in age progression.

In all tested animals, expression of ASYN was strong compared to wt littermates across the investigated brain regions (Figure 12). Additional to ASYN, we chose three markers related to the lysosome and lysosomal degradation: Rab7, GAPDH, a well-known substrate of the lysosome, and CatD indicative of lysosomal function. All protein levels were normalized against β-actin, which acted as loading control and only then compared for differences.

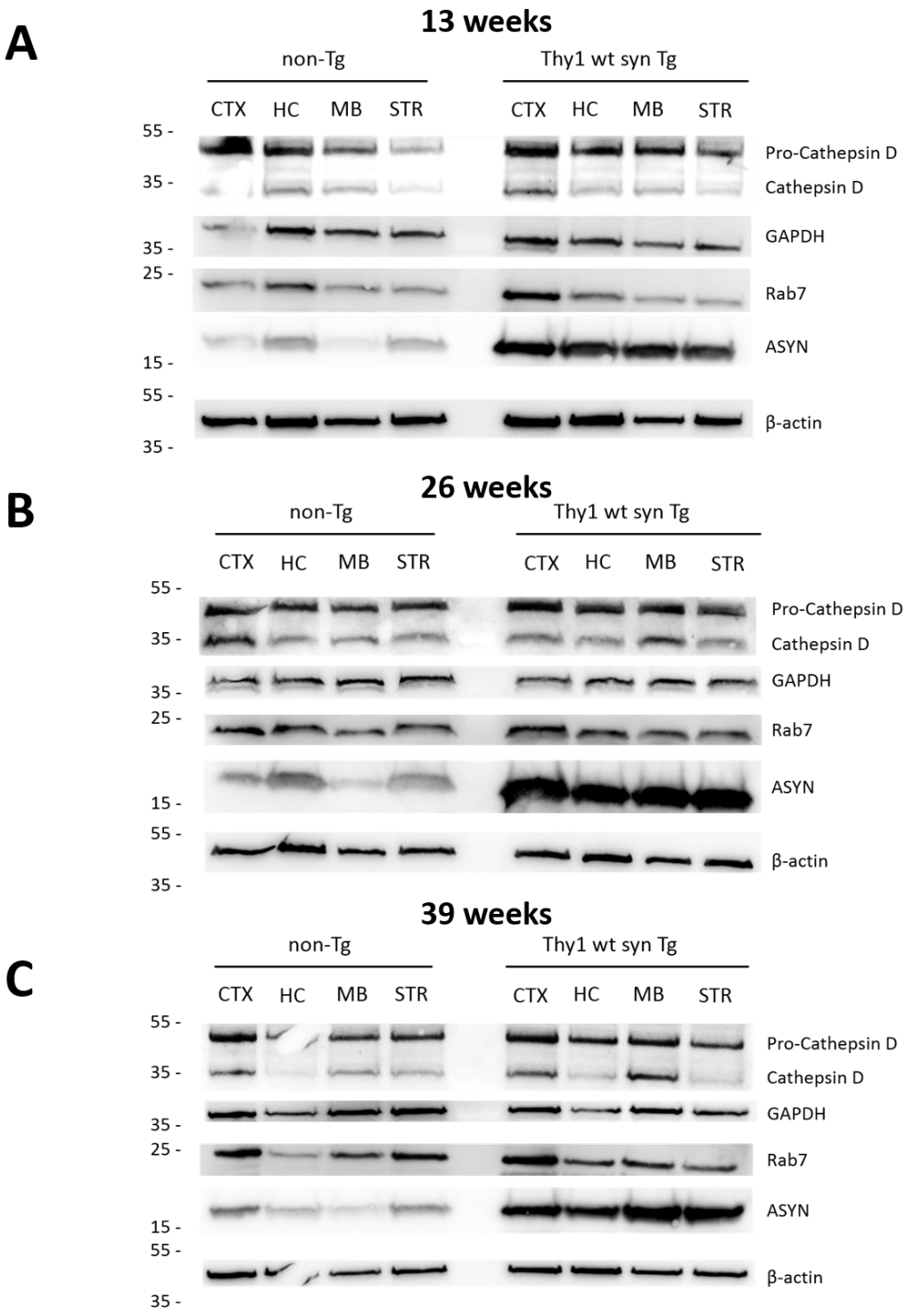


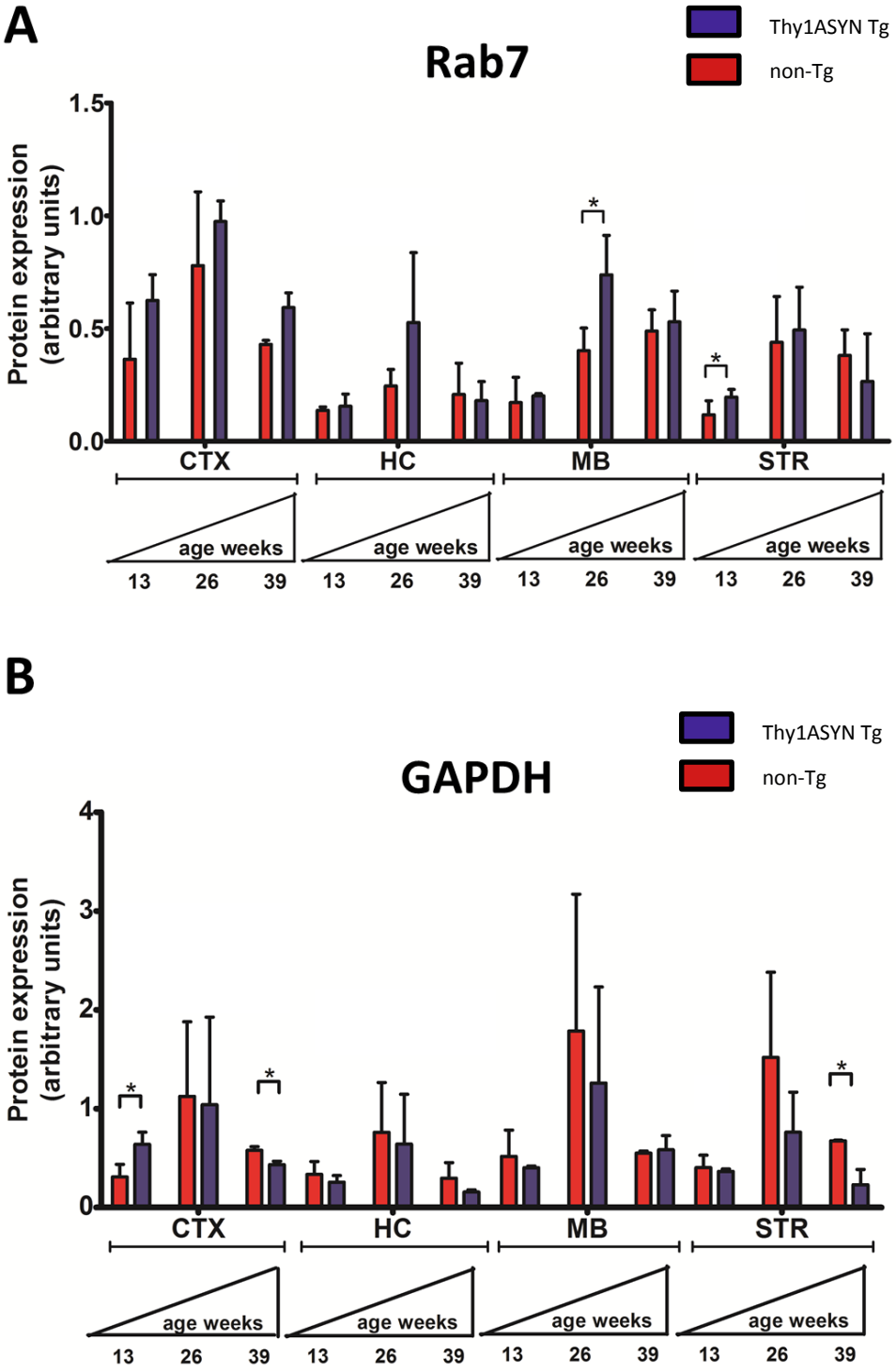
Figure 12: Representative immunoblots for degradation markers of transgenic animals and wt littermate controls at different ages. (A) Representative immunoblot of 13 week old ASYN transgenic animal and wt littermate control probed for Rab7, CatD, ASYN and GAPDH, as well as β-actin as loading control. (B) Representative immunoblot of 26 week old ASYN transgenic animal and wt littermate control probed for Rab7, CatD, ASYN and GAPDH, as well as β-actin as loading control. (C) Representative immunoblot of 39 week old ASYN transgenic animal and wt littermate control probed for Rab7, CatD, ASYN and GAPDH, as well as β-actin as loading control.

In the cortex of Thy1-ASYN Tg animals, Rab7 is elevated compared to wt littermate controls at all three ages (Figure 13A). Increased expression of lysosomal marker Rab7 is therefore evident in the cortex across adulthood and into advanced age. In the hippocampus of ASYN transgenic mice, Rab7 expression is upregulated two-fold compared to littermate controls at 26 weeks of age (Figure 13A). In 13 week old animals we can detected a slight elevation, while in 39 week old animals, Rab7 expression is indistinguishable between transgenic and non-transgenic animals (Figure 13A). In the midbrain we can detect a similar pattern, as we can detect slight elevations of Rab7 levels in 13 week and 39 week old animals, but we see a significant increase of Rab7 in 26 week old animals Figure 13A. In the striatum, Rab7 levels are elevated at 13 weeks of age by 66% in transgenic mice (Figure 13A). In 26 week old animals, Rab7 levels are almost equal between transgenic and non-transgenic animals, while in 39 week old animals, we can detect a slight decrease of Rab7 levels in animals carrying the transgene compared to wt controls.

The degradation pathway of GAPDH proceeds via the lysosome (Aniento et al., 1993), hence it can be used to assess lysosomal function. In cortices of 13 week old animals we determined an increase in GAPDH levels in ASYN transgenic animals compared to littermate controls (Figure 13B). Levels remain the same, if with high variances, at 26 weeks of age, but drop below levels of littermate controls at 39 weeks of age (Figure 13B). In the hippocampus no change in GAPDH levels is evident at 13 weeks and 26 weeks of age, but GAPDH levels drop below those of littermate controls at 39 weeks of age (Figure 13B). In midbrain, GAPDH levels are unchanged between ASYN transgenic animals and wt littermate controls. In striatum, GAPDH levels are unchanged at 13 weeks of age, but at 26 weeks old, GAPDH levels in ASYN transgenic animals are lower than in wt littermate controls (Figure 13B). At 39 weeks of age, GAPDH levels in ASYN transgenic animals are significantly lower than in wt control animals (Figure 13B).

CatD is the main lysosomal enzyme responsible for ASYN degradation (Qiao et al., 2008; Sevlever et al., 2008; Crabtree et al., 2014). CatD is cleaved only after association with the lysosome, where it matures from an inactive precursor protein to the proteolytically active form (Hasilik et al., 1982; Fusek and Vetvicka, 2005; Guha and Padh, 2008), which is why it can also indicate lysosomal function. Total levels of CatD are mildly increased in all brain regions of transgenic animals, although the variances are very high (Figure 12, Figure 13C). We see a slight increase of total CatD levels in cortices of ASYN transgenic

mice in all ages (Figure 13C). On the other hand, levels of cleaved, mature CatD are elevated in cortices of 13 week old ASYN transgenic mice (Figure 13D). Levels of mature CatD in the cortices of 26 week old mice are not distinguishable and at 39 weeks old, levels of mature CatD are slightly elevated in the cortices of transgenic mice. In hippocampi total and cleaved levels of CatD are slightly elevated in transgenic mice at 13 weeks and 26 weeks of age (Figure 13C, D). In 39 week old animals, levels of cleaved CatD drop sharply below levels in wt littermate controls, while total levels remain the same (Figure 13C, D). In midbrain, total levels of CatD are elevated in all ages in ASYN transgenic animals and significantly so at 39 weeks of age (Figure 13C). CatD cleavage remains unchanged in 13 weeks old transgenic animals compared to littermate controls but is mildly increased in 26 week old animals and significantly increased in 39 week old animals (Figure 13D). In the striatum, levels of total CatD remain on par between transgenic and non-transgenic animals and we are unable to observe a difference in the genotypes at any age (Figure 13C). The same is true for mature CatD and we are unable to observe genotypic differences, although a mild decrease in cleaved CatD levels might be determined (Figure 13D).


A
Rab7
Thy1ASYN Tg
non-Tg
Protein expression (arbitrary units)
1.5
1.0
0.5
0.0
*
*
CTX
HC
MB
STR
age weeks
13
26
39
B
GAPDH
Thy1ASYN Tg
non-Tg
Protein expression (arbitrary units)
4
3
2
1
0
*
*
*
CTX
HC
MB
STR
age weeks
13
26
39

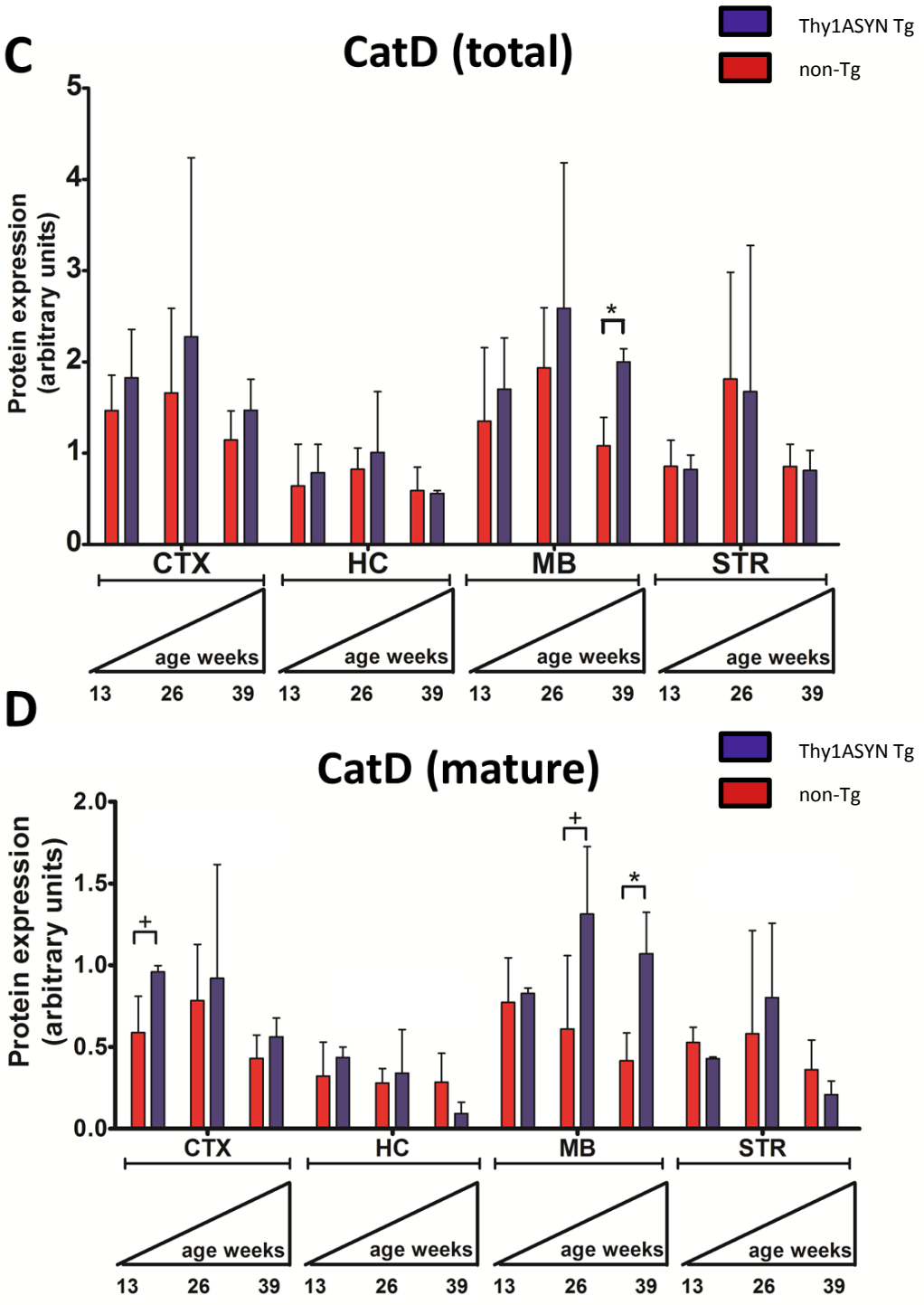

C
CatD (total)
Thy1ASYN Tg
non-Tg
Protein expression (arbitrary units)
0
1
2
3
4
5
*
CTX
HC
MB
STR
age weeks
13
26
39
D
CatD (mature)
Thy1ASYN Tg
non-Tg
Protein expression (arbitrary units)
0.0
0.5
1.0
1.5
2.0
+
+
*
CTX
HC
MB
STR
age weeks
13
26
39

Figure 13: Degradation markers are dysregulated in ASYN transgenic animals. (A) Quantification of immunoblots for Rab7 probe, compared between ASYN transgenic and wt animals at three different ages. Upregulation in ASYN transgenic mice is especially clear in young and middle aged mice and decreases in aged animals. (B) Quantification of immunoblots for GAPDH probe, compared between ASYN transgenic and wt animals at three different ages. Levels are increased or equal in ASYN transgenic mice and downregulated in aged ASYN transgenic mice. (C) Quantification of immunoblots for total CatD, compared between ASYN transgenic and wt animals at three different ages. Total levels of CatD are increased in ASYN transgenic animals. (D) Quantification of immunoblots for mature CatD, compared between ASYN transgenic and wt animals at three different ages. CatD cleavage is increased in young and middle aged ASYN transgenic animals but decreases in aged ASYN transgenic animals when compared to wt littermate controls. Results are representative of n=14 individuals with mean ± S.D.; + $0.1 < p < 0.05$, * $p \leq 0.05$ and all expressions are relative to β-actin levels in the different brain areas.

5. Discussion

ASYN, the main component of LBs, is a major player in PD pathology. For reasons that are not clearly understood, ASYN experiences a toxic gain of function upon mutation or increased expression. Several genetic screens have revealed gene clusters associated with intracellular trafficking to be effective modulators of ASYN toxicity.

Vesicle trafficking genes were first identified as modulators of ASYN toxicity in screens based on the model organisms *Saccharomyces cerevisiae* and *C. elegans* (Outeiro and Lindquist, 2003; Willingham et al., 2003; Hamamichi et al., 2008; van Ham et al., 2008). Synaptic vesicle trafficking has also been identified as one of factors of neuronal dysfunction that arise with ASYN pathology in other studies (Nemani et al., 2010; Scott et al., 2010). ASYN has been shown to interact with proteins of the trafficking gene families of SNAREs (Chandra et al., 2005; Burré et al., 2010; Thayanidhi et al., 2010, 2012) and Rab GTPases (Dalfó et al., 2004a, 2004b; Dalfó and Ferrer, 2005; Soper et al., 2008, 2011; Liu et al., 2009; Sancenon et al., 2012; Chen et al., 2013). Other genetic screens in these organisms could show further impact on ASYN modulated toxicity by the overexpression of Rab GTPases (Cooper et al., 2006; Gitler et al., 2008) or show an influence of ASYN on cellular Rab homeostasis (Gitler et al., 2008; Soper et al., 2011). Recently, we could also show in our own lab that ASYN interacts with Rab GTPases *in vivo* outside of a transgenic of a pathological context (Chutna et al., 2014; Yin et al., 2014).

In this study, we investigated the interplay of ASYN with a group of intracellular trafficking genes collectively known as Rab GTPases that are involved in a multitude of vesicular trafficking pathways (Stenmark, 2009). In order to achieve this, we utilized native, untagged ASYN as well as a C-terminal modified version of ASYN, that forms protein inclusions with LB-like characteristics (McLean et al., 2001; Klucken et al., 2012; Yin et al., 2014), and a library of EGFP-tagged Rab GTPases, as well as a mutants of selected Rab proteins without the ability to hydrolyze GTP or have a higher affinity to GDP (Bucci et al., 1992, 2000; Vitelli et al., 1997). We also made use of a mouse model overexpressing human ASYN under the control of a Thy1 promoter, resulting in a pre-PD-like phenotype with motor and cognitive impairment for our *in vivo* protein analysis (Rockenstein et al., 2002; Lam et al., 2011; Chesselet et al., 2012).

5.1 A screen in cells accumulating ASYN inclusions reveals mislocalized trafficking markers

While various studies have confirmed the interaction of ASYN with Rab GTPases, we present here a comprehensive screen of a majority of members of the Rab GTPase family with a variant of ASYN prone to inclusion formation in a mammalian cell line. Modifications of the C-terminus of ASYN have been shown to regulate ASYN aggregation and protein-protein interaction in the past (Chandra et al., 2005; Garcia-Reitböck et al., 2010; Levitan et al., 2011; Yin et al., 2014) and this is also the case in this variant of ASYN, termed SynT (McLean et al., 2001; Klucken et al., 2012).

Expressed alone alongside an empty vector, all investigated Rab proteins distribute in accordance to their cellular function (Hutagalung and Novick, 2011). Overexpression of Rab GTPases sometimes results in overly large target vesicles or clustering, but this has not been found to be of negative effect to the cell by itself (Barbieri et al., 1996; Bucci et al., 2000; Chan et al., 2011).

Most Rab proteins were mislocalized, away from their normal cellular compartment, upon inclusion formation (compare Table 6). In cells without inclusions, SynT lacked any obvious effect on the morphology of Rab proteins. Upon formation of inclusions positive for SynT, however, most Rab proteins were localized away from their target membranes and was diffusely distributed through the cytoplasm. Also, Rab proteins tended to colocalize with the SynT mediated inclusions regardless of their original pathway. This hints at pathological ASYN disturbing at least one trafficking pathway, possibly one central to all, or most other cellular vesicle trafficking pathways. Intriguingly, this tendency of Rab proteins to localize to SynT positive inclusions did not affect Rab7, a Rab GTPase localized to late endosomes and lysosomes and an important factor for autophagosome formation. On the other hand, other Rab GTPases associated with autophagosome formation, Rab5A (Ravikumar et al., 2008; Ao et al., 2014) and Rab33B (Itoh et al., 2008), did colocalize with SynT inclusions at least partially. The colocalization of Rab GTPases with ASYN mediated aggregates has been reported previously for yeast (Soper et al., 2011), but never in this scope in mammalian cells. Further, in this cited study, the yeast homologue of Rab7, Ypt7, also colocalized with the aggregates, which is the not the case in our investigations.

Contrary to this, most Rab GTPases did not have any obvious effect on the formation of SynT inclusions. While the initial screen was not quantitative and only targeted obvious changes in either Rab proteins or SynT distribution and behavior, it became obvious that inclusion formation was increased with the expression of Rab7. Interestingly, this is also the Rab protein which did not localize to the SynT mediated inclusions. In the case of Rab8A, a decrease in the amount of condensed nuclei indicating cellular toxicity, and an effect on ASYN toxicity has been observed in the past (Gitler et al., 2008).

We eventually selected three major candidates for further investigation, all of which are located in the endosomal pathway: Rab5A, Rab7 and Rab8A.

Rab5A is located at early endosomes and a regulator of clathrin mediated endocytosis (Bucci et al., 1992; Zeigerer et al., 2012), as well as endocytic sorting (Deinhardt et al., 2006; Zeigerer et al., 2012) and has been found to interact with mutant A30P ASYN in transgenic mice (Dalfó et al., 2004b). Rab5A positive compartments turn into Rab7 positive, late endosomal and finally lysosomal vesicles during a process termed Rab conversion (Rink et al., 2005; Poteryaev et al., 2010; Zeigerer et al., 2012). Cargo in the early endosome does not so much leave the vesicle as the whole vesicle matures into the degradation pathway. The discrepancy in our results in regards to Rab5A and Rab7 in the presence of SynT positive protein inclusions is therefore intriguing, and these two candidates were selected for their different behavior despite their localization in adjacent pathways. Rab8A is localized at tubular recycling endosomes in the TGN secretory pathway (Hattula et al., 2006; Henry and Sheff, 2008) and we were curious whether the previously discovered alleviation of toxicity (Gitler et al., 2008) would also be replicable in our model.

5.2 Endosomal Rab proteins alter the size and pattern of ASYN inclusions

We observed inclusion formation of ASYN in our cell model in the presence and absence of EGFP-fused Rab5A and Rab7. We could establish a strong trend towards an increase in inclusion formation with co-expression with Rab5A and a significant increase of inclusions when Rab7 was co-expressed. We could also show an increase in inclusion formation in the presence of Rab8A which acted protective in light of SynT mediated toxicity (Yin et al., 2014). Similar results were recently obtained in a study with Rab11A,

another Rab GTPase located at recycling endosomes (Chutna et al., 2014). It is striking that we could determine an increase in inclusion formation with Rab7, which does not localize with ASYN mediated inclusions, but not an alteration in cellular toxicity. While Rab5A, Rab7 and Rab8A all localize to endosomal compartments, the trafficking pathways are different and Rab8A is involved in exocytosis while Rab5A and Rab7 are located in the sorting and degradation pathway. On the other hand, we could determine a strong colocalization with Rab7 and untagged, native ASYN. While ASYN is often present as a free, unstructured protein in the cytosol, we could determine discreet punctate structures in the cells. Upon investigation in our colocalization experiments these were found to be of endosomal nature due to their strong colocalization with endosomal Rab5A. While ASYN has been found in in vesicles of unknown origin (Lee et al., 2005), and also has been found localized to endosomal structures in HEK293T and SH-SY5Y cells (Hasegawa et al., 2011) before, we here present the direct comparison between these two pathways. It has been proposed before that ASYN monomers and intermediate structures are cleared via the lysosome (Lee et al., 2004; Hasegawa et al., 2011) and our experiments show that native ASYN can be transported via endosomes to the lysosomal pathway. This is also concurrent with our results that both Rab5A and Rab7 are able to alter particulate size of native ASYN in a cellular context to different degrees. As ASYN oligomers could be shown to be degraded by the lysosome (Sevlever et al., 2008; Crabtree et al., 2014), the expression of both Rab GTPases might enhance this process. We can see higher molecular weight oligomer fractions with the co-expression of Rab5A, which might mean an increased sorting activity and clearance by the degradation pathway, as we see a comparatively stronger signal with the expression of Rab7.

The opposite results we observed in our model of inclusion formation likely present a disturbance of this sorting pathway. A likely scenario for the effect of ASYN inclusions on Rab5A might be that Rab5A positive endosomes get caught in the inclusions formed by ASYN, while lysosomes do not suffer this fate. This might also explain why Rab5A positive compartments are enlarged in ASYN transgenic animals and human cases of DLB (Sancenon et al., 2012). It is also possible that by overexpressing the Rab proteins we push the system in this direction, i.e. the increased amount of endosomes in the cell due to Rab5 overexpression leads to molecular crowding and endosomes stick to the inclusions. On the other hand, by overexpressing Rab7 we increase the number of lysosomes and might also increase protein turnover. This could also explain the increase

in inclusion formation as misfolded proteins are often spatially sequestered into compartment like e.g. Q-bodies (Escusa-Toret et al., 2013) in an attempt to improve cellular fitness. In axons, the retrograde transport of compartments positive for Rab7 as well as autophagosomes was found to be impaired by the formation of Lewy neurites (Volpicelli-Daley et al., 2014). However, this study utilized recombinant pre-formed fibrils rather than a genetically encoded inclusion model.

5.3 Recycling of ASYN to the plasma membrane is influenced by endosomal Rab proteins

The secretion of ASYN has been a topic of much discussion, and while it is indisputable that ASYN can be endocytosed (Sung et al., 2001; Liu et al., 2009), the question of exocytosis is not concluded. Prion-like transmission, exosomes, regulated exocytosis and tunneling nanotubes have been proposed (Kordower et al., 2008a, 2008b; Steiner et al., 2011), a hypothesis that might explain Braak staging of the disorder (Braak et al., 2002a, 2002b, 2004). The expression of a dominant negative version of vacuolar protein 4 led to an increase in ASYN release (Hasegawa et al., 2011) and dominant negative mutant of Rab11A, which is located at recycling endosomes, had the same effect (Chutna et al., 2014). Rab11A overexpression also potentially alleviates ASYN mediated toxicity at the synapse, restoring the non-pathological phenotype and regulating neurotransmitter release (Breda et al., 2014). Taking all of this into account, we wanted to know whether one of our candidates would be able to modulate ASYN secretion either in our inclusion model or with native ASYN.

While Rab5A is involved in endocytosis in general and endocytosis of ASYN in particular (Sung et al., 2001), data about the effect of Rab5A overexpression on sorting activity is yet missing. Interestingly, when ASYN and Rab5A wt or the constitutively active mutant Q79L are overexpressed, we can see an increase in ASYN release. This might be consistent with an increase in sorting activity into the recycling or exocytic pathway, although a previous study could not determine alterations in membrane recycling upon expression of constitutive active Rab5A (Ceresa et al., 2001). However, ASYN is not only present as a free protein in endosomes, but also occurs in multivesicular bodies (MVBs) and is exocytosed in exosomes (Emmanouilidou et al., 2010; Steiner et al., 2011) and Rab5 regulates the motility of endosomes alongside microtubuli (Nielsen et al., 1999).

On the other hand, knockdown of Rab11A or expression of the dominant negative mutant led to an increase of ASYN secretion, hinting at a different pathway of ASYN recycling (Chutna et al., 2014). We therefore think that native ASYN is recycled to the plasma membrane via a pathway that is at least partially Rab5A dependent. This is supported by our observations that upon expression of the dominant negative form of Rab5A, secretion is reduced, though not abolished. On the other hand, our observations could hint at a mechanism of compensation for excessive potentially harmful protein within the cell. In transgenic animals, an increase in size of Rab5A positive compartments was observed (Sancenon et al., 2012) and Rab5A is regionally upregulated in mild cognitive disorder and AD (Ginsberg et al., 2010, 2011). Further, we can see a reduction of secretion of ASYN in our cellular inclusion model with expression of the constitutive active mutant while it remains unaffected when the pathway is impaired with dominant negative Rab5A N133I. Since in both cases we investigate a model that is based on overexpression, it would be interesting to see results from cells that natively secrete ASYN while at the same time overexpressing Rab5A Q79L or N133I. Overall, it is possible that Rab5A is able to help recycle oligomeric ASYN, but not higher molecular weight inclusions or aggregates.

With Rab7 we observed a decrease for all three variants in both models. In the case of Rab7 wt and constitutive active Q67L we can probably explain it with higher protein turnover due to an increase in lysosome number and size. Interesting to note is that one study found a slight increase in ASYN secretion when Rab7 was co-expressed (Ejlerskov et al., 2013), but this was in a cellular model employing the A30P mutant, which is known to not possess the same membrane binding capabilities as wt ASYN (Jo et al., 2004; Wislet-Gendebien et al., 2008). We also have to take into account that while ASYN was shown to be secreted into the extracellular space, the ELISA method only measures free ASYN. On the other hand, ASYN is also released from the cell in exosomes (Emmanouilidou et al., 2010; Danzer et al., 2012), which derive from MVBs and surrounded by a membrane (Emmanouilidou et al., 2010). In a previous study with Rab11A, alterations of the activity state of this Rab protein associated with recycling endosomes did not change secretion of ASYN via exosomes (Chutna et al., 2014). On the other hand, MVBs are positive for Rab7 (Bucci et al., 2000) and Rab7 positive structures increase with Rab7 overexpression (Bucci et al., 2000). This why in our study with overexpression of wt and constitutive active Rab7 might lead to an increase of ASYN secretion via this pathway. Further, we know that dominant negative Rab7 leads to a

loss of lysosomal acidification (Bucci et al., 2000) and expression of dominant negative vacuolar protein 4 lead to an increase in secretion of ASYN (Hasegawa et al., 2011). We therefore also expected an increase or no change in ASYN secretion and our findings did not support those expectations. For native ASYN as well as for the inclusion model we also see a decrease in secretion upon co-expressing Rab7 T22N. One explanation is that ASYN is retained in the cell, as one study found that Rab7 T22N inhibits secretion of Flightless (Lei et al., 2012), an actin remodeling protein usually secreted via the lysosomal pathway. While the authors found increased levels of Flightless in the cells, we cannot determine the same for intracellular ASYN in immunoblot analysis, which might be due to the metabolic differences between fibroblasts and H4 cells. On the other hand, in rat dorsal root ganglion neurons, the expression of dominant negative Rab7 had no effect on the secretory pathway (Saxena et al., 2005). As a final explanation, ASYN might be degraded via other pathways, like the UPS, when the lysosomal pathway is disturbed or AYN might be retained in autophagic vacuoles that form with Rab7 T22N expression as previously investigated (Jäger et al., 2004). We can exclude, however, that the changes in ASYN secretion are correlated with cell death, which points at an active pathway regulated by the endocytic recycling and degradation machinery.

Altogether, we found evidence that Rab5A and Rab7 are involved in the secretory and degradation pathway in H4 neuroglioma cells.

5.4 Constitutive ASYN expression *in vivo* leads to dysregulation of the degradation pathway

Finally, we wanted to investigate our observations in regards to the degradation pathway in an *in vivo* model of ASYN pathology. For this purpose, we used a mouse model that overexpresses full length human ASYN under the control of the Thy1 promoter (Rockenstein et al., 2002; Chesselet et al., 2012). We initially found an upregulation of Rab7 in 26 week old mice. This is not concurrent with our findings in either of our cell cultures models, where expression of transiently overexpressed Rab7 was unaffected by the expression of ASYN. However, it is hard to model an age related condition in cell culture and even a model like our model of inclusion formation that mimics certain aspects of the pathology can only supply a limited amount of

information. In order to determine whether the increase in Rab7 was an age-related phenomenon, we chose to investigate individuals at two additional ages and also take more markers of the lysosomal pathway into account.

GAPDH is a well-known substrate of the lysosome and can be used as an indicator of lysosomal function (Aniento et al., 1993) and we used it to assess protein degradation in the investigated brain areas. In young and middle aged mice, soluble GAPDH is elevated or remains level with the amounts found in wt littermates, although in aged mice, levels decrease in most brain areas. GAPDH possesses two surface cysteines that are known to be oxidized to disulfide bonds under conditions of oxidative stress and form insoluble aggregates (Nakajima et al., 2007). In our investigations, we only considered the soluble fraction and therefore cannot evaluate insoluble GAPDH levels. It is interesting to note, that the formation of the disulfide bond also increases oxidative stress and this aggregate formation participates in cell death (Nakajima et al., 2009). Further, GAPDH is translocated into the nucleus under conditions of oxidative stress (Dastoor and Dreyer, 2001) and these have been observed in PD and ASYN pathologies (Dexter et al., 1987; Fariello, 1988; Spina and Cohen, 1989; Sofic et al., 1992; Sian et al., 1994; Hashimoto et al., 1999; Nakaso et al., 2013). Our observations in regards to GAPDH only target the cytoplasmatic fraction and we therefore cannot make any claims in regards to the levels of GAPDH in the nucleus. However, GAPDH has been identified as a potential therapeutic target in neurodegenerative diseases, PD among them (Berry, 2004). We suggest that our findings in regards to GAPDH should be further investigated to also take the nuclear fraction into account.

An overexpression of Rab7 is concurrent with an increase in number and size of lysosomes (Bucci et al., 2000) and it is striking that the increased expression of Rab7 is present at all ages and brain regions we investigated. Regionally increased expression of Rab7 has been found in human cases of AD and mild cognitive impairment during the investigation of *post mortem* brains (Ginsberg et al., 2010, 2011) and pathological mutation in Charchot-Mary-Tooth Disease Type2B usually leads to a constitutively activated phenotype (Spinosa et al., 2008). It is also important to note that expression of constitutively active Rab7 rescues receptor degradation impairment in LRRK2 pathology (Gómez-Suaga et al., 2014). Given our own observations and this study about another PD-associated gene, it seems likely that the degradation pathway is key to teasing apart the molecular underpinnings of the disease. We therefore were curious whether the

increase in Rab7 expression resulted in a functional change of the lysosome *in vivo*. Therefore we investigated the amount of total as well as cleaved CatD, the protease mainly responsible for ASYN degradation (Sevlever et al., 2008; Cullen et al., 2009; Crabtree et al., 2014). We found an increase of CatD cleavage in transgenic animals compared to their wt littermate controls, which hints at an increase activity of the lysosomes. It is interesting to note that inactivation of CatD leads to the formation of ASYN positive inclusions and higher molecular weight species (Qiao et al., 2008; Cullen et al., 2009; Crabtree et al., 2014). However, CatD ablation is also associated with lysosomal storage disease (Koike et al., 2000; Vitner et al., 2010), while increased CatD activity has been linked to neurodegenerative conditions (Hetman et al., 1995; Amritraj et al., 2013). In association with PD, elevated CatD levels were found in a chronic MPTP monkey model distal from the site of insult where they caused apoptotic events (Yelamanchili et al., 2011). While the authors conclude that elevation of CatD activity might be causative of the pathology, we have to note that the monkeys were 6-8 years of age before receiving MPTP, while our mouse model expresses human ASYN from the first postnatal week onwards (Chesselet et al., 2012). Also, since we are evaluating a mouse model exhibiting genetic ASYN pathology rather than drug-induced parkinsonism, we are confident that our findings are not due to an initial increase CatD level. It is also interesting to note that elevated CatD levels have been observed cases of AD (Cataldo et al., 1995) and positive modulation of lysosomal enzymes has been proposed as a mechanism of treating protein accumulation diseases (Bahr et al., 2012), as which synucleinopathies are indubitably categorized. As already noted, the ASYN transgene is expressed from the first postnatal week onwards and by the age of 13 weeks, cells might try to compensate for the increased expression of a potentially harmful protein in two ways: i) sequestering the protein into inclusions which were observed during extensive characterization of the mouse line (Chesselet et al., 2012) and ii) by increasing the activity of the degradation machinery that we can show both with the elevated expression of Rab7 and the increased cleavage of CatD. Both of these observations would also explain our findings in the inclusion model, where overexpression of Rab7 resulted in an increase in inclusion formation. Since we also could not observe a change in toxicity levels, taken together, these findings indicate that the formation of ASYN deposits are the attempt by cells to maintain homeostasis. Intriguingly, we see a decrease of cleaved CatD levels in aged mice compared to littermate controls. We think these findings might indicate a progressive failure of the

degradation machinery and maintenance in proteostasis in aged mice. This hypothesis concurrent with the observation that transgenic mice of this line do not show an increased mortality until the age of 14 months, after which survival rates drop sharply compared to wt littermates (Chesselet et al., 2012).

6. Conclusions and Outlook

In this study, we showed the ability of ASYN to influence the distribution pattern of Rab GTPases upon formation of inclusions with LB-like characteristics. We also showed that depending on their intracellular function, Rab GTPases can act as genetic modifiers for ASYN inclusion formation and toxicity. With the overexpression of wt ASYN, endosomal Rab GTPases can change particulate size and influence the secretion of ASYN, which has potential implications on spreading of ASYN pathology between cells. In an *in vivo* model of ASYN pathology we showed the significant modulation of degradation markers at different ages, suggesting a compensatory mechanism to remove excess ASYN. Our study illustrates ASYN pathology in the endo-lysosomal pathway, which is one of the most important and most critical of the cell.

To expand our findings, further *in vivo* evaluations in models of ASYN pathology will be necessary, including an analysis of insoluble fractions of brain lysates. Rab GTPases are molecular switches, therefore downstream targets of Rab5A and Rab7 should be screened for dysregulation in our employed models, as this might only present the start of a cascade of dysregulation. We also suggest a functional investigation of the Rab conversion step from Rab5A to Rab7 via fluorescent endocytic markers destined for the degradation pathway. This will shed light on whether lysosomal biogenesis is impaired in ASYN pathology.

PD is an age-related disorder that is classically related to motor symptoms. However, recent research has shown that the pathology also spreads into unrelated systems causing symptoms that range from loss olfaction to sleep disorders. These non-classical symptoms often precede the clinical onset of the disease by decades and hint at an underlying biological explanation of PD pathology. Protein trafficking to and from target membranes is of great importance in all cell types, but neurons are highly specialized and polarized cells. In such strongly compartmentalized structures, disrupting the fine-tuned homeostasis of protein trafficking has to result in debilitating consequences. In this case, the PD-associated vulnerability of dopaminergic neurons located in the substantia nigra is specific to their biology and the consequent loss of dopamine in the striatum a debilitating side effect of the disease. This is especially true as a disruption in vesicle trafficking will affect vesicles carrying dopamine, which is known to produce reactive oxygen species. Our finding that the endo-lysosomal system is impaired by ASYN pathology, and that modifying trafficking proteins located in this pathway alters

ASYN mediated toxicity, provide important insight into the molecular underpinnings of PD. Our observations regarding the modulation in secretion of ASYN also highlight an important element of PD pathology transmission to neighboring neurons. By investigating the protein transport pathways, identifying dysregulated trafficking steps and modifiers of ASYN toxicity, we have taken tentative strides towards the identification of novel targets for therapeutic intervention.

Abbreviations

%	Percent
µg	Micro gram
µl	Micro liter
A	Alanine
AD	Alzheimer's disease
APS	Ammoniumpersulfate
ASYN	alpha-synuclein
Atg	Autophagy-related-gene
ATP	Adenosinetriphosphate
AAV	Adeno-associated virus
bp	Base pairs
BSA	Bovine serum albumin
C	Carboxy
C	Celcius
CA	Constitutive active
$CaCl_2$	Calcium chloride
$CaPO_4$	Calcium phosphate
CatD	Cathepsin D
cm	Centimeter
CMA	Chaperone mediated autophagy
CO_2	Carbondioxide
CTX	Cortex
D	Aspartic Acid
dd	Double destilled
DLB	Dementia with Lewy bodies
DN	Dominant negative
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
E	Glutamic acid
e.g.	exempli gratia
ECL	Enhanced chemoluminescence
EDTA	Ethylenediaminetetraacetic acid

EGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
FCS	Fetal calf serum
G	Glycine
g	Grams
g	Gravitational constant
GAP	GTPase activating protein
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GARP	Golgi-associated retrograde protein
GDI	GTPase dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GFP	Green Fluorescent Protein
GTP	Guanosine triphosphate
H	Histidine
h	hour
H_2O	Dihydrogen monoxide, water
HC	Hippocampus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IgG	Immunoglobuline G
K	Lysine
kb	Kilo base
KCl	Potassium chloride
kDa	Kilo Dalton
L	Leucine
LB	Lewy body
LB	Lysogeny broth
LDH	Lactate dehydrogenase
LRRK2	Leucine-rich-repeat-kinase 2
M	Molar
mA	Milli ampere

MB	Midbrain
mg	Milli grams
MgCl2	Magnesium chloride
min	Minute
mg	Milli gram
ml	Milli liter
mM	Milli molar
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MSA	Multiple systems atrophy
MBV	Multivesicular body
N	Amino
N	Asparagine
$Na_2HPO_4*2H_2O$	Dihydro-Disodium hydrogen phosphate
NaCl	Natrium chloride
$NaH_2PO_4*H_2O$	Hydro-Disodium hydrogen phosphate
nM	Nano molar
nm	Nano meter
P	Phosphate
P	Proline
PAGE	Polyachrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
pH	potentia Hydrogenii
PI	Phosphoinositide
PM	Plasma membrane
PRA	Prenylated Rab acceptor
PrP	Prion protein
Q	Glutamine
Rab	Ras gene from rat brain
RIPA	Radioimmuniprecipitation assay
RNA	Ribnucleic acid
RNAi	Ribonucleic acid interference

ROI	Region of interest
rpm	Rounds per minute
RT	Room temperature
S	Serine
S	Svedberg
S.D.	Standard deviation
SDS	Sodium-Dodecyl-Sulfate
SEC	Size exclusion chromatography
SOC	Super Optimal Broth
SNAP	Synaptosomal-associated protein
SNARE	Soluble NSF Attachment Protein Receptor
STR	Striatum
t	Target
T	Threonine
TBS	Tris buffered saline
Tg	Transgene
Tg	Transgenic
TEMED	Tetramethylethylenediamine
TGN	Trans-Golgi network
TMB	3,3’,5,5’-Tetramethylbenzidine
UPS	Ubiquitin-proteasome-system
v	Vesicle
V	Volt
VAMP	Vesicle-associated membrane protein 2
wt	wildtype
α	alpha
β	Beta
γ	Gamma

List of Figures

List of Tables

References

Aloisi AL, Bucci C (2013) Rab GTPases-cargo direct interactions: fine modulators of intracellular trafficking. Histol Histopathol.

Al-Wandi A, Ninkina N, Millership S, Williamson SJM, Jones PA, Buchman VL (2010) Absence of alpha-synuclein affects dopamine metabolism and synaptic markers in the striatum of aging mice. Neurobiol Aging 31:796–804.

Amritraj A, Wang Y, Revett TJ, Vergote D, Westaway D, Kar S (2013) Role of cathepsin D in U18666A-induced neuronal cell death: potential implication in Niemann-Pick type C disease pathogenesis. J Biol Chem 288:3136–3152.

Angot E, Steiner J a, Hansen C, Li J-Y, Brundin P (2010) Are synucleinopathies prion-like disorders? Lancet Neurol 9:1128–1138.

Aniento F, Roche E, Cuervo AM, Knecht E (1993) Uptake and degradation of glyceraldehyde-3-phosphate dehydrogenase by rat liver lysosomes. J Biol Chem 268:10463–10470.

Ao X, Zou L, Wu Y (2014) Regulation of autophagy by the Rab GTPase network. Cell Death Differ 21:348–358.

Bahr BA, Wisniewski ML, Butler D (2012) Positive lysosomal modulation as a unique strategy to treat age-related protein accumulation diseases. Rejuvenation Res 15:189–197.

Bandyopadhyay U, Kaushik S, Varticovski L, Cuervo AM (2008) The chaperone-mediated autophagy receptor organizes in dynamic protein complexes at the lysosomal membrane. Mol Cell Biol 28:5747–5763.

Barbieri MA, Li G, Mayorga LS, Stahl PD (1996) Characterization of Rab5:Q79L-stimulated endosome fusion. Arch Biochem Biophys 326:64–72.

Barone P et al. (2009) The PRIAMO study: A multicenter assessment of nonmotor symptoms and their impact on quality of life in Parkinson's disease. Mov Disord 24:1641–1649.

Bartels T, Choi JG, Selkoe DJ (2011) α-Synuclein occurs physiologically as a helically folded tetramer that resists aggregation. Nature 477:107–110.

Bedford L, Hay D, Devoy A, Paine S, Powe DG, Seth R, Gray T, Topham I, Fone K, Rezvani N, Mee M, Soane T, Layfield R, Sheppard PW, Ebendal T, Usoskin D, Lowe J, Mayer RJ (2008) Depletion of 26S proteasomes in mouse brain neurons causes neurodegeneration and Lewy-like inclusions resembling human pale bodies. J Neurosci 28:8189–8198.

Ben Gedalya T, Loeb V, Israeli E, Altschuler Y, Selkoe DJ, Sharon R (2009) Alpha-synuclein and polyunsaturated fatty acids promote clathrin-mediated endocytosis and synaptic vesicle recycling. Traffic 10:218–234.

Berry MD (2004) Glyceraldehyde-3-phosphate dehydrogenase as a target for small-molecule disease-modifying therapies in human neurodegenerative disorders. J Psychiatry Neurosci 29:337–345.

Betarbet R, Canet-Aviles RM, Sherer TB, Mastroberardino PG, McLendon C, Kim J-H, Lund S, Na H-M, Taylor G, Bence NF, Kopito R, Seo BB, Yagi T, Yagi A, Klinefelter G, Cookson MR, Greenamyre JT (2006) Intersecting pathways to neurodegeneration in Parkinson's disease: effects of the pesticide rotenone on DJ-1, alpha-synuclein, and the ubiquitin-proteasome system. Neurobiol Dis 22:404–420.

Bétemps D, Verchère J, Brot S, Morignat E, Bousset L, Gaillard D, Lakhdar L, Melki R, Baron T (2014) Alpha-synuclein spreading in M83 mice brain revealed by detection of pathological α-synuclein by enhanced ELISA. Acta Neuropathol Commun 2:29.

Binolfi A, Theillet F-X, Selenko P (2012) Bacterial in-cell NMR of human α-synuclein: a disordered monomer by nature? Biochem Soc Trans 40:950–954.

Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MCJ, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, Heutink P (2003) Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 299:256–259.

Braak H, Del Tredici K, Bratzke H, Hamm-Clement J, Sandmann-Keil D, Rüb U (2002a) Staging of the intracerebral inclusion body pathology associated with idiopathic Parkinson's disease (preclinical and clinical stages). J Neurol 249 Suppl :III/1–5.

Braak H, Del Tredici K, Rüb U, de Vos RAI, Jansen Steur ENH, Braak E (2002b) Staging of brain pathology related to sporadic Parkinson's disease. Neurobiol Aging 24:197–211.

Braak H, Ghebremedhin E, Rüb U, Bratzke H, Del Tredici K (2004) Stages in the development of Parkinson's disease-related pathology. Cell Tissue Res 318:121–134.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Breda C, Nugent ML, Estranero JG, Kyriacou CP, Outeiro TF, Steinert JR, Giorgini F (2014) Rab11 modulates α-synuclein mediated defects in synaptic transmission and behaviour. Hum Mol Genet.

Bucci C, De Luca M (2012) Molecular basis of Charcot-Marie-Tooth type 2B disease. Biochem Soc Trans 40:1368–1372.

Bucci C, Parton RG, Mather IH, Stunnenberg H, Simons K, Hoflack B, Zerial M (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70:715–728.

Bucci C, Thomsen P, Nicoziani P, McCarthy J, van Deurs B (2000) Rab7: a key to lysosome biogenesis. Mol Biol Cell 11:467–480.

Burré J, Sharma M, Tsetsenis T, Buchman V, Etherton MR, Südhof TC (2010) Alpha-synuclein promotes SNARE-complex assembly in vivo and in vitro. Science 329:1663–1667.

Cabin DE, Shimazu K, Murphy D, Cole NB, Gottschalk W, McIlwain KL, Orrison B, Chen A, Ellis CE, Paylor R, Lu B, Nussbaum RL (2002) Synaptic vesicle depletion correlates with attenuated synaptic responses to prolonged repetitive stimulation in mice lacking alpha-synuclein. J Neurosci 22:8797–8807.

Cataldo AM, Barnett JL, Berman SA, Li J, Quarless S, Bursztajn S, Lippa C, Nixon RA (1995) Gene expression and cellular content of cathepsin D in Alzheimer's disease brain: evidence for early up-regulation of the endosomal-lysosomal system. Neuron 14:671–680.

Ceresa BP, Lotscher M, Schmid SL (2001) Receptor and membrane recycling can occur with unaltered efficiency despite dramatic Rab5(q79l)-induced changes in endosome geometry. J Biol Chem 276:9649–9654.

Chan C-C, Scoggin S, Wang D, Cherry S, Dembo T, Greenberg B, Jin EJJ, Kuey C, Lopez A, Mehta SQQ, Perkins TJJ, Brankatschk M, Rothenfluh A, Buszczak M, Hiesinger PRR (2011) Systematic discovery of Rab GTPases with synaptic functions in Drosophila. Curr Biol 21:1704–1715.

Chandra S, Chen X, Rizo J, Jahn R, Südhof TC (2003) A broken alpha -helix in folded alpha -Synuclein. J Biol Chem 278:15313–15318.

Chandra S, Gallardo G, Fernández-Chacón R, Schlüter OM, Südhof TC (2005) Alpha-synuclein cooperates with CSPalpha in preventing neurodegeneration. Cell 123:383–396.

Chartier-Harlin MC et al. (2011) Translation initiator EIF4G1 mutations in familial parkinson disease. Am J Hum Genet 89:398–406.

Chartier-Harlin M-C, Kachergus J, Roumier C, Mouroux V, Douay X, Lincoln S, Levecque C, Larvor L, Andrieux J, Hulihan M, Waucquier N, Defebvre L, Amouyel P, Farrer M, Destée A (2004) Alpha-synuclein locus duplication as a cause of familial Parkinson's disease. Lancet 364:1167–1169.

Chavrier P, Parton RG, Hauri HP, Simons K, Zerial M (1990a) Localization of low molecular weight GTP binding proteins to exocytic and endocytic compartments. Cell 62:317–329.

Chavrier P, Vingron M, Sander C, Simons K, Zerial M (1990b) Molecular cloning of YPT1/SEC4-related cDNAs from an epithelial cell line. Mol Cell Biol 10:6578–6585.

Chen RHC, Wislet-Gendebien S, Samuel F, Visanji NP, Zhang G, Marsilio D, Langman T, Fraser PE, Tandon A (2013) α-Synuclein membrane association is regulated by the Rab3a recycling machinery and presynaptic activity. J Biol Chem 288:7438–7449.

Chesselet M-F, Richter F, Zhu C, Magen I, Watson MB, Subramaniam SR (2012) A progressive mouse model of Parkinson's disease: the Thy1-aSyn ("Line 61") mice. Neurotherapeutics 9:297–314.

Chou AP, Li S, Fitzmaurice AG, Bronstein JM (2010) Mechanisms of rotenone-induced proteasome inhibition. Neurotoxicology 31:367–372.

Chutna O, Gonçalves S, Villar-Piqué A, Guerreiro P, Marijanovic Z, Mendes T, Ramalho J, Emmanouilidou E, Ventura S, Klucken J, Barral DC, Giorgini F, Vekrellis K, Outeiro TF (2014) The small GTPase Rab11 co-localizes with α-synuclein in intracellular inclusions and modulates its aggregation, secretion and toxicity. Hum Mol Genet.

Conway KA, Harper JD, Lansbury PT (1998) Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease. Nat Med 4:1318–1320.

Cookson MR (2005) The biochemistry of Parkinson's disease. Annu Rev Biochem 74:29–52.

Cooper AA, Gitler AD, Cashikar A, Haynes CM, Hill KJ, Bhullar B, Liu K, Xu K, Strathearn KE, Liu F, Cao S, Caldwell KA, Caldwell GA, Marsischky G, Kolodner RD, Labaer J, Rochet J, Bonini NM, Lindquist S (2006) Alpha-synuclein blocks ER-Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. Science 313:324–328.

Crabtree D, Dodson M, Ouyang X, Boyer-Guittaut M, Liang Q, Ballestas ME, Fineberg N, Zhang J (2014) Over-expression of an inactive mutant cathepsin D increases endogenous alpha-synuclein and cathepsin B activity in SH-SY5Y cells. J Neurochem 128:950–961.

Cuervo AM, Dice JF (1998) Lysosomes, a meeting point of proteins, chaperones, and proteases. J Mol Med (Berl) 76:6–12.

Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, Sulzer D (2004) Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. Science 305:1292–1295.

Cullen V, Lindfors M, Ng J, Paetau A, Swinton E, Kolodziej P, Boston H, Saftig P, Woulfe J, Feany MB, Myllykangas L, Schlossmacher MG, Tyynelä J (2009) Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo. Mol Brain 2:5.

Dalfó E, Barrachina M, Rosa JL, Ambrosio S, Ferrer I (2004a) Abnormal alpha-synuclein interactions with rab3a and rabphilin in diffuse Lewy body disease. Neurobiol Dis 16:92–97.

Dalfó E, Ferrer I (2005) Alpha-synuclein binding to rab3a in multiple system atrophy. Neurosci Lett 380:170–175.

Dalfó E, Gómez-Isla T, Rosa JL, Nieto Bodelón M, Cuadrado Tejedor M, Barrachina M, Ambrosio S, Ferrer I (2004b) Abnormal alpha-synuclein interactions with Rab

proteins in alpha-synuclein A30P transgenic mice. J Neuropathol Exp Neurol 63:302–313.

Danzer KM, Kranich LR, Ruf WP, Cagsal-Getkin O, Winslow AR, Zhu L, Vanderburg CR, McLean PJ (2012) Exosomal cell-to-cell transmission of alpha synuclein oligomers. Mol Neurodegener 7:42.

Dastoor Z, Dreyer JL (2001) Potential role of nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase in apoptosis and oxidative stress. J Cell Sci 114:1643–1653.

Dauer W, Przedborski S (2003) Parkinson's disease: mechanisms and models. Neuron 39:889–909.

Davie CA (2008) A review of Parkinson's disease. Br Med Bull 86:109–127.

De Lau LML, Breteler MMB (2006) Epidemiology of Parkinson's disease. Lancet Neurol 5:525–535.

Deinhardt K, Salinas S, Verastegui C, Watson R, Worth D, Hanrahan S, Bucci C, Schiavo G (2006) Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway. Neuron 52:293–305.

Desplats P, Lee H-J, Bae E-J, Patrick C, Rockenstein E, Crews L, Spencer B, Masliah E, Lee S-J (2009) Inclusion formation and neuronal cell death through neuron-to-neuron transmission of alpha-synuclein. Proc Natl Acad Sci U S A 106:13010–13015.

Dexter DT, Wells FR, Agid F, Agid Y, Lees AJ, Jenner P, Marsden CD (1987) Increased nigral iron content in postmortem parkinsonian brain. Lancet 2:1219–1220.

Diekmann Y, Seixas E, Gouw M, Tavares-Cadete F, Seabra MC, Pereira-Leal JB (2011) Thousands of rab GTPases for the cell biologist. PLoS Comput Biol 7:e1002217.

Dodson MW, Zhang T, Jiang C, Chen S, Guo M (2012) Roles of the Drosophila LRRK2 homolog in Rab7-dependent lysosomal positioning. Hum Mol Genet 21:1350–1363.

Edvardson S, Cinnamon Y, Ta-Shma A, Shaag A, Yim YI, Zenvirt S, Jalas C, Lesage S, Brice A, Taraboulos A, Kaestner KH, Greene LE, Elpeleg O (2012) A deleterious mutation in DNAJC6 encoding the neuronal-specific clathrin-uncoating Co-chaperone auxilin, is associated with juvenile parkinsonism. PLoS One 7.

Eisbach SE, Outeiro TF (2013) alpha-Synuclein and intracellular trafficking: impact on the spreading of Parkinson's disease pathology. J Mol Med (Berl) 91:693–703.

Ejlerskov P, Rasmussen I, Nielsen TT, Bergström A-L, Tohyama Y, Jensen PH, Vilhardt F (2013) Tubulin polymerization-promoting protein (TPPP/p25α) promotes unconventional secretion of α-synuclein through exophagy by impairing autophagosome-lysosome fusion. J Biol Chem 288:17313–17335.

El-Agnaf OMA, Salem SA, Paleologou KE, Cooper LJ, Fullwood NJ, Gibson MJ, Curran MD, Court JA, Mann DMA, Ikeda S, Cookson MR, Hardy J, Allsop D (2003) Alpha-synuclein implicated in Parkinson's disease is present in extracellular biological fluids, including human plasma. FASEB J 17:1945–1947.

Emmanouilidou E, Melachroinou K, Roumeliotis T, Garbis SD, Ntzouni M, Margaritis LH, Stefanis L, Vekrellis K (2010) Cell-produced alpha-synuclein is secreted in a calcium-dependent manner by exosomes and impacts neuronal survival. J Neurosci 30:6838–6851.

Engelender S (2008) Ubiquitination of alpha-synuclein and autophagy in Parkinson's disease. Autophagy 4:372–374.

Erickson RP, Larson-Thomé K, Valenzuela RK, Whitaker SE, Shub MD (2008) Navajo microvillous inclusion disease is due to a mutation in MYO5B. Am J Med Genet A 146A:3117–3119.

Erlanger DM, Kutner KC, Barth JT, Barnes R (1999) Neuropsychology of sports-related head injury: Dementia Pugilistica to Post Concussion Syndrome. Clin Neuropsychol 13:193–209.

Escusa-Toret S, Vonk WIM, Frydman J (2013) Spatial sequestration of misfolded proteins by a dynamic chaperone pathway enhances cellular fitness during stress. Nat Cell Biol 15:1231–1243.

Fariello RG (1988) Experimental support for the implication of oxidative stress in the genesis of parkinsonian syndromes. Funct Neurol 3:407–412.

Fauvet B, Fares M-B, Samuel F, Dikiy I, Tandon A, Eliezer D, Lashuel HA (2012a) Characterization of semisynthetic and naturally Nα-acetylated α-synuclein in vitro and in intact cells: implications for aggregation and cellular properties of α-synuclein. J Biol Chem 287:28243–28262.

Fauvet B, Kamdem MM, Fares M-B, Desobry C, Michael S, Ardah MT, Tsika E, Coune P, Prudent M, Lion N, Eliezer D, Moore DJ, Schneider B, Aebischer P, El-Agnaf OM, Masliah E, Lashuel HA (2012b) Alpha-synuclein in the central nervous system and from erythrocytes, mammalian cells and E. coli exists predominantly as a disordered monomer. J Biol Chem.

Ferrer I (2011) Neuropathology and neurochemistry of nonmotor symptoms in Parkinson's disease. Parkinsons Dis 2011:708404.

Figueroa C, Taylor J, Vojtek AB (2001) Prenylated Rab acceptor protein is a receptor for prenylated small GTPases. J Biol Chem 276:28219–28225.

Fischer von Mollard G, Stahl B, Li C, Südhof TC, Jahn R (1994a) Rab proteins in regulated exocytosis. Trends Biochem Sci 19:164–168.

Fischer von Mollard G, Stahl B, Walch-Solimena C, Takei K, Daniels L, Khoklatchev A, De Camilli P, Südhof TC, Jahn R (1994b) Localization of Rab5 to synaptic vesicles

identifies endosomal intermediate in synaptic vesicle recycling pathway. Eur J Cell Biol 65:319–326.

Fischer von Mollard G, Südhof TC, Jahn R (1991) A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. Nature 349:79–81.

Fleming SM, Salcedo J, Fernagut P-O, Rockenstein E, Masliah E, Levine MS, Chesselet M-F (2004) Early and progressive sensorimotor anomalies in mice overexpressing wild-type human alpha-synuclein. J Neurosci 24:9434–9440.

Fornai F, Schlüter OM, Lenzi P, Gesi M, Ruffoli R, Ferrucci M, Lazzeri G, Busceti CL, Pontarelli F, Battaglia G, Pellegrini A, Nicoletti F, Ruggieri S, Paparelli A, Südhof TC (2005) Parkinson-like syndrome induced by continuous MPTP infusion: convergent roles of the ubiquitin-proteasome system and alpha-synuclein. Proc Natl Acad Sci U S A 102:3413–3418.

Fortin DL, Troyer MD, Nakamura K, Kubo S, Anthony MD, Edwards RH (2004) Lipid rafts mediate the synaptic localization of alpha-synuclein. J Neurosci 24:6715–6723.

Freed CR, Greene PE, Breeze RE, Tsai WY, DuMouchel W, Kao R, Dillon S, Winfield H, Culver S, Trojanowski JQ, Eidelberg D, Fahn S (2001) Transplantation of embryonic dopamine neurons for severe Parkinson's disease. N Engl J Med 344:710–719.

Freundt EC, Maynard N, Clancy EK, Roy S, Bousset L, Sourigues Y, Covert M, Melki R, Kirkegaard K, Brahic M (2012) Neuron-to-neuron transmission of α-synuclein fibrils through axonal transport. Ann Neurol 72:517–524.

Fujiwara H, Hasegawa M, Dohmae N, Kawashima A, Masliah E, Goldberg MS, Shen J, Takio K, Iwatsubo T (2002) alpha-Synuclein is phosphorylated in synucleinopathy lesions. Nat Cell Biol 4:160–164.

Fusek M, Vetvicka V (2005) Dual role of cathepsin D: ligand and protease. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 149:43–50.

Garcia-Reitböck P, Anichtchik O, Bellucci A, Iovino M, Ballini C, Fineberg E, Ghetti B, Della Corte L, Spano P, Tofaris GK, Goedert M, Spillantini MG (2010) SNARE protein redistribution and synaptic failure in a transgenic mouse model of Parkinson's disease. Brain 133:2032–2044.

Gasser T (2009) Molecular pathogenesis of Parkinson disease: insights from genetic studies. Expert Rev Mol Med 11:e22.

Gasser T, Müller-Myhsok B, Wszolek ZK, Oehlmann R, Calne DB, Bonifati V, Bereznai B, Fabrizio E, Vieregge P, Horstmann RD (1998) A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 18:262–265.

Geppert M, Bolshakov VY, Siegelbaum SA, Takei K, De Camilli P, Hammer RE, Südhof TC (1994) The role of Rab3A in neurotransmitter release. Nature 369:493–497.

Giasson BI, Duda JE, Quinn SM, Zhang B, Trojanowski JQ, Lee VM-Y (2002) Neuronal alpha-synucleinopathy with severe movement disorder in mice expressing A53T human alpha-synuclein. Neuron 34:521–533.

Gimenez MC, Rodríguez Aguirre JF, Colombo MI, Delgui LR (2015) Infectious Bursal Disease Virus uptake Involves Macropinocytosis and Trafficking to Early Endosomes in a Rab5-dependent Manner. Cell Microbiol.

Ginsberg SD, Mufson EJ, Alldred MJ, Counts SE, Wuu J, Nixon RA, Che S (2011) Upregulation of select rab GTPases in cholinergic basal forebrain neurons in mild cognitive impairment and Alzheimer's disease. J Chem Neuroanat 42:102–110.

Ginsberg SD, Mufson EJ, Counts SE, Wuu J, Alldred MJ, Nixon RA, Che S (2010) Regional selectivity of rab5 and rab7 protein upregulation in mild cognitive impairment and Alzheimer's disease. J Alzheimers Dis 22:631–639.

Gitler AD, Bevis BJ, Shorter J, Strathearn KE, Hamamichi S, Su LJ, Caldwell KA, Caldwell GA, Rochet J, McCaffery JM, Barlowe C, Lindquist S (2008) The Parkinson's disease protein alpha-synuclein disrupts cellular Rab homeostasis. Proc Natl Acad Sci U S A 105:145–150.

Glickman MH, Ciechanover A (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 82:373–428.

Goedert M (2001) Alpha-synuclein and neurodegenerative diseases. Nat Rev Neurosci 2:492–501.

Golbe LI, Di Iorio G, Bonavita V, Miller DC, Duvoisin RC (1990) A large kindred with autosomal dominant Parkinson's disease. Ann Neurol 27:276–282.

Gómez-Suaga P, Rivero-Ríos P, Fdez E, Blanca Ramírez M, Ferrer I, Aiastui A, López De Munain A, Hilfiker S (2014) LRRK2 delays degradative receptor trafficking by impeding late endosomal budding through decreasing Rab7 activity. Hum Mol Genet.

Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18:2714–2723.

Guha S, Padh H (2008) Cathepsins: fundamental effectors of endolysosomal proteolysis. Indian J Biochem Biophys 45:75–90.

Gutierrez MG, Munafó DB, Berón W, Colombo MI (2004) Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. J Cell Sci 117:2687–2697.

Haas AL, Siepmann TJ (1997) Pathways of ubiquitin conjugation. FASEB J 11:1257–1268.

Hamamichi S, Rivas RN, Knight AL, Cao S, Caldwell KA, Caldwell GA (2008) Hypothesis-based RNAi screening identifies neuroprotective genes in a Parkinson's disease model. Proc Natl Acad Sci U S A 105:728–733.

Hansen C, Angot E, Bergström A-L, Steiner JA, Pieri L, Paul G, Outeiro TF, Melki R, Kallunki P, Fog K, Li J-Y, Brundin P (2011) α-Synuclein propagates from mouse brain to grafted dopaminergic neurons and seeds aggregation in cultured human cells. J Clin Invest 121:715–725.

Hasegawa T, Konno M, Baba T, Sugeno N, Kikuchi A, Kobayashi M, Miura E, Tanaka N, Tamai K, Furukawa K, Arai H, Mori F, Wakabayashi K, Aoki M, Itoyama Y, Takeda A (2011) The AAA-ATPase VPS4 regulates extracellular secretion and lysosomal targeting of α-synuclein. PLoS One 6:e29460.

Hashimoto M, Hsu LJ, Xia Y, Takeda A, Sisk A, Sundsmo M, Masliah E (1999) Oxidative stress induces amyloid-like aggregate formation of NACP/alpha-synuclein in vitro. Neuroreport 10:717–721.

Hasilik A, von Figura K, Conzelmann E, Nehrkorn H, Sandhoff K (1982) Lysosomal enzyme precursors in human fibroblasts. Activation of cathepsin D precursor in vitro and activity of beta-hexosaminidase A precursor towards ganglioside GM2. Eur J Biochem 125:317–321.

Hatano Y et al. (2004) PARK6-linked autosomal recessive early-onset parkinsonism in Asian populations. Neurology 63:1482–1485.

Hattula K, Furuhjelm J, Tikkanen J, Tanhuanpää K, Laakkonen P, Peränen J (2006) Characterization of the Rab8-specific membrane traffic route linked to protrusion formation. J Cell Sci 119:4866–4877.

Henry L, Sheff DR (2008) Rab8 regulates basolateral secretory, but not recycling, traffic at the recycling endosome. Mol Biol Cell 19:2059–2068.

Hetman M, Filipkowski RK, Domagala W, Kaczmarek L (1995) Elevated cathepsin D expression in kainate-evoked rat brain neurodegeneration. Exp Neurol 136:53–63.

Hodge GK, Butcher LL (1980) Pars compacta of the substantia nigra modulates motor activity but is not involved importantly in regulating food and water intake. Naunyn Schmiedebergs Arch Pharmacol 313:51–67.

Hutagalung AH, Novick PJ (2011) Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91:119–149.

Itoh T, Fujita N, Kanno E, Yamamoto A, Yoshimori T, Fukuda M (2008) Golgi-resident small GTPase Rab33B interacts with Atg16L and modulates autophagosome formation. Mol Biol Cell 19:2916–2925.

Jäger S, Bucci C, Tanida I, Ueno T, Kominami E, Saftig P, Eskelinen E-L (2004) Role for Rab7 in maturation of late autophagic vacuoles. J Cell Sci 117:4837–4848.

Jang A, Lee H-J, Suk J-E, Jung J-W, Kim K-P, Lee S-J (2010) Non-classical exocytosis of alpha-synuclein is sensitive to folding states and promoted under stress conditions. J Neurochem 113:1263–1274.

Jankovic J (2008) Parkinson's disease: clinical features and diagnosis. J Neurol Neurosurg Psychiatry 79:368–376.

Jao CC, Der-Sarkissian A, Chen J, Langen R (2004) Structure of membrane-bound alpha-synuclein studied by site-directed spin labeling. Proc Natl Acad Sci U S A 101:8331–8336.

Jo E, Darabie AA, Han K, Tandon A, Fraser PE, McLaurin J (2004) alpha-Synuclein-synaptosomal membrane interactions: implications for fibrillogenesis. Eur J Biochem 271:3180–3189.

Kang L, Moriarty GM, Woods LA, Ashcroft AE, Radford SE, Baum J (2012) N-terminal acetylation of α-synuclein induces increased transient helical propensity and decreased aggregation rates in the intrinsically disordered monomer. Protein Sci 21:911–917.

Kiely AP, Asi YT, Kara E, Limousin P, Ling H, Lewis P, Proukakis C, Quinn N, Lees AJ, Hardy J, Revesz T, Houlden H, Holton JL (2013) α-Synucleinopathy associated with G51D SNCA mutation: a link between Parkinson's disease and multiple system atrophy? Acta Neuropathol 125:753–769.

Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, Shimizu N (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 392:605–608.

Klucken J, Poehler A-M, Ebrahimi-Fakhari D, Schneider J, Nuber S, Rockenstein E, Schlötzer-Schrehardt U, Hyman BT, McLean PJ, Masliah E, Winkler J (2012) Alpha-synuclein aggregation involves a bafilomycin A 1-sensitive autophagy pathway. Autophagy 8:754–766.

Koike M, Nakanishi H, Saftig P, Ezaki J, Isahara K, Ohsawa Y, Schulz-Schaeffer W, Watanabe T, Waguri S, Kametaka S, Shibata M, Yamamoto K, Kominami E, Peters C, von Figura K, Uchiyama Y (2000) Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons. J Neurosci 20:6898–6906.

Kordower JH, Chu Y, Hauser R a, Freeman TB, Olanow CW (2008a) Lewy body-like pathology in long-term embryonic nigral transplants in Parkinson's disease. Nat Med 14:504–506.

Kordower JH, Chu Y, Hauser RA, Olanow CW, Freeman TB (2008b) Transplanted dopaminergic neurons develop PD pathologic changes: a second case report. Mov Disord 23:2303–2306.

Krebs CE, Karkheiran S, Powell JC, Cao M, Makarov V, Darvish H, Di Paolo G, Walker RH, Shahidi GA, Buxbaum JD, De Camilli P, Yue Z, Paisán-Ruiz C (2013) The sac1 domain of SYNJ1 identified mutated in a family with early-onset progressive parkinsonism with generalized seizures. Hum Mutat 34:1200–1207.

Krüger R, Kuhn W, Müller T, Woitalla D, Graeber M, Kösel S, Przuntek H, Epplen JT, Schöls L, Riess O (1998) Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. Nat Genet 18:106–108.

Kuwahara T, Koyama A, Koyama S, Yoshina S, Ren C-H, Kato T, Mitani S, Iwatsubo T (2008) A systematic RNAi screen reveals involvement of endocytic pathway in neuronal dysfunction in alpha-synuclein transgenic C. elegans. Hum Mol Genet 17:2997–3009.

Kuzuhara S, Mori H, Izumiyama N, Yoshimura M, Ihara Y (1988) Lewy bodies are ubiquitinated. A light and electron microscopic immunocytochemical study. Acta Neuropathol 75:345–353.

Lam HA, Wu N, Cely I, Kelly RL, Hean S, Richter F, Magen I, Cepeda C, Ackerson LC, Walwyn W, Masliah E, Chesselet M-F, Levine MS, Maidment NT (2011) Elevated tonic extracellular dopamine concentration and altered dopamine modulation of synaptic activity precede dopamine loss in the striatum of mice overexpressing human α-synuclein. J Neurosci Res 89:1091–1102.

Larsen KE, Schmitz Y, Troyer MD, Mosharov E, Dietrich P, Quazi AZ, Savalle M, Nemani V, Chaudhry F a, Edwards RH, Stefanis L, Sulzer D (2006) Alpha-synuclein overexpression in PC12 and chromaffin cells impairs catecholamine release by interfering with a late step in exocytosis. J Neurosci 26:11915–11922.

Laurén J, Gimbel DA, Nygaard HB, Gilbert JW, Strittmatter SM (2009) Cellular prion protein mediates impairment of synaptic plasticity by amyloid-beta oligomers. Nature 457:1128–1132.

Lautier C, Goldwurm S, Dürr A, Giovannone B, Tsiaras WG, Pezzoli G, Brice A, Smith RJ (2008) Mutations in the GIGYF2 (TNRC15) Gene at the PARK11 Locus in Familial Parkinson Disease. Am J Hum Genet 82:822–833.

Lee HJ, Kang SJ, Lee K, Im H (2011) Human α-synuclein modulates vesicle trafficking through its interaction with prenylated Rab acceptor protein 1. Biochem Biophys Res Commun 412:526–531.

Lee H-J, Khoshaghideh F, Patel S, Lee S-J (2004) Clearance of alpha-synuclein oligomeric intermediates via the lysosomal degradation pathway. J Neurosci 24:1888–1896.

Lee H-J, Patel S, Lee S-J (2005) Intravesicular localization and exocytosis of alpha-synuclein and its aggregates. J Neurosci 25:6016–6024.

Lee M-TG, Mishra A, Lambright DG (2009) Structural mechanisms for regulation of membrane traffic by rab GTPases. Traffic 10:1377–1389.

Lei N, Franken L, Ruzehaji N, Offenhäuser C, Cowin AJ, Murray RZ (2012) Flightless, secreted through a late endosome/lysosome pathway, binds LPS and dampens cytokine secretion. J Cell Sci 125:4288–4296.

Levitan K, Chereau D, Cohen SIA, Knowles TPJ, Dobson CM, Fink AL, Anderson JP, Goldstein JM, Millhauser GL (2011) Conserved C-terminal charge exerts a profound influence on the aggregation rate of α-synuclein. J Mol Biol 411:329–333.

Li J-Y, Englund E, Holton JL, Soulet D, Hagell P, Lees AJ, Lashley T, Quinn NP, Rehncrona S, Björklund A, Widner H, Revesz T, Lindvall O, Brundin P (2008) Lewy bodies in

grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. Nat Med 14:501–503.

Li Y-J et al. (2002) Age at onset in two common neurodegenerative diseases is genetically controlled. Am J Hum Genet 70:985–993.

Liu J, Zhang J-P, Shi M, Quinn T, Bradner J, Beyer R, Chen S, Zhang J (2009) Rab11a and HSP90 regulate recycling of extracellular alpha-synuclein. J Neurosci 29:1480–1485.

Liu J, Zhou Y, Wang Y, Fong H, Murray TM, Zhang J (2007) Identification of proteins involved in microglial endocytosis of alpha-synuclein. J Proteome Res 6:3614–3627.

Liu Y, Fallon L, Lashuel HA, Liu Z, Lansbury PT (2002) The UCH-L1 gene encodes two opposing enzymatic activities that affect α-synuclein degradation and Parkinson's disease susceptibility. Cell 111:209–218.

Luk KC, Kehm V, Carroll J, Zhang B, O'Brien P, Trojanowski JQ, Lee VM-Y (2012) Pathological α-synuclein transmission initiates Parkinson-like neurodegeneration in nontransgenic mice. Science 338:949–953.

Lundblad M, Decressac M, Mattsson B, Björklund A (2012) Impaired neurotransmission caused by overexpression of α-synuclein in nigral dopamine neurons. Proc Natl Acad Sci U S A.

Magen I, Fleming SM, Zhu C, Garcia EC, Cardiff KM, Dinh D, De La Rosa K, Sanchez M, Torres ER, Masliah E, Jentsch JD, Chesselet M-F (2012) Cognitive deficits in a mouse model of pre-manifest Parkinson's disease. Eur J Neurosci 35:870–882.

Maltsev AS, Ying J, Bax A (2012) Impact of N-terminal acetylation of α-synuclein on its random coil and lipid binding properties. Biochemistry 51:5004–5013.

Maroteaux L, Campanelli JT, Scheller RH (1988) Synuclein: a neuron-specific protein localized to the nucleus and presynaptic nerve terminal. J Neurosci 8:2804–2815.

Marzella L, Ahlberg J, Glaumann H (1981) Autophagy, heterophagy, microautophagy and crinophagy as the means for intracellular degradation. Virchows Arch B Cell Pathol Incl Mol Pathol 36:219–234.

Masliah E, Rockenstein E, Veinbergs I, Mallory M, Hashimoto M, Takeda A, Sagara Y, Sisk A, Mucke L (2000) Dopaminergic loss and inclusion body formation in alpha-synuclein mice: implications for neurodegenerative disorders. Science 287:1265–1269.

Masuda-Suzukake M, Nonaka T, Hosokawa M, Oikawa T, Arai T, Akiyama H, Mann DMA, Hasegawa M (2013) Prion-like spreading of pathological α-synuclein in brain. Brain 136:1128–1138.

McCray BA, Skordalakes E, Taylor JP (2010) Disease mutations in Rab7 result in unregulated nucleotide exchange and inappropriate activation. Hum Mol Genet 19:1033–1047.

McLean PJ, Kawamata H, Hyman BT (2001) Alpha-synuclein-enhanced green fluorescent protein fusion proteins form proteasome sensitive inclusions in primary neurons. Neuroscience 104:901–912.

McNaught KS, Jenner P (2001) Proteasomal function is impaired in substantia nigra in Parkinson's disease. Neurosci Lett 297:191–194.

McNaught KSP, Olanow CW (2006) Proteasome inhibitor-induced model of Parkinson's disease. Ann Neurol 60:243–247.

Mendez MF (1995) The neuropsychiatric aspects of boxing. Int J Psychiatry Med 25:249–262.

Mizushima N, Ohsumi Y, Yoshimori T (2002) Autophagosome formation in mammalian cells. Cell Struct Funct 27:421–429.

Mukhopadhyay A (1997) Rab7 Regulates Transport from Early to Late Endocytic Compartments in Xenopus Oocytes. J Biol Chem 272:13055–13059.

Murphy DD, Rueter SM, Trojanowski JQ, Lee VM (2000) Synucleins are developmentally expressed, and alpha-synuclein regulates the size of the presynaptic vesicular pool in primary hippocampal neurons. J Neurosci 20:3214–3220.

Nachury M V, Loktev A V, Zhang Q, Westlake CJ, Peränen J, Merdes A, Slusarski DC, Scheller RH, Bazan JF, Sheffield VC, Jackson PK (2007) A core complex of BBS proteins cooperates with the GTPase Rab8 to promote ciliary membrane biogenesis. Cell 129:1201–1213.

Nakajima H, Amano W, Fujita A, Fukuhara A, Azuma Y-T, Hata F, Inui T, Takeuchi T (2007) The active site cysteine of the proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase is essential in oxidative stress-induced aggregation and cell death. J Biol Chem 282:26562–26574.

Nakajima H, Amano W, Kubo T, Fukuhara A, Ihara H, Azuma Y-T, Tajima H, Inui T, Sawa A, Takeuchi T (2009) Glyceraldehyde-3-phosphate dehydrogenase aggregate formation participates in oxidative stress-induced cell death. J Biol Chem 284:34331–34341.

Nakaso K, Tajima N, Ito S, Teraoka M, Yamashita A, Horikoshi Y, Kikuchi D, Mochida S, Nakashima K, Matsura T (2013) Dopamine-Mediated Oxidation of Methionine 127 in α-Synuclein Causes Cytotoxicity and Oligomerization of α-Synuclein. PLoS One 8:e55068.

Nemani VM, Lu W, Berge V, Nakamura K, Onoa B, Lee MK, Chaudhry FA, Nicoll RA, Edwards RH (2010) Increased expression of alpha-synuclein reduces neurotransmitter release by inhibiting synaptic vesicle reclustering after endocytosis. Neuron 65:66–79.

Ng EL, Gan BQ, Ng F, Tang BL (2012) Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes. Cell Biochem Funct 30:515–523.

Nielsen E, Severin F, Backer JM, Hyman AA, Zerial M (1999) Rab5 regulates motility of early endosomes on microtubules. Nat Cell Biol 1:376–382.

Outeiro TF, Lindquist S (2003) Yeast cells provide insight into alpha-synuclein biology and pathobiology. Science 302:1772–1775.

Paisan-Ruiz C, Bhatia KP, Li A, Hernandez D, Davis M, Wood NW, Hardy J, Houlden H, Singleton A, Schneider SA (2009) Characterization of PLA2G6 as a locus for dystonia-parkinsonism. Ann Neurol 65:19–23.

Pankratz N, Nichols WC, Uniacke SK, Halter C, Rudolph A, Shults C, Conneally PM, Foroud T (2002) Genome screen to identify susceptibility genes for Parkinson disease in a sample without parkin mutations. Am J Hum Genet 71:124–135.

Parkinson J (1817) An essay on the shaking palsy. Whittingham and Rowland.

Pasanen P, Myllykangas L, Siitonen M, Raunio A, Kaakkola S, Lyytinen J, Tienari PJ, Pöyhönen M, Paetau A (2014) A novel α-synuclein mutation A53E associated with atypical multiple system atrophy and Parkinson's disease-type pathology. Neurobiol Aging 35:2180.e1–e5.

Pereira-Leal JB, Seabra MC (2001) Evolution of the Rab family of small GTP-binding proteins. J Mol Biol 313:889–901.

Petroi D, Popova B, Taheri-Talesh N, Irniger S, Shahpasandzadeh H, Zweckstetter M, Outeiro TF, Braus GH (2012) Aggregate clearance of α-synuclein in Saccharomyces cerevisiae depends more on autophagosome and vacuole function than on the proteasome. J Biol Chem 287:27567–27579.

Pfeffer SR (2001) Rab GTPases: specifying and deciphering organelle identity and function. Trends Cell Biol 11:487–491.

Polymeropoulos MH (1997) Mutation in the -Synuclein Gene Identified in Families with Parkinson's Disease. Science (80-) 276:2045–2047.

Polymeropoulos MH et al. (1997) Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 276:2045–2047.

Polymeropoulos MH, Higgins JJ, Golbe LI, Johnson WG, Ide SE, Di Iorio G, Sanges G, Stenroos ES, Pho LT, Schaffer AA, Lazzarini AM, Nussbaum RL, Duvoisin RC (1996) Mapping of a gene for Parkinson's disease to chromosome 4q21-q23. Science 274:1197–1199.

Poteryaev D, Datta S, Ackema K, Zerial M, Spang A (2010) Identification of the switch in early-to-late endosome transition. Cell 141:497–508.

Potokar M, Lacovich V, Chowdhury HH, Kreft M, Zorec R (2012) Rab4 and Rab5 GTPase are required for directional mobility of endocytic vesicles in astrocytes. Glia 60:594–604.

Proukakis C, Dudzik CG, Brier T, MacKay DS, Cooper JM, Millhauser GL, Houlden H, Schapira AH (2013) A novel α-synuclein missense mutation in Parkinson disease. Neurology 80:1062–1064.

Qiao L et al. (2008) Lysosomal enzyme cathepsin D protects against alpha-synuclein aggregation and toxicity. Mol Brain 1:17.

Quadri M et al. (2013) Mutation in the SYNJ1 gene associated with autosomal recessive, early-onset parkinsonism. Hum Mutat 34:1208–1215.

Ramirez A, Heimbach A, Gründemann J, Stiller B, Hampshire D, Cid LP, Goebel I, Mubaidin AF, Wriekat A-L, Roeper J, Al-Din A, Hillmer AM, Karsak M, Liss B, Woods CG, Behrens MI, Kubisch C (2006) Hereditary parkinsonism with dementia is caused by mutations in ATP13A2, encoding a lysosomal type 5 P-type ATPase. Nat Genet 38:1184–1191.

Ravikumar B, Imarisio S, Sarkar S, O'Kane CJ, Rubinsztein DC (2008) Rab5 modulates aggregation and toxicity of mutant huntingtin through macroautophagy in cell and fly models of Huntington disease. J Cell Sci 121:1649–1660.

Rendón WO, Martínez-Alonso E, Tomás M, Martínez-Martínez N, Martínez-Menárguez JA (2013) Golgi fragmentation is Rab and SNARE dependent in cellular models of Parkinson's disease. Histochem Cell Biol 139:671–684.

Rink J, Ghigo E, Kalaidzidis Y, Zerial M (2005) Rab conversion as a mechanism of progression from early to late endosomes. Cell 122:735–749.

Rockenstein E, Mallory M, Hashimoto M, Song D, Shults CW, Lang I, Masliah E (2002) Differential neuropathological alterations in transgenic mice expressing alpha-synuclein from the platelet-derived growth factor and Thy-1 promoters. J Neurosci Res 68:568–578.

Rodrigues e Silva AM, Geldsetzer F, Holdorff B, Kielhorn FW, Balzer-Geldsetzer M, Oertel WH, Hurtig H, Dodel R (2010) Who was the man who discovered the "Lewy bodies"? Mov Disord 25:1765–1773.

Sancenon V, Lee S-A, Patrick C, Griffith J, Paulino A, Outeiro TF, Reggiori F, Masliah E, Muchowski PJ (2012) Suppression of α-synuclein toxicity and vesicle trafficking defects by phosphorylation at S129 in yeast depends on genetic context. Hum Mol Genet 21:2432–2449.

Satake W et al. (2009) Genome-wide association study identifies common variants at four loci as genetic risk factors for Parkinson's disease. Nat Genet 41:1303–1307.

Saxena S, Bucci C, Weis J, Kruttgen A (2005) The small GTPase Rab7 controls the endosomal trafficking and neuritogenic signaling of the nerve growth factor receptor TrkA. J Neurosci 25:10930–10940.

Schlossmacher MG, Frosch MP, Gai WP, Medina M, Sharma N, Forno L, Ochiishi T, Shimura H, Sharon R, Hattori N, Langston JW, Mizuno Y, Hyman BT, Selkoe DJ,

Kosik KS (2002) Parkin localizes to the Lewy bodies of Parkinson disease and dementia with Lewy bodies. Am J Pathol 160:1655–1667.

Scott D, Roy S (2012) α-Synuclein inhibits intersynaptic vesicle mobility and maintains recycling-pool homeostasis. J Neurosci 32:10129–10135.

Scott DA, Tabarean I, Tang Y, Cartier A, Masliah E, Roy S (2010) A pathologic cascade leading to synaptic dysfunction in alpha-synuclein-induced neurodegeneration. J Neurosci 30:8083–8095.

Sevcsik E, Trexler AJ, Dunn JM, Rhoades E (2011) Allostery in a disordered protein: oxidative modifications to α-synuclein act distally to regulate membrane binding. J Am Chem Soc 133:7152–7158.

Sevlever D, Jiang P, Yen S-HC (2008) Cathepsin D is the main lysosomal enzyme involved in the degradation of alpha-synuclein and generation of its carboxy-terminally truncated species. Biochemistry 47:9678–9687.

Shojaee S, Sina F, Banihosseini SS, Kazemi MH, Kalhor R, Shahidi GA, Fakhrai-Rad H, Ronaghi M, Elahi E (2008) Genome-wide Linkage Analysis of a Parkinsonian-Pyramidal Syndrome Pedigree by 500 K SNP Arrays. Am J Hum Genet 82:1375–1384.

Sian J, Dexter DT, Lees AJ, Daniel S, Agid Y, Javoy-Agid F, Jenner P, Marsden CD (1994) Alterations in glutathione levels in Parkinson's disease and other neurodegenerative disorders affecting basal ganglia. Ann Neurol 36:348–355.

Silverman JS, Schwartz KJ, Hajduk SL, Bangs JD (2011) Late endosomal Rab7 regulates lysosomal trafficking of endocytic but not biosynthetic cargo in Trypanosoma brucei. Mol Microbiol 82:664–678.

Singleton AB et al. (2003) alpha-Synuclein locus triplication causes Parkinson's disease. Science 302:841.

Sofic E, Lange KW, Jellinger K, Riederer P (1992) Reduced and oxidized glutathione in the substantia nigra of patients with Parkinson's disease. Neurosci Lett 142:128–130.

Sofic E, Riederer P, Heinsen H, Beckmann H, Reynolds GP, Hebenstreit G, Youdim MB (1988) Increased iron (III) and total iron content in post mortem substantia nigra of parkinsonian brain. J Neural Transm 74:199–205.

Sönnichsen B, De Renzis S, Nielsen E, Rietdorf J, Zerial M (2000) Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. J Cell Biol 149:901–914.

Soper JH, Kehm V, Burd CG, Bankaitis VA, Lee VM-Y (2011) Aggregation of α-synuclein in S. cerevisiae is associated with defects in endosomal trafficking and phospholipid biosynthesis. J Mol Neurosci 43:391–405.

Soper JH, Roy S, Stieber A, Lee E, Wilson RB, Trojanowski JQ, Burd CG, Lee VM-Y (2008) Alpha-synuclein-induced aggregation of cytoplasmic vesicles in Saccharomyces cerevisiae. Mol Biol Cell 19:1093–1103.

Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, Goedert M (1997) Alpha-synuclein in Lewy bodies. Nature 388:839–840.

Spina MB, Cohen G (1989) Dopamine turnover and glutathione oxidation: implications for Parkinson disease. Proc Natl Acad Sci U S A 86:1398–1400.

Spinosa MR, Progida C, De Luca A, Colucci AMR, Alifano P, Bucci C (2008) Functional characterization of Rab7 mutant proteins associated with Charcot-Marie-Tooth type 2B disease. J Neurosci 28:1640–1648.

Steiner J a, Angot E, Brundin P (2011) A deadly spread: cellular mechanisms of α-synuclein transfer. Cell Death Differ 18:1425–1433.

Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10:513–525.

Stenmark H, Parton RG, Steele-Mortimer O, Lütcke A, Gruenberg J, Zerial M (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J 13:1287–1296.

Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Müller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Krüger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Hum Mol Genet 14:2099–2111.

Sung JY, Kim J, Paik SR, Park JH, Ahn YS, Chung KC (2001) Induction of neuronal cell death by Rab5A-dependent endocytosis of alpha-synuclein. J Biol Chem 276:27441–27448.

Thayanidhi N, Helm JR, Nycz DC, Bentley M, Liang Y, Hay JC (2010) Alpha-synuclein delays endoplasmic reticulum (ER)-to-Golgi transport in mammalian cells by antagonizing ER/Golgi SNAREs. Mol Biol Cell 21:1850–1863.

Thayanidhi N, Liang Y, Hasegawa H, Nycz DC, Oorschot V, Klumperman J, Hay JC (2012) R-SNARE ykt6 resides in membrane-associated protease-resistant protein particles and modulates cell cycle progression when over-expressed. Biol Cell 104:397–417.

Ulmer TS, Bax A, Cole NB, Nussbaum RL (2005) Structure and dynamics of micelle-bound human alpha-synuclein. J Biol Chem 280:9595–9603.

Utskarpen A, Slagsvold HH, Iversen T-G, Wälchli S, Sandvig K (2006) Transport of ricin from endosomes to the Golgi apparatus is regulated by Rab6A and Rab6A'. Traffic 7:663–672.

Van der Putten H, Wiederhold KH, Probst A, Barbieri S, Mistl C, Danner S, Kauffmann S, Hofele K, Spooren WP, Ruegg MA, Lin S, Caroni P, Sommer B, Tolnay M, Bilbe G

(2000) Neuropathology in mice expressing human alpha-synuclein. J Neurosci 20:6021–6029.

Van Ham TJ, Thijssen KL, Breitling R, Hofstra RMW, Plasterk RHA, Nollen EAA (2008) C. elegans model identifies genetic modifiers of alpha-synuclein inclusion formation during aging. PLoS Genet 4:e1000027.

Vitelli R, Santillo M, Lattero D, Chiariello M, Bifulco M, Bruni CB, Bucci C (1997) Role of the Small GTPase RAB7 in the Late Endocytic Pathway. J Biol Chem 272:4391–4397.

Vitner EB, Dekel H, Zigdon H, Shachar T, Farfel-Becker T, Eilam R, Karlsson S, Futerman AH (2010) Altered expression and distribution of cathepsins in neuronopathic forms of Gaucher disease and in other sphingolipidoses. Hum Mol Genet 19:3583–3590.

Volpicelli-Daley LA, Gamble KL, Schultheiss CE, Riddle DM, West AB, Lee VM-Y (2014) Formation of α-Synuclein Lewy Neurite-like aggregates in Axons Impedes the Transport of Distinct Endosomes. Mol Biol Cell.

Volpicelli-Daley LA, Luk KC, Patel TP, Tanik SA, Riddle DM, Stieber A, Meaney DF, Trojanowski JQ, Lee VM-Y (2011) Exogenous α-synuclein fibrils induce Lewy body pathology leading to synaptic dysfunction and neuron death. Neuron 72:57–71.

Wang W et al. (2011) A soluble α-synuclein construct forms a dynamic tetramer. Proc Natl Acad Sci U S A 108:17797–17802.

Webb JL, Ravikumar B, Atkins J, Skepper JN, Rubinsztein DC (2003) Alpha-Synuclein is degraded by both autophagy and the proteasome. J Biol Chem 278:25009–25013.

Wider C, Skipper L, Solida A, Brown L, Farrer M, Dickson D, Wszolek ZK, Vingerhoets FJG (2008) Autosomal dominant dopa-responsive parkinsonism in a multigenerational Swiss family. Park Relat Disord 14:465–470.

Willingham S, Outeiro TF, DeVit MJ, Lindquist SL, Muchowski PJ (2003) Yeast genes that enhance the toxicity of a mutant huntingtin fragment or alpha-synuclein. Science 302:1769–1772.

Winner B, Jappelli R, Maji SK, Desplats PA, Boyer L, Aigner S, Hetzer C, Loher T, Vilar M, Campioni S, Tzitzilonis C, Soragni A, Jessberger S, Mira H, Consiglio A, Pham E, Masliah E, Gage FH, Riek R (2011) In vivo demonstration that alpha-synuclein oligomers are toxic. Proc Natl Acad Sci U S A 108:4194–4199.

Wislet-Gendebien S, Visanji NP, Whitehead SN, Marsilio D, Hou W, Figeys D, Fraser PE, Bennett SAL, Tandon A (2008) Differential regulation of wild-type and mutant alpha-synuclein binding to synaptic membranes by cytosolic factors. BMC Neurosci 9:92.

Wong E, Cuervo AM (2010) Integration of clearance mechanisms: the proteasome and autophagy. Cold Spring Harb Perspect Biol 2:a006734.

Yelamanchili S V, Chaudhuri AD, Flynn CT, Fox HS (2011) Upregulation of cathepsin D in the caudate nucleus of primates with experimental parkinsonism. Mol Neurodegener 6:52.

Yin G, Lopes da Fonseca T, Eisbach SE, Anduaga AM, Breda C, Orcellet ML, Szegő ÉM, Guerreiro P, Lázaro DF, Braus GH, Fernandez CO, Griesinger C, Becker S, Goody RS, Itzen A, Giorgini F, Outeiro TF, Zweckstetter M, Szegő EM (2014) α-Synuclein interacts with the switch region of Rab8a in a Ser129 phosphorylation-dependent manner. Neurobiol Dis 70C:149–161.

Zarranz JJ, Alegre J, Gómez-Esteban JC, Lezcano E, Ros R, Ampuero I, Vidal L, Hoenicka J, Rodriguez O, Atarés B, Llorens V, Gomez Tortosa E, del Ser T, Muñoz DG, de Yebenes JG (2004) The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. Ann Neurol 55:164–173.

Zeigerer A, Gilleron J, Bogorad RL, Marsico G, Nonaka H, Seifert S, Epstein-Barash H, Kuchimanchi S, Peng CG, Ruda VM, Del Conte-Zerial P, Hengstler JG, Kalaidzidis Y, Koteliansky V, Zerial M (2012) Rab5 is necessary for the biogenesis of the endolysosomal system in vivo. Nature 485:465–470.

Zerial M, Sönnichsen B (n.d.) Confocal images of Rab4-, Rab5- and Rab11-labeling.

Zhu M, Li J, Fink AL (2003) The association of alpha-synuclein with membranes affects bilayer structure, stability, and fibril formation. J Biol Chem 278:40186–40197.

Acknowledgements

My genuine thanks go to Prof. Dr. Tiago Outeiro for the opportunity to conduct my doctoral studies in his lab and providing me with the time and space to do so.

I also want to thank my thesis committee, Prof. Dr. Markus Zweckstetter and Dr. Sebastian Kügler, for their patience, their open door and the opportunity for collaborations.

This thesis would never have seen completion if not for the continued moral, scientific and technical support of Dr. Ellen Gerhardt, Dr. Éva Szegő, Dr. Anna Villar-Piqué and Christiane Fahlbusch. Their help was invaluable many times over and I couldn't have asked for more. Thank you Tomás Fonseca and Raquel Pinho for your support and sharing food and coffee. Also thank you to all the other people who joined the lab over the years, for however long a time it was, for the diversity. Thank you to Anke Gregus for the help running those blots, as well as Sonja Reisenauer and Omar Diaz for keeping everything else running.

I owe more than just my thanks to Elisabeth, who was my saving grace and an enormous emotional support through the rough patches, and who occasionally kicked me out of inertia. She also always dealt admirably with my obsessions as well as my shortcomings. I want to thank Sue-Ann for living life in parallel and for ending the Great Radio Silence. Thank you Manuel for reminding me always that everything can be less complicated. Thank you Nathalie for the coffee and sharing my hockey passion.

The first to ever inspire me for research was Diane, and I'm grateful she reminded me recently how hard it is to re-evaluate decisions. I've known Anna, Katharina, Kristina, Miriam and Monika for the majority of my life now and their continued friendship and support are valued beyond what they might believe. Thank you Cassie for sharing law school madness and all things Canada, and Sam for providing perspective in recent months. When I came to Göttingen I never would have dreamed of meeting all of these amazing people who I was lucky to spend time with. Their generosity and open mindedness have always been something to aspire to. It's impossible to thank all of them, but thank you to my girls—Andonia, Oliwia, Silvia and Tina—for movie dates and dancing through the night, and to Andrea for making introductions and for all the long talks. Thank you to Luis, who made a difference. My thanks also go to Peter Watts for keeping me reminded of the other end of the spectrum.

I want to thank William, Calle, along with the other Pappenheimer (my studies in both harmony and hereditary insanities), as well as Kris and Alex (my teachers in perseverance), and James (my eternal pragmatist) for opening their lives to me. I owe them for the distraction they provided. I learned more with them than I would ever have expected.

I have to thank Peter Loose and Reinhold Schrieck for being lion tamers and not giving up on us in general, and me in particular, when they had good reason to. I am who I am in part due to their faith in me and believing in me when even I didn't anymore, back then and in more recent times.

Finally I owe my deepest gratitude and more to my mother, Monika Ahrens, and grandmother, Elisabeth Ahrens, who wanted me to succeed and do well, even when they didn't understand what I was doing for the most part. Ultimately it was their support and their unquestioning love that helped me finish this and come all this way.

Curriculum Vitae

Name: Sibylle Elisabeth Eisbach
Date of birth: 06. July 1984
Place of birth Wickede (Ruhr)
Nationality: German
Marital status: Single

Education:

08.2011 – present University of Göttingen, DFG PhD Program Programm "Molecular Physiology of the Brain" (Göttingen Graduate School for Neuroscience, Biophysics and Molecular Bioscience)
Thesis: "The interplay between α-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies"
Supervisor: Prof. Dr. Tiago Outeiro

10.2008 – 10.2010 **Master of Science (MSc)** "Molecular Cell Biology", University of Bielefeld
Thesis: "Identification of Neuronal NF-κB Targets in the Mouse"
Department for Animal Cell Biology
supervisor: apl. Prof. Dr. Barbara Kaltschmidt

10.2005 – 09.2008 **Bachelor of Science (BSc),** University of Bielefeld
Thesis: "Characterisation of redox-sensitive fluorescent proteins and establishing of redox potential measurements in living cells"
Department for Biochemistry and Physiology of Plants
supervisor: Jun. Prof. Dr. Thorsten Seidel

09.1995 – 07.2004 Walburgisgymnasium Menden

Engagement:

Organization committee 2nd PhD Symposium of DFG PhD Program "Molecular Physiology of the Brain" of the Göttingen Graduate School for Neuroscience, Biophysics and Molecular Bioscience; esp. Booklet

Additional Qualifications:

Languages German (native speaker), English (fluent), French (basic), proof of Latin

Certificates "Writing in the Sciences" Stanford Online, with distinction

Publications:

2014 Yin G, Lopes da Fonseca T, **Eisbach SE**, Anduaga AM, Breda C, Orcellet ML, Szegő ÉM, Guerreiro P, Lázaro DF, Braus GH, Fernandez CO, Griesinger C, Becker S, Goody RS, Itzen A, Giorgini F, Outeiro TF, Zweckstetter M, Szegő EM (2014) α-Synuclein interacts with the switch region of Rab8a in a Ser129 phosphorylation-dependent manner. Neurobiol Dis 70C:149–161.

Fonseca-Ornelas L, **Eisbach SE**, Paulat M, Giller K, Fernández CO, Outeiro TF, Becker S, Zweckstetter M (2014) Small molecule-mediated stabilization of vesicle-associated helical α-synuclein inhibits pathogenic misfolding and aggregation. Nat Commun 5:5857.

2013 **Eisbach SE,** Outeiro TF (2013) alpha-Synuclein and intracellular trafficking: impact on the spreading of Parkinson's disease pathology. J Mol Med (Berl) 91:693–703.

Posters:

1) 10th Göttingen Meeting if the Neuroscience Society; Eisbach, S.E., Outeiro, T.F. "The interplay between alpha-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies"

2) Neurizons 2013 PhD Conference; Eisbach, S.E., Outeiro, T.F. "The interplay between alpha-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies"

3) PhD Symposium "Game of Brains"; Eisbach, S.E., Outeiro T.F. "The interplay between alpha-synuclein and Rab GTPases in Parkinson's disease"

4) GGNB Science Day 2013; Eisbach, S.E., Szegő, E.M., Outeiro T.F. "The interplay between alpha-synuclein and Rab GTPases in Parkinson's disease"

5) Neuroscience Students Work Shop (NeuroDoWo 2013) Eisbach, S.E., Szegő, E.M., Outeiro, T.F. "The interplay between alpha-synuclein and Rab GTPases: Insights into the molecular basis of synucleinopathies"