Amyloid-beta Causes Autophagy Dysfunction by Inhibiting Protein Prenylation

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

Centre for Neuroscience
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Abstract

There is ample evidence that autophagy is affected in Alzheimer's disease (AD) but the causes, the nature of the dysfunction and the mechanisms of autophagy impairment are unclear. Autophagy depends on vesicular trafficking and membrane fusion, events that rely on several protein complexes and small GTPases. Previously our laboratory demonstrated that a neurotoxic mechanism of amyloid-β oligomers (Aβ) is inhibition of protein prenylation. Reduced protein prenylation results in impairment of intracellular and axonal trafficking. The Rab family of small GTPases are prenylated proteins required for normal trafficking, membrane fusion and autophagy. We hypothesize that defective autophagy in AD is due to inhibited protein prenylation and restoring protein prenylation will normalize the autophagic pathway and prevent neuronal death. We performed *in vitro* experiments to determine the nature of autophagy dysfunction. A biomarker of autophagy is the microtubule-associated protein light chain 3-II (LC3-II), which associates with the autophagosome membrane. Neurons challenged with Aβ accumulated LC3-II when analyzed by western blot. LC3-II increase is ambiguous, since it could represent induced autophagy or blocked lysosomal degradation of LC3-II. To differentiate between these possibilities we directly examined autophagic flux by expressing mCherry-GFP-LC3 in cultured cells. Autophagic flux was decreased in cultured cells treated with Aβ, and was recovered by rescuing protein prenylation with geranylgeranylpyrophosphate. Similarly, simvastatin and psoromic acid, two agents that inhibit protein prenylation, also blocked autophagic flux in a prenylation-dependent manner. Among prenylated proteins we focus on Rab7, which is essential

in autophagy progression and lysosomal biogenesis, and is altered in brains of AD patients. During autophagy, Rab7 localizes to autophagosomes together with LC3. Treatment with Aβ reduced Rab7 co-localization with LC3. Normalization of protein prenylation restored colocalization of Rab7 and LC3.

Significantly, reversing autophagy dysfunction has been validated as an innovative therapeutic strategy in AD. Yet, the lack of knowledge on the nature and cause(s) of autophagy dysfunction prevents the development of selective autophagy-targeted strategies with disease-modifying value. Our work will provide an essential evidence base for potential therapeutic developments that target autophagy flux in the CNS.

Acknowledgements

I was fortunate for the opportunity to perform graduate studies at the University of Alberta within the Neuroscience and Mental Health Institute, and the Department of Pharmacology. There are several people that made this possible and contributed to the work in this thesis, and it is my pleasure to thank them.

First and foremost, I am forever thankful to my supervisor, Prof. Elena Posse de Chaves for accepting me in her laboratory and guiding me from start to finish throughout my Master of Science program. Elena taught me valuable lessons on critical thinking, experimental design and scientific writing, and has opened my mind to new worlds of knowledge. I was very fortunate to travel to Halifax, Santiago, Ottawa, and Chicago during my graduate program both for my thesis and for science in general; my supervisor promoted and enabled these scientific travels and I am indebted to her for my experiences around the world.

Thank you to my supervisory committee: Prof. William Colmers and Prof. Satyabrata Kar for the valuable insights in our meetings and throughout my graduate studies, and for each of your classes that enriched my knowledge. Also thank you Dr. Colmers for your expresso machine. Thank you Prof. Thomas Simmen for being my external examiner for my thesis defense and the useful feedback. Thank you Prof. Claudio Hetz for welcoming me into your laboratory at the University of Chile in order to learn a technique for this thesis.

I owe much gratitude to the past and present members of the Posse de Chaves laboratory. This thesis would not have been possible without the hard work of Neha

Sharma, Sarah Samuelson, Dr. Amany Mohamed, and Dr. Elizabeth Garcia. They each taught me valuable lessons at the bench and helped create an enjoyable working environment.

Thank you to Sheldon Michaelson, James MacKay, Luis Carlos, Danny Galleguillos, Vicente Valenzuela, Melanie Alpaugh, Katherine Mifflin, Jennifer Ling, and Kevin Thorburn for the technical assistance, scientific discussions, and many laughs.

I am very grateful to the Canadian Institutes of Health Research for the Frederick Banting and Charles Best graduate scholarship; to my supervisor's funding from the Alzheimer Society of Canada and CIHR; and to the Graduate Student Association and Faculty of Graduate Studies and Research for the student travel awards.

Last but not least, I am deeply thankful to my beloved friends and family who push me to be my best and support me at my worst. You know who you are and I am lucky to have you.

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AD	Alzheimer's disease
AICD	APP intracellular C-terminal domain
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ATG	Autophagy-related genes
Atg	Autophagy-related proteins
AV	Autophagic vesicle
Aβ	Amyloid-beta peptides
BafA1	Bafilomycin A1
BBB	Blood-brain-barrier
CSF	Cerebrospinal fluid
CTF	Carboxy-terminal fragment
DHC	Dehydrocholesterol
ER	Endoplasmic reticulum
FPP	Farnesyl pyrophosphate
FTase	FPP transferase enzyme
FYCO	FYVE and coiled-coil domain containing protein
GAP	GTPase activating protein
GDI	GDP-dissociation inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GGPP	Geranylgeranyl pyrophosphate
GGTase	GGPP trasferase enzyme
GTP	Guanosine triphosphate
HMGR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HOPS	Homotypic fusion and vacuole protein sorting
ICV	Intracerebroventricular
IPP	Isopentenyl pyrophosphate
LAMP	Lysosome-associated membrane protein
LC3	Microtubule-associated protein light chain 3
MCI	Mild cognitive impairment
MTOC	Microtubule organizing centre
mTOR	Mammalian target of rapamycin
MVB	Multivesicular body
NFT	Neurofibrillary tangle

oAβ	Amyloid beta oligomers
PA	Psoromic acid
PI3K	Phosphoinositide 3-kinase
PS1	Presenilin-1
REP	Rab escort protein
RILP	Rab-interacting lysosomal protein
ROS	Reactive oxygen species
SREBP	Sterol regulatory element-binding protein
TGN	Trans-Golgi network
tLC3	mCherry-GFP-LC3 fluorescent protein
Vps	Vacuolar protein sorting

1. Introduction

1.1. Alzheimer's disease

Age-related dementia is most commonly caused by Alzheimer's disease (AD), and is estimated to contribute to about 75% of all cases (Barker et al., 2002). The Alzheimer's Disease International report of 2015 approximated that 46.8 million people are currently living with AD or related dementias worldwide, and that this number will reach 131.5 million by 2050 due to the increased proportion of elderly (Prince et al., 2015). In Canada there are 747 thousand individuals living with AD and related dementias, and this number is predicted to double by the year 2031 (Alzheimer-Society, 2015). The total worldwide economic cost was estimated at 818 billion USD annually in 2015, and was projected to rise to 2 trillion USD by 2030 (Prince et al., 2015). These substantial numbers include direct and indirect monetary costs and the hundreds of millions of hours sacrificed by informal care-givers, who face many emotional and physical difficulties later in life.

AD has been studied for over a century and, although the pathogenesis of AD is incompletely understood, important insights have enabled an initial understanding of the causes behind this progressive neurodegenerative disease. AD exist in two forms: the early-onset, familial form, which accounts for approximately 1-5% of cases and occurs in the fourth to fifth decade of life; and the late-onset sporadic form that occurs in the seventh or eighth decade, for which age is the strongest risk factor (Alzheimer-Society, 2010; Pimplikar, 2009). While less is known about the etiology of sporadic AD, both types appear to be due to an imbalance in Aβ production and

clearance (Hardy and Selkoe, 2002) and have the same neurological and histopathological features (Pimplikar, 2009). The familial form is driven by overproduction of amyloid beta peptides (Aβ) that are prone to aggregate and cause neurotoxicity (Selkoe, 1991). In contrast, Aβ production is not significantly altered in sporadic AD. Instead, defects in the clearance of Aβ play a key role in sporadic AD development (Saido, 2013; Selkoe and Schenk, 2003). Overwhelming evidence suggests that AD pathogenesis is driven by aberrant protein aggregation (Holtzman et al., 2011).

1.1.1. AD is a disorder of protein aggregation

AD is named after Alois Alzheimer, a German psychiatrist, who, in the early 20th century, studied a 51 year-old female patient that developed rapid memory loss and behavioural deterioration over a 4.5 year period (Alzheimer et al., 1995). Alzheimer's examination of the patient's brain post-mortem revealed general brain atrophy, arteriosclerotic vascular tissue, adipose saccules in glial cells, tangles of neurofibrils, and miliary foci scattered throughout the cortex (Alzheimer et al., 1995).

With the advancement of molecular staining and microscopy methods, it became clear that AD is a disorder of protein aggregation in which accumulation of Aβ and microtubule-associated protein tau drive pathogenesis (Holtzman et al., 2011). The two classical hallmarks of AD are known as neurofibrillary tangles (NFTs) and senile plaques (Selkoe et al., 2004), which correspond to intracellular and extracellular protein aggregations, respectively. NFTs are composed of the hyper-

phosphorylated microtubule-associated protein tau which forms paired helical filaments (PHFs) that aggregate into tangles (Goedert et al., 2006; Lee and Trojanowski, 1992). A large body of evidence supports a central role for NFTs in AD pathogenesis as well as other diseases collectively known as tauopathies (Iqbal and Grundke-Iqbal, 2008; Medina and Avila, 2014; Spillantini and Goedert, 2013), but there is some evidence that NFT pathology is triggered by Aβ accumulation (Golde et al., 2011; Jin et al., 2011). This thesis is focused on Aβ pathology.

The senile plaques are large and complex in composition, containing dystrophic neuronal processes, activated glia cells, and other inflammatory components that surround a compact deposit of $A\beta_{42}$ fibrils (Divry and Florkin, 1927; Glenner and Wong, 1984; Schneider, 1998). Fibrillar deposits of Aβ are usually observed at the centre of, and surrounding, senile plaques, in brain parenchyma and leptomeningeal blood vessels (reviewed in (Mohamed et al., 2011)). Non-fibrillar aggregates are present as diffuse deposits through the gray matter, and represent the early stages of Aβ aggregation (reviewed in (Mohamed et al., 2011)). Cholesterol and its transport protein, apolipoprotein E (apoE) have also been found in senile plaques (Burns et al., 2003; Namba et al., 1991). There are many proteins that associate with amyloid in senile plaques. Some proteins inhibit while others enhance Aβ fibrillization (Castillo et al., 1997; Fraser et al., 1993). For example, the heat-shock protein (Hsp) chaperones interact with Aβ, and their overexpression protect against $A\beta_{42}$-induced toxicity (Magrane et al., 2005). It was suggested that the formation and toxicity of Aβ aggregates may depend on the chaperone-to-Aβ ratio and on the nature of the Aβ assembly involved in the interaction (reviewed in (Mohamed et al., 2011)).

An interesting proposal, known as the 'inside-out hypothesis,' is that senile plaques may result from the lysis of neurons caused by intraneuronal Aβ accumulation (D'Andrea et al., 2001; Pensalfini et al., 2014). Comparably, it is accepted that Aβ is released from nerve terminals of synapses and is deposited extracellularly (Lazarov et al., 2002; Lundgren et al., 2014).

1.1.2. The cholinergic hypothesis of AD

Another hallmark of AD is the degeneration of basal forebrain cholinergic neurons (Davies and Maloney, 1976), which project to the cortex and hippocampus, and are important in memory function (Drachman and Leavitt, 1974; Volpicelli-Daley et al., 2003). The dysfunction of cholinergic innervation is the most consistent neuropathological feature in AD, and is the strongest correlate to the degree of dementia (Cummings and Back, 1998; Schneider, 1998). The selective degeneration of the cholinergic systems may result from toxicity caused by the NFT and $A\beta_{42}$ aggregations (Schneider, 1998). This was inferred from the apparent pathogenic spread of NFTs and senile plaques that begin accumulating in medial temporal regions, and advance to the basal forebrain cholinergic nucleus (Schneider, 1998). Acetylcholinesterase inhibitors were the first drugs used clinically to ameliorate cognitive dysfunction in AD (Galimberti and Scarpini, 2011); they were created to prevent the decreased cholinergic neurotransmission in AD (Galimberti and Scarpini, 2011). However, the treatment does not delay or prevent the underlying neurodegeneration, which is the goal for novel therapeutics (Strittmatter, 2012).

1.1.3. Putative etiology of AD

The amyloid protein (i.e., $A\beta_{42}$) was discovered in senile plaques (Glenner et al., 1984; Masters et al., 1985). It was later found to be produced via enzymatic cleavage of amyloid precursor protein (APP) (Goldgaber et al., 1987; Kang et al., 1987). Thereafter, different missense mutations of APP were identified by studying pedigrees with cases of familial AD (Goate et al., 1991; Murrell et al., 1991; Naruse et al., 1991). These mutations are responsible for the over-production of $A\beta_{42}$ in familial AD (Citron et al., 1992), and there are now over 25 APP mutations known to be causative of familial AD (Thinakaran and Koo, 2008). Moreover, mutations in the APP processing enzymes that generate $A\beta_{42}$ have also been identified (St George-Hyslop et al., 1992), which also promote $A\beta_{42}$ over-production (Selkoe and Wolfe, 2007). Additionally, middle-aged adults with Trisomy-21 also have abnormally high $A\beta_{42}$ load resulting in AD-like pathology and often symptoms of dementia due to the localization of APP in chromosome 21 (St George-Hyslop et al., 1987). These discoveries were crucial in forming the dominant theory for AD pathogenesis: the amyloid-cascade hypothesis, which posits that the accumulation of $A\beta_{42}$ in senile plaques triggers a cascade of cell stress, inflammation, and toxicity that causes neurodegeneration (Hardy and Allsop, 1991; Selkoe, 1991).

1.2. Amyloid-β peptides

1.2.1. The amyloid-cascade hypothesis

The foundation of this hypothesis came from studies of infrequent autosomal dominant forms of AD in which overproduction of Aβ invariably results in the disease

(reviewed in (Musiek and Holtzman, 2015)). The original version of the amyloid hypothesis postulated that Aβ is the causative agent in AD and that NFTs, neuron loss, vascular damage, and dementia result from Aβ deposition into senile plaques (Hardy and Higgins, 1992). However, an early event that causes pathology and neuronal dysfunction in AD is the intracellular accumulation of soluble Aβ in neurons (D'Andrea et al., 2001; Gouras et al., 2000). Thus, the hypothesis was revised to include this key event (reviewed in (Wirths and Bayer, 2012; Wirths et al., 2004)).

1.2.2. Aβ production

Aβ is a group of hydrophobic peptides derived from sequential proteolytic cleavage of the ubiquitously expressed type-I transmembrane glycoprotein, APP (Kang et al., 1987; Maulik et al., 2013; Selkoe et al., 2004). APP processing occurs through the non-amyloidogenic pathway and the amyloidogenic pathway (**Figure 1**) (reviewed in (Holtzman et al., 2011; Maulik et al., 2013)). In the predominant non-amyloidogenic pathway, APP is cleaved by the α-secretase protease within its Aβ peptide region to produce soluble APPα and the α-C-terminal fragment (CTF) (i.e., C83). α-CTF is then cleaved by the endomembrane γ-secretase protease complex to yield the cytoplasmic APP intracellular C-terminal domain (AICD) and the extracellular P3 peptide (i.e., a significantly truncated Aβ) Explicitly, the α-secretase cleavage precludes Aβ production (Selkoe, 1991). Several enzymes including tumor necrosis factor-α-converting enzyme/A disintegrin and metalloproteinase domain containing protein-17 (TACE/ADAM-17), ADAM-9, and ADAM-10 can cleave APP

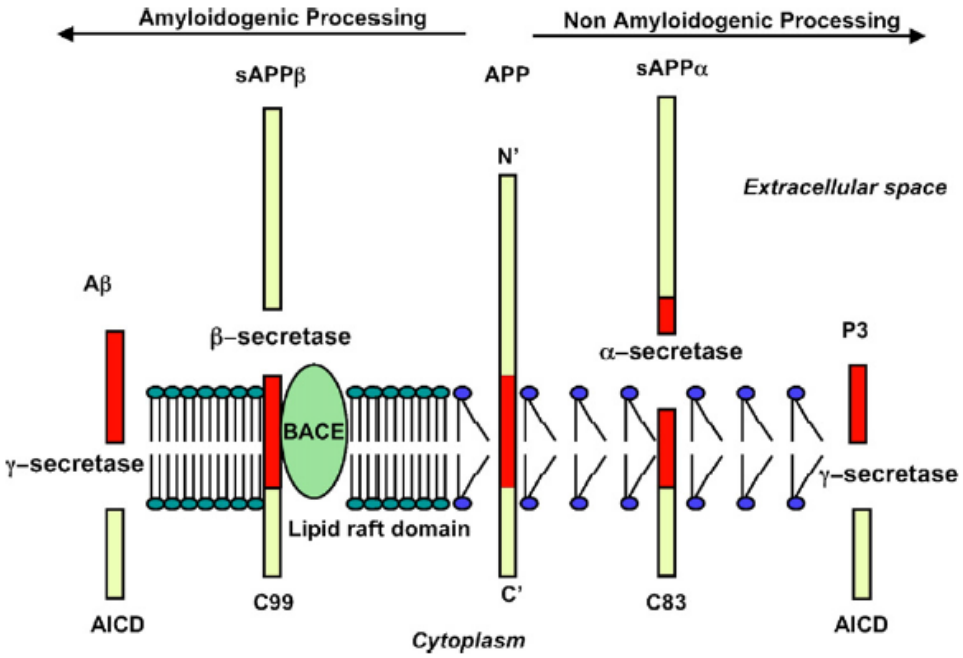


Figure 1. The amyloidogenic and non-amyloidogenic pathways for APP processing. α-secretase cleaves APP in cholesterol- and sphingolipid-poor domains and by β-secretase (BACE) in lipid raft domains generating the correspondent C-terminal fragments (CTFs) C83 and C99 and the large ectomains sAPPα and sAPPβ, respectively. The α-secretase cleaves within the Aβ region to preclude amyloidogenic processing. γ-secretase cleavage of C83 results in the release of P3. Aβ is the result of γ-secretase activity on C99. In both pathways γ-secretase releases APP intracellular domain (AICD), which may be involved in nuclear signaling. Taken from Posse de Chaves, E.I. (2006) *Biochim Biophys Acta* **1758**, 1995-2015, copyright license 3751670554703.

via α-secretase activity. On the other hand, the γ-secretase protease complex comprises the aspartyl protease presenilin 1 (PS1) or presenilin 2 (PS2) and three cofactors [i.e., nicastrin, presenilin enhancer protein 2 (PEN-2), and anterior pharynx defective 1 (APH-1)]. In the amyloidogenic pathway, APP is cleaved by the β-secretase protease to produce sAPPβ and the β-CTF (i.e., C99); the β-CTF is then cleaved by γ-secretase to yield the Aβ peptides (reviewed in (Maulik et al., 2013)). β-secretase is an aspartyl protease called β -site APPcleaving enzyme 1 (BACE1). The γ-secretase protease cleaves APP imprecisely to produce Aβ peptides ranging from 38-43 residues; however, $A\beta_{40}$ and $A\beta_{42}$ are the most common and approximately make up 90% and 10% of Aβ production, respectively (Selkoe et al., 2004). Both the non-amyloidogenic and amyloidogenic pathways of APP processing occur under normal conditions in all cells (reviewed in (Haass et al., 1992; Mohamed et al., 2011)). The Aβ peptides are continuously secreted and are found in cerebrospinal fluid and plasma (Seubert et al., 1992). Notably, the non-amyloidogenic and amyloidogenic pathways of APP processing occur at different cellular sites (Thinakaran and Koo, 2008). The non-amyloidogenic pathway occurs primarily at the plasma membrane (Sisodia, 1992), whereas amyloidogenic processing occurs inside neurons in the endocytic vesicles, trans-Golgi network, and endoplasmic reticulum (ER) (Greenfield et al., 1999; Koo and Squazzo, 1994; Langui et al., 2004). Thus, Aβ peptides may have pathogenic opportunities within neurons before reaching the extracellular space and senile plaques.

1.2.3. Assemblies of Aβ

Originally, Aβ was characterized as the subunit of fibrils found in senile plaques (Glenner and Wong, 1984; Masters et al., 1985). The monomeric Aβ peptides are initially unstructured, but undergo a solubility-dependent conformational change and gain secondary structure rich in β-sheets (Shen and Murphy, 1995). This secondary structure allows Aβ to stack orthogonally into protofilaments, which then laterally associate into fibrils via cross β-sheets (reviewed in (Mohamed et al., 2011)). Because the concentrations of Aβ found in human CSF and conditioned media from human brain cells is in the nanomolar range (Seubert et al., 1992), whereas micromolar concentrations of Aβ were required for *in vitro* aggregation (Lomakin et al., 1997; Mohamed et al., 2011), a seeding mechanism was proposed to account for the initial polymerization of Aβ peptides (Harper and Lansbury, 1997). Potential seeds could be trace metal ions, membrane gangliosides, or other proteins (reviewed in (Masters and Selkoe, 2012)).

Among the structural requirements of Aβ aggregation, a penta-residue sequence, KLVFF, near the middle of Aβ is essential to the aggregation propensity of Aβ peptides (Tjernberg et al., 1996). Furthermore, the C-terminal residues of Aβ are mainly hydrophobic and promote aggregation of Aβ (reviewed in (Mohamed et al., 2011)). It was confirmed that $A\beta_{42}$ is more prevalent in senile plaques than shorter Aβ peptides due to its stronger hydrophobicity (Iwatsubo et al., 1994; Jarrett et al., 1993; Snyder et al., 1994).

The amyloid fibrils may be considered as an end-state assembly of Aβ peptides due to their compact and insoluble nature. However, there are several intermediate

assemblies of Aβ peptides that exist between the monomeric and fibrillar forms (reviewed in (Mohamed et al., 2011)). In particular, Aβ peptides aggregate into soluble, low- and high-molecular weight oligomers, ranging from dimers to >250-mers, that take various forms such as trimers, globulomers, amylospheroids, and protofibrils (reviewed in (Benilova et al., 2012; Mohamed et al., 2011)). These various oligomers exist in a complex equilibrium with monomers and fibrils; and likely all contribute to Aβ toxicity (Benilova et al., 2012), although perhaps with different potencies (Townsend et al., 2006). Soluble oligomeric species of Aβ (oAβ) were extracted from AD brain tissue (Gong et al., 2003; Kayed et al., 2003; Lacor et al., 2004; McLean et al., 1999), and were also generated *in vitro* (Dahlgren et al., 2002). The neurotoxicity of cell-derived and synthetic oAβ are known to differ: the former causes toxicity in the nanomolar range, whereas the latter requires micromolar concentrations to cause neuronal toxicity (Benilova et al., 2012; Reed et al., 2011). The reasons for this disparity are unclear, but may involve heterogeneity in the cell-derived (i.e., "natural") oAβ assemblies; that is, multiple oligomeric conformations acting synergistically as a toxic 'oligomer soup' (Benilova et al., 2012). In contrast, the well-defined conditions in which synthetic oAβ is generated may allow for the characterization of assembly-specific effects (Benilova et al., 2012). The experiments in this thesis used a synthetic preparation of oAβ that has been previously characterized to mainly contain dimers, trimers, and tetramers (Dahlgren et al., 2002; Saavedra et al., 2007).

1.2.4. Intraneuronal Aβ

Thirty years ago it was predicted that "the amyloid is first deposited in the neuron" (Masters et al., 1985). Later, the accumulation of intraneuronal Aβ in AD was observed in immunohistochemical studies with Aβ-specific antibodies (Gouras et al., 2000; Langui et al., 2004). Interestingly, the intraneuronal Aβ immunoreactivity was more abundant than NFT and senile plaque pathology, and was suggested to precede these two classical hallmarks (Gouras et al., 2000). In support of this notion, intraneuronal Aβ granules preceded extracellular plaque depositions in APPxPS1 transgenic AD mice (Langui et al., 2004). Perhaps expectedly, it was specifically the $A\beta_{42}$ peptide that accumulated within neurons (D'Andrea et al., 2001; Gouras et al., 2000).

Intraneuronal accumulation of $A\beta_{42}$ is now regarded as one of the earliest pathological issues in AD (reviewed in (Mohamed and Posse de Chaves, 2011; Wirths et al., 2004)). Interestingly, the extracellular and intracellular pools of Aβ likely exist in dynamic equilibrium (LaFerla et al., 2007). For instance, Aβ immunotherapy in the 3xTg-AD mouse model reduced extracellular plaque load, which was followed by clearance of intraneuronal Aβ (Oddo et al., 2004). Furthermore, in the same animal model, intraneuronal Aβ accumulation preceded the re-emergence of extracellular Aβ plaques after immunotherapy was ceased, which suggested that intraneuronal Aβ is a source for the extracellular pool (Oddo et al., 2006). Relatedly, the intraneuronal pool of Aβ originates from both inside and outside the cell. Inside neurons, amyloidogenic processing of APP occurs in endosomes, trans-Golgi network (TGN), multivesicular bodies (MVB), and ER (Greenfield et al., 1999; Koo and Squazzo, 1994;

Langui et al., 2004). In addition oAβ_{42} can be internalized from the extracellular space by several mechanisms (reviewed in (Mohamed and Posse de Chaves, 2011)), including a dynamin-dependent endocytic mechanism in axons that is independent of clathrin (Saavedra et al., 2007). After internalization, the oAβ_{42} traffics to MVBs and mixes with endogenously generated Aβ (Mohamed and Posse de Chaves, 2011; Rajendran et al., 2006). The intraneuronal Aβ peptides may be secreted via exosomes and contribute to the extracellular Aβ pool (Rajendran et al., 2006). Moreover, Aβ is released from synapses into the extracellular pool (Schroeder and Koo, 2005). This thesis examined some of the consequences of intracellular oAβ_{42} accumulation.

1.2.5. Mechanisms of Aβ toxicity

Although senile plaques are a classic feature of AD, and senile plaque load is consistently higher in affected AD brain areas compared to the corresponding areas in normal aged brains (Selkoe, 1994), senile plaque load does not correlate with the severity of disease (Braak and Braak, 1991). Conversely, soluble Aβ_{42} levels were highly correlated with the severity of AD (McLean et al., 1999), and there are much higher levels of soluble oligomeric forms of Aβ_{42} in AD brains compared to normal brains (i.e., a 12-fold difference) (Kuo et al., 1996). An interesting idea is that senile plaques represent relatively inert Aβ reservoirs in equilibrium with soluble oAβ assemblies, being the latter the true culprits of neurotoxicity (Hardy and Selkoe, 2002). The type of soluble oAβ assembly may influence its mechanism of toxicity, which could be important for potential treatments aiming to prevent Aβ-induced neurodegeneration (Benilova et al., 2012; Mohamed et al., 2011).

Neurons are highly differentiated, post-mitotic cells that form many connections (i.e., synapses) with other cells in order to function. There is abundant evidence that oAβ damages dendritic spines and impairs plasticity mechanisms such as long-term potentiation (Lambert et al., 1998; Walsh et al., 2002; Wu et al., 2010a). Rats that received intracerebroventricular (ICV) injections of oAβ showed cognitive impairments and memory dysfunction (Cleary et al., 2005). These 'subtle alterations' do not account for the dramatic neuron-loss in AD (Hardy, 2009); however, this kind of synaptic damage may account for the early cognitive problems in people with AD, which has an insidious progression and accelerated neurodegeneration that likely arises from accumulated Aβ stress and NFT pathology (Benilova et al., 2012).

As reviewed by Benilova *et al*. (2012), there are a few general mechanisms by which oAβ initiates synaptotoxicity and neurodegenation: 1) by binding directly to receptors and altering their signaling properties; 2) by inducing pore formation at the plasma membrane, which causes ion imbalances; and 3) by causing non-specific membrane disruptions that lead to oxidative stress. Previous work in our laboratory revealed a novel mechanism of oAβ-induced cytotoxicity: oAβ inhibited the maturation of Sterol Regulatory Element Binding Protein 2 (SREBP2), an important transcriptional regulator for the mevalonate pathway, which decreased production of isoprenoid lipids, and prevented protein prenylation (Mohamed et al., 2012). The inhibition of protein prenylation caused by oAβ may represent a biological pathway that could be targeted for disease-modifying therapy. This thesis investigated a mechanism by which impairment of protein prenylation may lead to neuronal death.

1.3. The Mevalonate Pathway

Cholesterol biosynthesis was elucidated in the first half of the twentieth century through separate investigations by Konrad Bloch and Feodor Lynen, which earned them the Nobel Prize for Physiology or Medicine of 1964 (Zetterstrom, 2009). The process is called the mevalonate pathway (**Figure 2**). It involves the concerted action of over twenty enzymes for the energy-dependent synthesis of cholesterol and isoprenoids from acetyl-CoA (Gaylor, 2002; Goldstein and Brown, 1990). SREBP2 is the most important transcriptional regulator of the mevalonate pathway enzymes (Goldstein et al., 2006; Horton et al., 2003). The rate-limiting enzyme of the pathway is 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), which is the target of the widely used cholesterol-lowering drugs called statins (Goldstein and Brown, 1990; Rodwell et al., 1976).

Enzymes of the mevalonate pathway are expressed in the brain (Valdez et al., 2010), which accounts for 2% of total body weight, yet contains 25% of the body's total cholesterol (Dietschy and Turley, 2004; O'Brien and Sampson, 1965). Brain cholesterol is separated from the peripheral pool of cholesterol by the blood-brain-barrier (BBB) which is normally impermeable to plasma lipoproteins (reviewed in (Dietschy, 2009)). This situation makes the mevalonate pathway particularly important for the brain to secure a very active *in situ* synthesis of cholesterol to meet demands for synaptogenesis, axonal development, and neurotransmitter release (Dietschy and Turley, 2004; Pfrieger and Ungerer, 2011). For this thesis, the mevalonate pathway is conceptually broken down into two main branches: the post-squalene pathway (the cholesterol pathway), and the non-sterol isoprenoids pathway

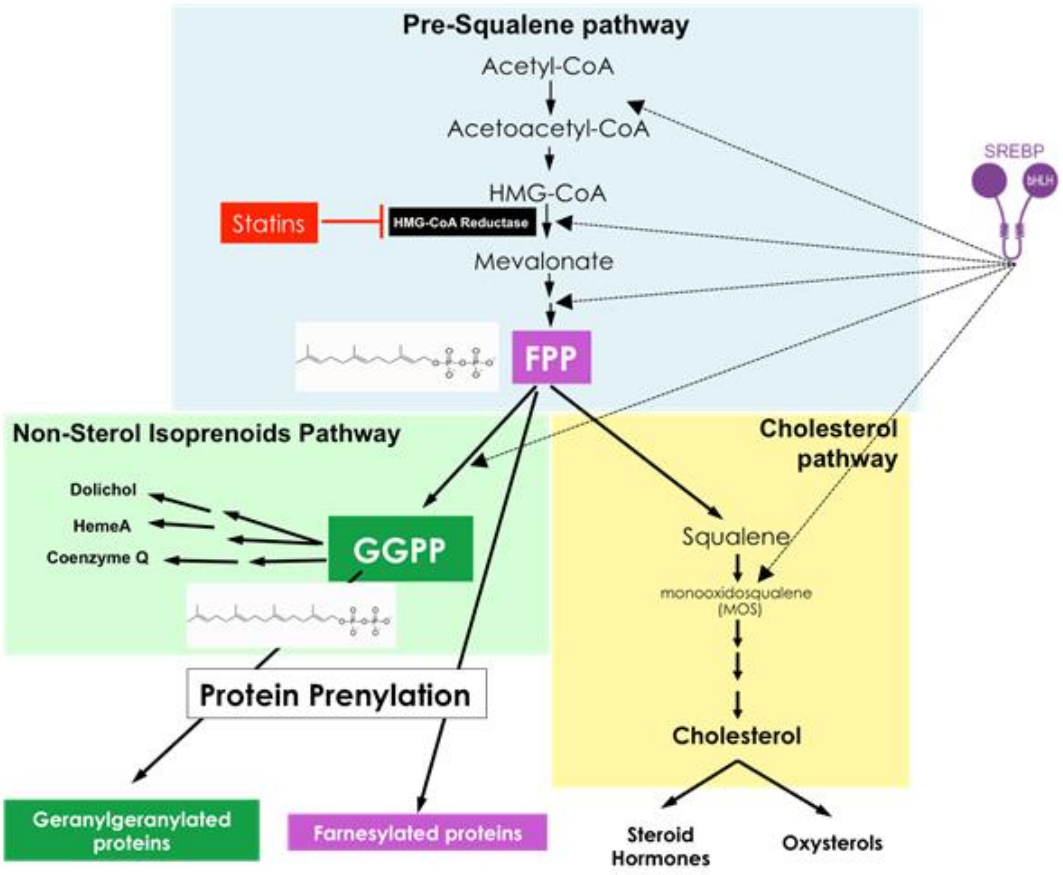


Figure 2. Simplified depiction of the mevalonate pathway showing the two main branches corresponding to isoprenoid synthesis (in green) and cholesterol synthesis (in yellow). SREBP-2 regulates the expression of the enzymes in the mevalonate pathway, including HMG-CoA reductase, the rate-limiting enzyme targeted by statins.

(**Figure 2**). The product of the early mevalonate pathway (i.e., pre-squalene pathway) is farnesyl pyrophosphate (FPP), a 15-carbon isoprenoid lipid, which initiates the branches for the post-squalene pathway and the non-sterol isoprenoids pathway (Mohamed et al., 2015).

1.3.1. Post-squalene pathway

The post-squalene pathway results in production of cholesterol. FPP is converted to squalene by the action of squalene synthase (Do et al., 2009), and this is the first step committed to cholesterol synthesis. Notably, deletion of squalene synthase is embryonic lethal in mice (Tozawa et al., 1999). Squalene is converted to mono-oxidosqualene, and then to lanosterol which is the fundamental carbon skeleton for all fungal and animal steroids. Lanosterol is then metabolized to cholesterol through 19 enzymatic steps in either the Kandutsch-Russell or Bloch pathways (Mohamed et al., 2015). The main difference between the pathways is in the precursors that are used for cholesterol synthesis: the Kandutsch-Russell pathway includes lathosterol and 7-dehydrocholesterol (7-DHC) as intermediates; while the Bloch pathway uses desmosterol as an intermediate (Lutjohann, 2006). Neurons contain precursors for the Kandutsch-Russel pathway (e.g., 7-DHC) whereas astrocytes contain precursors for the Bloch pathway (e.g., desmosterol) (Nieweg et al., 2009). Although the post-squalene pathway is not the focus of this thesis, it is essential for cholesterol production and, therefore, normal function of the central nervous system. In fact, disturbances in this pathway may result in brain replacement

of cholesterol with its precursors, which causes serious disorders of the nervous system (e.g., Smith-Lemli-Opitz syndrome) (Jira, 2013; Tint et al., 1997).

1.3.2. Post-squalene pathway in AD

There is little consensus about total brain cholesterol alterations in patients with AD (Cossec et al., 2010; Martin et al., 2010; Wood et al., 2014). Using different methods for measuring cholesterol, some studies found no change in cholesterol content in any portion of the brain (Edlund et al., 1992; Snipes and Suter, 1997) or the hippocampus in AD brains (Eckert et al., 2000), while other studies reported changes in cholesterol levels in specific brain areas, particularly in regions with extensive Aβ deposits and NFTs. Xiong and collaborators found an increase in cholesterol in the cortex of AD brains (Xiong et al., 2008), Heverin *et al*. described a significant increase of cholesterol concentration in the basal ganglia but not in other brain areas in a small group of AD brains (Heverin et al., 2004), and Cutler *et al*. reported accumulation of free cholesterol in the middle frontal gyrus and frontal cortex but not the unaffected cerebellum in AD brains (Cutler et al., 2004). It was also indicated that, as the severity of the disease progressed, there was an increase in membrane- and amyloid plaque-associated cholesterol (Cutler et al., 2004; Mori et al., 2001; Panchal et al., 2010). It is also possible that a change in the distribution of cholesterol inside brain cells rather than a change in total cholesterol content may influence AD pathology (Burns and Duff, 2002). Our laboratory showed that oAβ_{42} induces cholesterol sequestration within the neuronal endosomal/lysosomal system, and impairs intracellular trafficking (Mohamed et al., 2012). Clearly, cholesterol

homeostasis has important implications for AD; however, this thesis focused on the role of the non-sterol isoprenoids.

1.3.3. Non-sterol isoprenoids pathway

Isoprenoids are important biomolecules, synthesized by all lifeforms. They range in size and complexity from the 5-carbon isopentenyl pyrophosphate (IPP) to long-chain dolichols to ubiquinone, a redox mediator in aerobic respiration (Holstein and Hohl, 2004; McTaggart, 2006). The importance of the non-sterol isoprenoid pathway is emphasized by the number of diseases that are associated with its dysfunction, including AD, Parkinson's disease, cancer, and tuberculosis (Hooff et al., 2010b; Obiol-Pardo et al., 2011; Rajanikant et al., 2007; Wiemer et al., 2009). Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are 15- and 20-carbon isoprenoids, respectively.

The enzymes responsible for synthesis of FPP and its non-sterol derivatives are prenyltransferases that catalyze consecutive condensations of IPPs with allylic substrates to form linear backbones for all isoprenoid compounds (Wang and Ohnuma, 2000). The enzyme GGPPS (GGPP synthase) catalyzes the conversion of FPP into GGPP (Kandutsch et al., 1964). The main role of FPP and GGPP is in the post-translational prenylation (i.e., farnesylation and geranylgeranylation) of proteins (section 1.4). In mouse brain cytosol, FPPS (FPP synthase) and GGPPS activities were higher than those in the corresponding fractions from the liver, perhaps reflecting a higher demand for protein prenylation in the brain (Ericsson et al., 1993). FPPS and GGPPS activities were differentially distributed across various sub-regions of the

brain. FPPS activity was present in all brain regions as expected by the several products that derive from it (Runquist et al., 1995). The activity of GGPPS was ~100 fold lower than FPPS activity, which agrees with the more limited use of GGPP, mostly for protein prenylation and as a precursor of a limited number of metabolites. GGPPS activity was lowest in the cerebellum (Runquist et al., 1995). There have not been any reported cases of FPPS or GGPPS deficiency in humans (Holstein and Hohl, 2004). FPPS, but not GGPPS, is transcriptionally regulated by SREBP-2 (Ericsson et al., 1996; Horton et al., 2002). FPPS is post-translationally regulated by a product-feedback competitive inhibition as FPP (product) competes with GPP (substrate) for the active site (Laskovics et al., 1979; Lutz et al., 1992). Similarly, GGPPS activity is allosterically inhibited by GGPP (Lutz et al., 1992).

1.3.4. Coordination of the post-squalene and non-sterol isoprenoids branches

The mevalonate pathway ensures that non-sterol isoprenoids are produced constantly without over-accumulation of potentially toxic cholesterol. Cells have two alternative sources of cholesterol namely intracellular synthesis, and uptake, but only the intracellular synthesis provides non-sterol isoprenoids; therefore, the mevalonate pathway has to maintain the minimum requirement of isoprenoids at all times irrespective of cholesterol levels. Analysis of the affinity of the enzymes in the different branches of the pathway uncovered the mechanisms that mediate such regulation. The affinity of GGPPS for FPP (K_m value of 0.6 μM) (Ericsson et al., 1993) is much higher than the affinity of squalene synthase for FPP (K_m value of ~15 μM) (Biller et al., 1988). Moreover, both coenzyme Q and dolichol synthesis are saturated

at a much lower concentration of isoprene intermediates than the concentration required to saturate cholesterol synthesis (Gold and Olson, 1966; James and Kandutsch, 1980). Thus, under limited concentrations of mevalonate and FPP, the non-sterol isoprenoid branch will be favored. Furthermore, inhibition of the mevalonate production by statins will reduce FPP supply for the production of cholesterol first. Mohamed *et al.* showed that pravastatin reduced cholesterol synthesis in primary neurons without affecting protein prenylation (therefore, isoprenoid synthesis) (Mohamed et al., 2012); however, prenylation may be reduced by other statins that more potently inhibit the production of mevalonate due to increased lipophilicity and ability to enter cells and cross the BBB. Because of the very high affinity of protein farnesyl transferase for FPP (Km below 0.1 μM) (Sinensky et al., 1990), farnesylation is preserved under many statin treatments (Rauthan and Pilon, 2011; Winter-Vann and Casey, 2005) and would be favored over geranylgeranylation.

1.4. Protein Prenylation

1.4.1. A post-translational modification

Protein prenylation is the post-translational attachment of FPP or GGPP via a thioether linkage to a cysteine residue within a C-terminal motif, catalyzed by prenyltransferase enzymes (**Figure 3**) (Casey and Seabra, 1996). Three types of transferases exist based on the peptide motif they recognize and the isoprenoid moieties they attach to the protein substrates (reviewed in (Casey and Seabra, 1996; McTaggart, 2006)). Farnesyl transferase (FTase) and geranylgeranyl transferase type

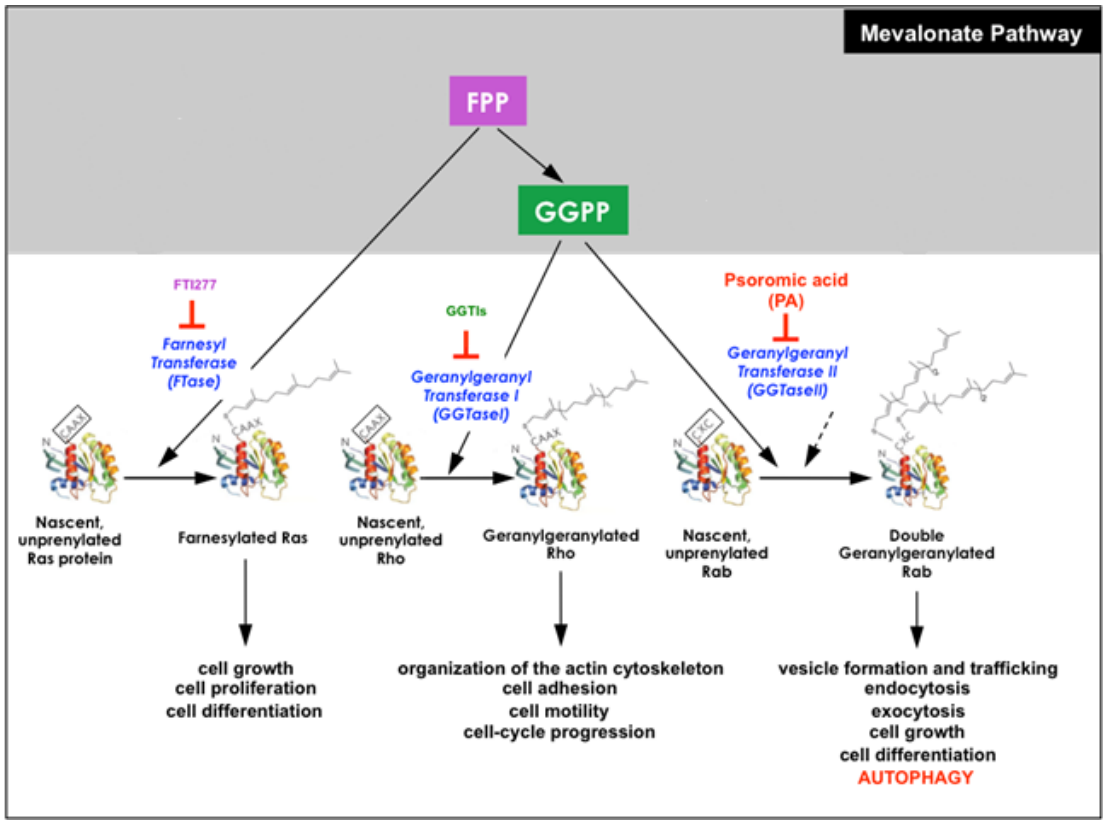


Figure 3. Scheme representing the post-translational modification of proteins by covalent attachment of farnesyl pyrophosphate (FPP) and/or geranylgeranyl pyrophosphate (GGPP) synthesized by the mevalonate pathway. The main prenyl transferases and the major functions of the prenylated products are listed. Some selective protein prenyltransferase inhibitors are also indicated. The focus of this thesis is on the geranylgeranylation of the Rab GTPases (right).

I (GGTase I) prenylate proteins with C-terminal "CAAX" motifs, where C is cysteine, A is often an aliphatic amino acid, and X at the C-terminus determines the specificity of protein prenylation. When X is a methionine or serine (e.g., Ras proteins), the protein is farnesylated and when X is a leucine residue (e.g., Rho family proteins), or a phenylalanine residue, the protein is geranylgeranylated by GGTase I. The Rab proteins are most commonly double-geranylgeranylated. The geranylgeranyl transferase type II (GGTase II) catalyzes attachment of GGPPs to the Rab family of small GTPases onto CC or CXC motifs (Casey and Seabra, 1996; McTaggart, 2006). Hypothetically there are around 300 human proteins that could be prenylated based on the C-terminal motifs (Sebti, 2005). Among them, heterotrimeric G protein subunits, nuclear lamins and small GTPases have been confirmed to be prenylated (McTaggart, 2006). Small GTPases represent the largest group of prenylated proteins.

1.4.2. Functions of prenylated small GTPases

The monomeric small GTPases are a superfamily of proteins that act as molecular switches in signaling pathways, and are active or inactive when bound to GTP or GDP, respectively (Stenmark, 2009). Active small GTPases interact with effector proteins promoting cellular signaling. There are over 100 known small GTPases structurally classified into five subfamilies: Ras, Rho/Rac, Ran, Sar1/ARF, and Rab (Takai et al., 2001). Many cellular activities are regulated by signaling cascades that involve small GTPase activity, including: cell cycle progression, cell adhesion, receptor signaling, gene expression, cytoskeletal organization, and membrane trafficking (Stenmark, 2009; Takai et al., 2001).

Importantly, prenylation provides a hydrophobic tail that promotes the localization of small GTPases to membranes and enables functional interactions of small GTPases with downstream effectors and soluble carrier proteins (e.g., GDP-dissociation inhibitors, GDIs) (Calero et al., 2003; McTaggart, 2006; Pylypenko et al., 2006; ten Klooster and Hordijk, 2007). As an example, some Rabs are mistargeted and dysfunctional when they are singly prenylated (Gomes et al., 2003). Moreover, the crucial role of prenylation and proper membrane localization for Rab function was confirmed by observations that, even in a constitutively active state (GTP-bound mutant Rabs), improperly prenylated Rabs could not achieve proper membrane localization and could not function normally (Gomes et al., 2003). In fact, the localization of small GTPases in distinct subcellular membranes determines their effectors and function and may be altered during disease.

The main focus of this thesis is on the role of Rab *prenylation* in autophagy, and its implications for the pathogenesis of AD.

1.4.3. FPP, GGPP and protein prenylation in AD

Up-regulation of 6 out of 10 genes of isoprenoid metabolism was found in autopsied hippocampus of patients with incipient AD (Blalock et al., 2004), which may represent an attempt to compensate the posttranslational inhibition of the mevalonate pathway during disease. There is limited information with respect to levels and regulation of FPP and GGPP in normal and AD brains. Recent studies showed that GGPP, FPP, and the mRNA of their respective synthases, FPPS and GGPPS, were elevated in brains of 13 male patients with AD (Eckert et al., 2009), in brains of

aged mice (Afshordel et al., 2014; Hooff et al., 2010b) and in neuroblastoma SH-SY5Y cells expressing APP695 (Hooff et al., 2010a). The significance of this elevation is still unknown because protein prenylation was not examined in these studies, and elevation of isoprenoids does not warrant an increase in protein prenylation. Indeed, even when GGPP levels were elevated in the aging mouse brain, the pools of Rac1, RhoA and Cdc42, associated to membranes were decreased, while Rab proteins had a mixed behavior (Afshordel et al., 2014). The reduction of the subunit β of GGTase I in the aging brain may be responsible for the decreased prenylation.

The roles of non-sterol isoprenoids and protein prenylation in AD have been identified mainly by using statins and inhibitors of protein prenyltransferases. FPP, GGPP and prenylated proteins are involved in diverse processes affected in AD pathology including APP metabolism, LTP and synaptic plasticity, Aβ toxicity, and intracellular membrane trafficking (Mohamed et al., 2015).

Statin-induced depletion of non-sterol isoprenoids and inhibition of protein prenyltransferases exert complex effects on APP/Aβ metabolism. In some cases treatment with statins or a FTase inhibitor stimulated the shedding of APP and the production of sAPPα in neuroblastoma cells overexpressing APPswe (Pedrini et al., 2005), while in other cases statins reduced the release of Aβ from cells but increased the intracellular accumulation of APP and Aβ, in a process prevented by GGPP (Cole et al., 2005; Ostrowski et al., 2007). The proteins affected by shortage of non-sterol isoprenoids, and responsible for the regulation APP/Aβ metabolism have been identified or proposed. The increase in APP shedding was mediated by Rho proteins (Pedrini et al., 2005). Rho may also be responsible for the reduction of brain Aβ levels

in CRND8 mice treated with statins, although there was no direct evidence that prenylation was affected (Chauhan et al., 2004). The accumulation of APP and Aβ within neurons that received statins was due to decreased delivery of Rab proteins to cell membranes (Ostrowski et al., 2007). It is known that Rabs participate in intracellular APP trafficking and processing (Jiang et al., 2014). A study of mice treated with statins has shown significant reduction of brain levels of Aβ and the C-terminal fragments (CTFs) due to enhanced trafficking of APP-CTFs to the lysosomes for degradation (Shinohara et al., 2010). The authors suggested that the process may involve a decrease in isoprenoids, and would be mediated by Rabs. However, Rab prenylation was not measured in this study and the conclusion of the involvement of isoprenoids resulted from experiments in cultured neurons in which mevalonate prevented the changes in trafficking. Unless the concentration of mevalonate is titrated to recover specifically the non-sterol isoprenoid pathway, mevalonate would also affect cholesterol levels. The regulation of APP cleavage and Aβ production by non-sterol isoprenoids and protein prenylation also involved APP secretases, although it is unclear if the decrease or the increase in isoprenoids and protein prenylation favors amyloidogenic processing of APP. Inhibition of farnesylation reduced the association of the β-secretase enzyme BACE1 with APP (although BACE itself is not farnesylated) and resulted in a dose-dependent decrease in Aβ release and production within the cell (Parsons and Austen, 2005). Moreover, statins caused inhibition of β-secretase dimerization into its more active form, which may be a mechanism of the reduction in Aβ production (Parsons et al., 2006). Statins also significantly decreased the association of the γ-secretase complex with lipid rafts and

GGOH prevented this (Urano et al., 2005). Contrary to this notion, in a separate study statins induced an increase of BACE levels in neurons, which was linked to the increase in Aβ production (Cole et al., 2005). GGOH, GGPP and FPP increased Aβ production by targeting γ-secretase (Kukar et al., 2005; Zhou et al., 2003; Zhou et al., 2008) but there is no consensus if this effect is dependent (Zhou et al., 2003) or independent (Kukar et al., 2005) of protein prenylation.

Aβ production is not significantly altered in sporadic forms of AD, which represent approximately 95% of cases (Clippingdale et al., 2001; Nathalie and Jean-Noel, 2008; Selkoe and Schenk, 2003). Instead, defects in Aβ removal may be key in the development of sporadic AD (Mawuenyega et al., 2010; Saido and Iwata, 2006). Statins and an FTase inhibitor promoted degradation of extracellular Aβ by microglia by stimulating the secretion of IDE (insulin degrading enzyme), an enzyme that degrades Aβ in the brain (Tamboli et al., 2010). The secretion of IDE from peripheral organs into the circulation was also increased in mice treated with statins (Tamboli et al., 2010). Moreover FTase but not GGTase I haplodeficiency in the APPPS1 mice increased steady-state levels of IDE (Cheng et al., 2013). The mechanisms by which farnesylation may regulate IDE secretion are still unclear.

Non-sterol isoprenoids have been associated with the regulation of neuroinflammation in AD. The role of inflammation in the AD brain is well known. The pro-inflammatory response mediated mainly by microglia may exacerbate and drive the pathogenic processes leading to neuronal loss. Microglia activation may occur as a response to Aβ accumulation in the brain. Statins inhibited the production

of IL-1β by monocytes after stimulation with Aβ, in a process that is independent of cholesterol but prevented by GGPP (Cordle and Landreth, 2005). The effect was mimicked by a GGTase I inhibitor and by inactivation of Rho proteins. Statins also induced cholesterol-independent inhibition of ROS production after stimulation with Aβ (Cordle and Landreth, 2005). Statin treatment of microglia resulted in perturbation of the cytoskeleton and morphological changes due to alteration in Rho family function (Cordle et al., 2005).

During the course of AD, tau is hyper-phosphorylated, detaches from the microtubules, and aggregates in the somatodendritic compartment in NFTs (Iqbal et al., 2010; Kidd, 1963). There is very limited information about the existence of any relationship between tau pathology and isoprenoids and/or protein prenylation. Statins caused changes in tau phosphorylation that were characteristic of those observed in preclinical stages of AD (Meske et al., 2003). These changes were mimicked by GGTase I inhibitors and compensated by GGPP suggesting that decreased prenylation of a Rho family member may be involved. The dose of statins seems to be critical in the effects on tau. In a cellular model of tauopathy, and in primary neurons, low-to-moderate doses of statins reduced total and phosphorylated tau levels, but high doses activated caspase 3 and increased levels of caspase-cleaved tau, which may facilitate tau toxicity/apoptosis (Hamano et al., 2012). A decrease in membrane localization of several small GTPases occurred concomitantly with tau reduction and GGPP reversed statin-induced decreases in tau levels. The authors focused their attention on RhoA, speculating that the statin-induced decrease in phosphorylated tau was caused by glycogen synthase kinase 3β (GSK3β) inactivation

through RhoA (Hamano et al., 2012).

Some recent work in genetically modified mice supported the concept that non-sterol isoprenoids and protein prenylation may have a detrimental role in AD and suggested that inhibition of protein prenylation could be a potential strategy for effectively treating AD. The increase of isoprenoids and protein prenylation has been suggested (although not tested) to contribute to tau pathology in a transgenic APP/PS1 mouse that constitutively overexpresses (P)SREBP-2 (Barbero-Camps et al., 2013). In a different mouse model the expression of protein prenyltransferases was genetically modified in order to reduce protein prenylation independent of non-sterol isoprenoids. Heterozygous deletion of FTase reduced Aβ deposition and neuroinflammation and rescued spatial learning and memory function in APPPS1 mice. Heterozygous deletion of GGTase I reduced the levels of Aβ and neuroinflammation but had no impact on learning and memory (Cheng et al., 2013). These studies in vivo are exciting but will benefit from direct measurement of brain levels of isoprenoids or protein prenylation. Based on the complex regulation of isoprenoid production, it will be important to determine if brain isoprenoid levels change in these mice since the existence of negative-feedback regulatory mechanisms downstream SREBP-2 argue that increased levels of active SREBP-2 does not warrant an increase in non-sterol isoprenoids.

A few prenylated proteins have been linked to AD. The contributions of Rho GTPases to AD are interesting. Rho-family GTPases are key proteins that integrate extracellular and intracellular signals. They are important regulators of the actin

cytoskeleton that play essential roles in orchestrating the development and remodeling of dendritic spines and synapses (Ramakers, 2002; Tolias et al., 2011). Precise spatio-temporal regulation of Rho GTPase activity is critical for their function. Aberrant Rho GTPase signaling due to mutations or other causes can cause spine and synapse defects resulting in abnormal neuronal connectivity and deficient cognitive functioning in humans (Ramakers, 2002; Tolias et al., 2011). Deregulation of Rho GTPases may contribute to dendritic spine loss during AD and might be a key pathogenic event contributing to synaptic deficits in AD (Bolognin et al., 2014; DeGeer and Lamarche-Vane, 2013).

Rab proteins regulate intracellular membrane trafficking, motility and fusion (Takai et al., 2001). In the nervous system, Rabs participate in important processes such as axonal endocytosis, retrograde transport of growth signals, synaptic function, and neurite growth (Ng and Tang, 2008). Rab7 and Rab5 protein levels were upregulated within basal forebrain, frontal cortex, and hippocampus but not in the less vulnerable cerebellum and striatum in MCI and AD (Ginsberg et al., 2011; Ginsberg et al., 2010). Importantly, this upregulation correlated with cognitive decline and neuropathological criteria for AD. The increase of Rab7 and Rab5 in AD brains was interpreted as over-activation of the endosomal pathway. In addition, increased levels of Rab7 have been found in CSF from AD patients and may represent a novel AD CSF biomarker (Armstrong et al., 2014). Previous work in our laboratory demonstrated that prenylation was reduced in primary neurons in culture treated with a toxic concentration of oAβ_{42} (Mohamed et al., 2012). Furthermore, prenylation of Rab7 was decreased in the TgCRND8 mouse model of AD (Mohamed et al., 2012).

Rab7 was investigated because of its association with AD, and its importance in membrane trafficking, a process that was also impaired in neurons treated with oAβ_{42} (Mohamed et al., 2012; Song et al., 2006). Interestingly, dysfunction in the endosomal pathway is recognized as an early neuropathological change in AD that likely induces oAβ_{42} production (Ginsberg et al., 2010; Nixon, 2005).

Autophagy is among the cellular processes that involve membrane trafficking mediated by Rabs (Ao et al., 2014). Autophagy is known to be dysfunctional in AD (Nixon et al., 2005) and is the main focus of this thesis.

1.5. Autophagy

1.5.1. A catabolic mechanism

Autophagy, literally meaning 'self-eating', comprises three major intracellular pathways in eukaryotic cells, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which share the process of lysosomal degradation, but are mechanistically different from one another, in particular in the way the substrates reach the lysosomal lumen (Cuervo, 2004a; Cuervo, 2004b). Macroautophagy (hereafter autophagy) is used to degrade long-lived proteins, aggregates, pathogens, and expired organelles, as well as to provide energy and amino acids (De Duve and Wattiaux, 1966; Klionsky and Emr, 2000; Nakagawa et al., 2004; Ravikumar et al., 2004; Yoshimori, 2004). Thus, autophagy occurs in steady-state conditions to maintain cytoplasm quality, and is induced by environmental stressors for energy and amino acid recycling. For example, induction of autophagy enables newborns to survive the neonatal starvation period between placental separation and initial

feeding (Kuma et al., 2004). Furthermore, neurodegeneration occurs in wild-type mice if they lack basal autophagy in neurons (Hara et al., 2006; Komatsu et al., 2006).

Microautophagy is not well-understood in mammals, but involves lysosomal membrane invagination around cargoes (Mijaljica et al., 2011); in CMA, proteins are shuttled by heat-shock cognate (Hsc) chaperones to the lysosome-associated membrane protein 2 (LAMP2) where they are unfolded and translocated into the lysosomal lumen for degradation (Cuervo and Dice, 1996). Of the three types, autophagy is the major pathway of cargo delivery to lysosomes (Klionsky and Emr, 2000) and is the focus of this thesis (**Figure 4**).

The dynamic process of autophagy requires an intracellular membrane source to sequester portions of cytoplasm for lysosomal delivery. Mammalian autophagosomes likely form randomly throughout the cytoplasm (Bento et al., 2013; Kimura et al., 2008), and there is evidence that ER, ER-mitochondria contact sites, and plasma membrane contribute to phagophore genesis (Hamasaki et al., 2013; Hayashi-Nishino et al., 2009; Ravikumar et al., 2010a). The term 'autophagic flux' is used to refer to the process of autophagosome formation, delivery of cargoes to the lysosome and degradation of the substrates, and is a more reliable indicator of autophagy activity than analysis of autophagosome numbers (Mizushima et al., 2010). Distinct vesicular compartments exist in autophagic flux: autophagosomes, produced from phagophore elongation and closure; amphisomes, produced from late endosome-autophagosome fusion; and autolysosomes, produced from lysosomal fusion with autophagosomes and/or amphisomes (Eskelinen, 2005; Gordon and Seglen, 1988; Mizushima et al., 2001).

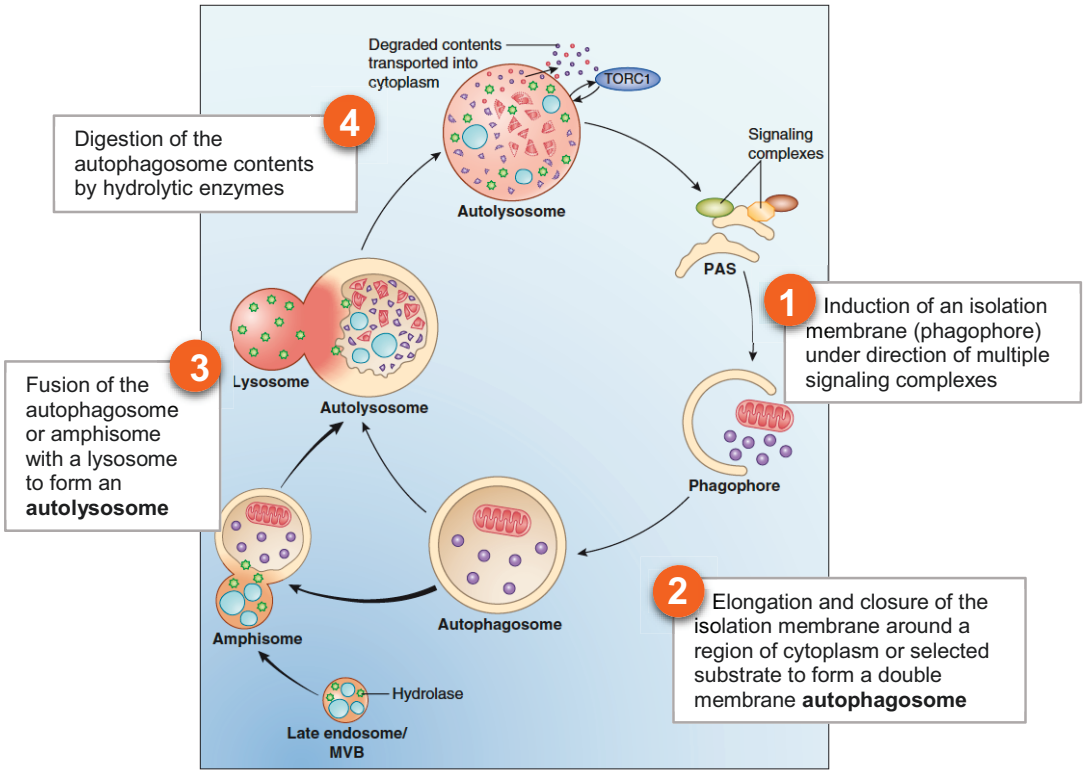


Figure 4. In a very simplified version, autophagy can be viewed as a cycle that involves trapping of selected substrates within a double membrane-limited vacuole (autophagosome), digestion of the substrate by lysosomal hydrolases and release of digestion products, some of which will control autophagy induction. The process is depicted as a cycle, since amino acids released during lysosomal digestion are one mechanism by which mTORC1 on lysosomes may be modulated. MVB, multivesicular body; PAS, preautophagosomal structure. Taken and adapted from Nixon (2013) *Nat Med* **19**, 983-997, copyright license 3746070915790.

1.5.2. Regulation of autophagy

Genetic screens in *Saccharomyces cerevisiae* (yeast) were crucial in discovering the molecular machinery that coordinates autophagic flux (Tsukada and Ohsumi, 1993). The autophagy-related genes and proteins are classified as ATG and Atg, respectively (Klionsky et al., 2003). Over 30 ATG genes have been identified in yeast, with many having mammalian orthologs (Ravikumar et al., 2010b). Interestingly, a proteomic analysis in human embryonic kidney cells revealed an "autophagy interaction network" comprising 751 interactions among 409 candidate proteins under basal autophagy (Behrends et al., 2010), highlighting the complexity of autophagy regulation in mammalian cells.

Induction of autophagy is regulated by two important protein complexes: the ULK1-Atg13-FIP200 complex, and the class III phosphoinositide 3-kinase (PI3K) Vps34-Beclin 1 complex (Bento et al., 2013; Ravikumar et al., 2010b). Their common function is to recruit Atg proteins to the phagophore to induce autophagosome formation. The PI3K pathway is highly regulated through various inhibitors and activators of Beclin 1 and the phosphoinositide cycle (Ravikumar et al., 2010b). The ULK1-Atg13-FIP200 complex is negatively regulated by mammalian target of rapamycin (mTOR) (Ravikumar et al., 2004), a serine/threonine kinase that integrates upstream signals (e.g., amino acid and growth factor signaling) to regulate downstream metabolic functions including autophagy and protein synthesis (Hay and Sonenberg, 2004). mTOR is found in two distinct complexes, mTOR complex 1 (mTORC1) and mTORC2; mTORC2 is a regulator of the actin cytoskeleton (Schmidt et al., 1996), whereas mTORC1 inhibition (e.g., with rapamycin) induces autophagy

(Hosokawa et al., 2009; Sarkar, 2013). mTORC1 will be referred to as mTOR in this thesis.

Important downstream Atg proteins are involved in two ubiquitin-like conjugation systems: the formation of Atg5-Atg12•Atg16L; and the activation and lipidation of LC3 to form LC3-II (microtubule-associated protein light chain 3) (Sarkar, 2013). These systems are crucial for phagophore elongation, cargo sequestration, and closure of the autophagosome. LC3-II is associated to the autophagosome membrane until its degradation in the lysosome, and is the most widely used protein marker of autophagosomes (Klionsky et al., 2012).

The bidirectional trafficking of autophagosomes is microtubule-dependent, and is achieved by the kinesin and dynein/dynactin motor complexes (reviewed in (Bento et al., 2013; Vale, 2003)). Dynein/dynactin activity transports the autophagosome toward the perinuclear microtubule organization centre (MTOC), where lysosomes are relatively abundant and likelihood of fusion is increased (Kimura et al., 2008). Importantly, Rabs regulate the movement of autophagosomes and lysosomes, thus being crucial in autophagy. Specifically Rab7 mediates kinesin and dynein-dependent trafficking of autophagosomes by interacting with specific effector proteins (Bento et al., 2013; Jordens et al., 2001). Moreover, autophagosome-lysosome fusion depends on activated Rab7 (**Figure 5**) (Gutierrez et al., 2004). Rab7 is regulated by guanine nucleotide dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs), and GTPase activating proteins (GAPs). The localization of Rab GTPases is crucial for proper interactions with their effectors, which is mediated in part by dual-geranylgeranylation (i.e., prenylation) (Calero et al., 2003; Gomes et

al., 2003). The C-terminal isoprenoid moieties enable Rabs to interact with GDIs, which function as cytosolic shuttles to transport Rabs between their functional membrane locations (Calero et al., 2003; Pfeffer et al., 1995; Pylypenko et al., 2006). Once in their proper membrane location, Rabs interactions with effectors is governed by their activation status (i.e., GTP- or GDP-bound) (Meresse et al., 1995). Thus, normal regulation of Rab GTPases is important for the progression of autophagic flux.

1.5.3. Autophagy in AD

Strong evidence for autophagy dysfunction in AD comes from Ralph Nixon's laboratory, who used immunogold labeling and electron microscopy to identify an accumulation of autophagic vacuoles (AVs) in the dystrophic neurites found in AD brains (Nixon et al., 2005). The authors suggested that later steps of autophagic flux (i.e., trafficking and fusion) may be impaired in the neurons that accumulated AVs, which was supported by a relatively higher amount of AVs in the distal axons and terminals compared to the cell bodies (Nixon et al., 2005). Nonetheless, aspects of autophagy in AD remain unclear, including its role in the disease process, and the nature of its dysfunction. Interestingly, the autophagy-lysosomal system may shift in AD from a functional and protective state to a pathological and deleterious state via $A\beta_{42}$ toxicity (Ling and Salvaterra, 2011).

There is evidence for several possible roles of autophagy in AD, such as: Aβ production (Yu et al., 2004), Aβ clearance (Hung et al., 2009), and Aβ secretion and plaque formation (Nilsson et al., 2013). Notably, endosomes and AVs contain APP and the secretase proteases that produce $A\beta_{42}$, and there is extensive convergence

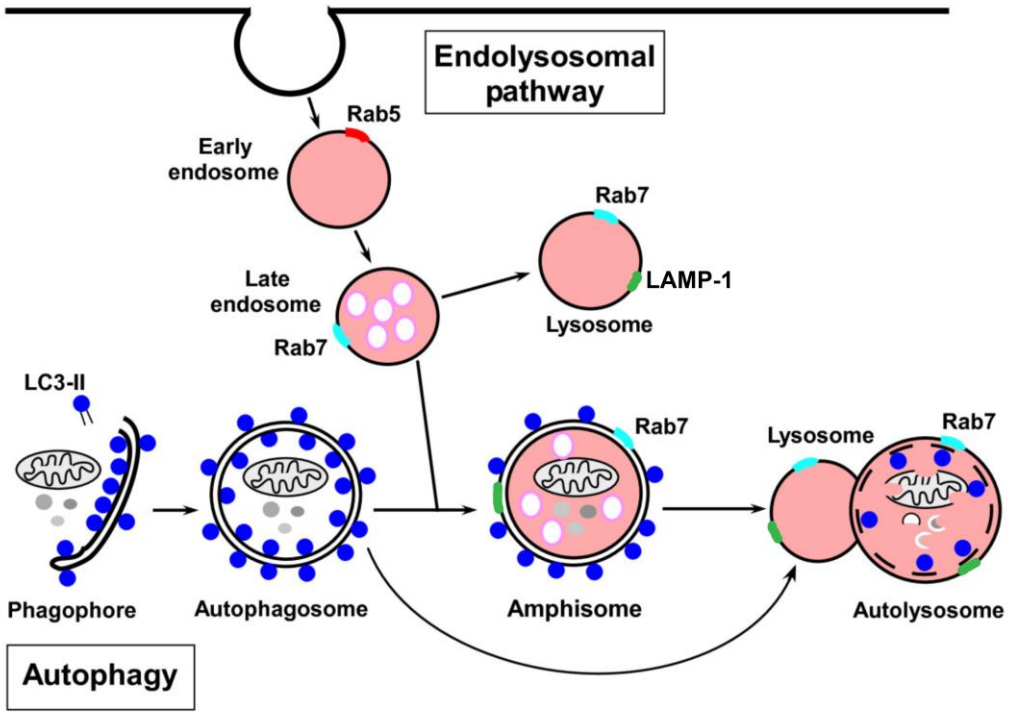


Figure 5. Early endosomes contain Rab5 on their surface. On the mature endosome (late endosome/multivesicular body) Rab5 exchanges with Rab7. The late endosome is fused with the lysosome (LY) and a hybrid organelle (on the right) is formed for the digestion of enclosed material. Autophagy is portrayed in the lower row. The phagophore membrane engulfs material to be enclosed in the autophagosome for degradation. Subsequently, a fully extended early autophagosome is formed. The autophagosome is characterized by the presence of the protein LC3-II. Thereafter, the mature autophagosome is labeled with the attachment of Rab7. The hybrid organelle (autolysosome) in the right is finally formed, when the autophagosome fuses with the lysosome. In the middle of the figure, in the fusion of the late autophagosome with the late endosome (or also with an early stage endosome) an amphisome is formed, which also fuses with the lysosome and becomes degraded. Taken from Hansen TE, Johansen T: Following autophagy step by step. *BMC Biology* 2011, **9**:39, Creative Commons license.

between the endocytic and autophagosomal pathways, especially during the later stages of flux (Funderburk et al., 2010). We focussed on deciphering the nature of autophagy dysfunction in AD, which is unclear due to conflicting data that indicated either impaired autophagy initiation (Pickford et al., 2008) or impaired cargo degradation (Boland et al., 2008; Nixon, 2007). Pickford *et al*.'s western blot analysis of gray matter from the midfrontal cortex of AD brains and age-matched control brains revealed a reduction in beclin-1 protein levels, an important component in the lipid kinase complex that initiates autophagy. Furthermore, AD transgenic mice that were heterozygous-deficient for the beclin-1 gene had increased amyloid pathology as observed with immunohistochemical staining (Pickford et al., 2008). Boland *et al*. treated primary cortical neurons with rapamycin and revealed that even in conditions of autophagy induction, that autophagosomes are transient and efficiently degraded in healthy neurons. They then blocked lysosomal function, and observed with electron microscopy that these treatments yielded similar observations to the AV accumulation in AD neurites (Boland et al., 2008).

There is also diverse evidence for Aβ's effect on autophagy (Cheung et al., 2011; Guglielmotto et al., 2014). Cheung *et al.* showed that low-molecular weight Aβ assemblies caused an induction of autophagy that was independent of beclin-1 (Cheung et al., 2011). Relatedly, Aβ was also shown to contemporaneously enhance autophagy induction and impair autophagosome clearance by blocking lysosomal degradation (Lipinski et al., 2010). Lipinski *et al.* used genome-wide analysis that revealed higher levels of autophagy induction in AD patients compared to age-matched controls, and postulated that it was a compensatory response to the toxic

action of Aβ (Lipinski et al., 2010). However, induction of autophagosome formation coupled with impaired lysosomal function may synergize pathologically to cause the accelerated neurodegeneration that characterizes AD. Thus, Aβ may be a key player in a pathological cycle in which it is both a substrate of autophagy and a cause of autophagy dysfunction.

A molecular mechanism for autophagy dysfunction in AD has not been definitively confirmed. Multiple lines of evidence suggest that deficiencies in beclin-1 signaling contribute to autophagy impairment in AD (reviewed in (Salminen et al., 2013)). Possible causes of beclin-1 deficiency include its non-functional sequestration in NFTs (Ma et al., 2010), cleavage by pathological caspase activation (Rohn et al., 2011), and inactivation due to phosphorylation of its interacting partners (e.g., Vps34) by cyclin-dependent kinases (CDK) which are linked to AD pathogenesis (Lopes and Agostinho, 2011). These events would impair autophagosome formation and render basal autophagy in neurons less efficient, possibly causing neurodegeneration (Hara et al., 2006; Komatsu et al., 2006). Additionally, it is known that lysosomal acidification is decreased in AD possibly due to impaired glycosylation of the V-ATPase proton pump that disrupts its subsequent trafficking from the ER (Lee et al., 2010; Wolfe et al., 2013), which may help to explain the impaired autophagosome clearance in AD. However, an alternative explanation for lysosome dysfunction in AD is based on lysosomal calcium dyshomeostasis that impairs lysosome fusion capacity (Coen et al., 2012). Evidently, the cause of lysosome incapacitation in AD is not fully understood. This thesis presents evidence that supports a role for protein prenylation in proper autophagosome-lysosome fusion,

and that Aβ-induced reduction of protein prenylation may be a pathological mechanism that leads to the autophagy dysfunction in AD.

Work from our laboratory demonstrated that treatment of primary neurons with oAβ_{42} causes intracellular cholesterol sequestration and impairs intracellular trafficking as a result of reduction of prenylation of Rabs (e.g., Rab7) (Mohamed et al., 2012). Inhibition of Rab7 prenylation may impair its interaction with GDI shuttling proteins and therefore its ability to properly localize to its target membranes (Calero et al., 2003). An improper localization of Rab7 could prevent proper dynein/dynactin-mediated autophagosome transport to the MTOC (Johansson et al., 2007; Kimura et al., 2008) and, furthermore, the Rab7 functionality in autolysosome formation (Gutierrez et al., 2004). Thus, Rab7 prenylation status may be critical for normal autophagic flux.

1.6. Hypothesis and Objectives

We hypothesize that defective autophagy in AD is due to inhibited protein prenylation and that restoring prenylation will prevent neurodegeneration. To test this hypothesis we:

1) Demonstrated that oAβ_{42} blocks autophagic flux;
2) Proved that inhibition of protein prenylation causes inhibition of autophagic flux;
3) Provided evidence that Rab7 hypo-prenylation leads to its intracellular mislocalization and autophagy impairment;
4) Present preliminary evidence that the pathological mechanism initiated by oAβ_{42} in cultured cells may occur in the brain.

2. Methodology

2.1. Reagents and antibodies

$A\beta_{42}$ was purchased from American Peptide Co. (Sunnyvale, CA). Reverse $oA\beta_{42}$ was purchased from Alpha Diagnostic Intl. Inc. Leibovitz's L-15-CO_2 culture medium was from Gibco (Catalogue #:21083-027). Immobilon polyvinylidene difluoride (PVDF) was from Bio-Rad. Chemiluminescence reagents were from Bio-Rad (170-5060). GGPP was purchased from Sigma-Aldrich (G6025). Bafilomycin A1 was from Sigma (B1793). Rapamycin was from LC Laboratories (R-5000). Simvastatin was from Calbiochem (567011). Psoromic acid was from Santa Cruz (sc-363581). DMEM (11995-065), Opti-MEM (31958-070), Neurobasal-A (10888-022), glutamax (35050-061), B27 (17504-044), N2 (175020-01), penstrep (15140-122), FBS (12483-020), and trypsin-EDTA (25200-072) were all purchased from Gibco. Primary antibodies used were β-Actin (1:2000, Cell Signaling Technology, 4967S), Rab7 (1:2000, Sigma, R8779), and LC3 (1:2000, Novus Biologicals, NB100-2220).

2.2. Cell culture

2.2.1. Culture of rat cortical neurons

Primary cortical neurons were prepared from newborn Sprague-Dawley rats (HSLAS, UofA) following a published protocol (Brewer and Torricelli, 2007). All procedures involving rats and mice were in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee: (Health Sciences Laboratory Animal Services, HSLAS) for the University of Alberta. Cortical tissue was dissected from the brain and enzymatically digested

with 1 mg/mL papain for 10 minutes at 37°C. DNase was added for the last 5 minutes of the incubation. Cells were suspended by mechanical dissociation with a sterile pipette, and the suspension was filtered through a 40 µm nylon cell strainer (Falcon). Neurons were plated on poly-D-lysine-coated wells at a density of 1.5 x 10^5 cells/well in 24-well plates. Neurons were maintained for 7 days in culture medium consisting of Neurobasal-A, B27 supplement, penstrep, and glutamax. Experiments began on the seventh day *in vitro* (DIV) using serum-free media supplemented with N2.

2.2.2. Culture of N2a cells

The N2a cell line (ATCC, CCL-131) was established from a neuroblastoma tumor of an albino mouse (Klebe and Ruddle, 1969). Cells were grown in a 75 cm^2 culture flask (Falcon, 353110) in culture medium consisting of DMEM, Opti-MEM, penstrep, and FBS. Cells were split into 24-well plates using 0.25% trypsin-EDTA at a density of 4 x 10^4 cells/well, and were allowed one day of growth before treatments. The same media without FBS was used for treatments with amyloid-β ($A\beta_{42}$) peptide. For immunofluorescence confocal experiments N2a cells were cultured on glass slides coated with poly-D-lysine and at a density of 4 x 10^4 cells/well.

2.3. Preparation of mouse brain lysates

Frontal cortices from 10-week old wild type and TgCRND8 mice (Chishti et al., 2001) were minced with scissors and homogenized in Tris-Triton X-100 buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton x-100, 1 mM EGTA) by 20 passages through a 22 gauge needle, as previously described (Mohamed et al., 2012). After 20 min on ice,

the homogenate was centrifuged at 1000 X *g* for 10 min at 4°C to clear unbroken cells and debris.

2.4. Preparation of oligomeric $A\beta_{42}$

Oligomeric $A\beta_{42}$ ($oA\beta_{42}$) was prepared following a published protocol (Dahlgren et al., 2002). $A\beta_{42}$ peptide was dissolved to 1 mM in hexafluoroisopropanol (HFIP) and aliquoted into sterile microcentrifuge tubes. A stream of nitrogen gas was used to remove the HFIP, and the peptide film was desiccated at -20°C. Twenty-four hours prior to use, the $A\beta_{42}$ peptide was resuspended in DMSO to a 5 mM concentration. Leibovitz's L-15-CO_2 medium was added to bring the peptide solution to a concentration of 100 μM, and this was incubated at 4°C for 24 h. All $oA\beta_{42}$ preparations used were similar to those characterized previously (Dahlgren et al., 2002; Saavedra et al., 2007). The neurotoxicity of this $oA\beta_{42}$ preparation has been characterized previously (Song et al., 2006).

2.5. Analysis of Autophagy Flux

2.5.1. Preparation of tandem LC3

The mCherry-GFP-LC3 (tLC3) plasmid containing the cytomegalovirus enhancer promoter was kindly provided by Claudio Hetz' laboratory (Castillo et al., 2013) (**Figure 6**). The plasmid was cloned into adeno-associated viral shuttle vectors containing serotype 2 inverted terminal repeats (AAV-tLC3) at a titre of 1 x 10^{12} genome copies per ml (GC/ml) by Welgen, Inc. (Worcester, MA).

2.5.2. Transduction of tLC3

The tLC3 plasmid was transduced into primary cortical neurons and N2a cells with the Lipofectamine® 3000 kit according to manufacturer's directions. Cells were allowed 24 hr for expression before undergoing treatments.

Transduction *in vivo* was achieved using intracerebroventricular (ICV) injection of the AAV-tLC3 contstruct, as previously published (Castillo et al., 2013). Briefly, Newborn C57BL/6 mouse pups were cryo-anesthetized (3-5 min) and then mounted on a cold styrofoam board wrapped in foil. An LED cold lamp was positioned overhead, and the injection-site was marked 1 mm lateral and 1 mm posterior to bregma. Each pup received one injection of 2.4 µl AAV-tLC3 into their left cerebral lateral ventricle using a 29G insulin syringe (BD, 309306) directed approximately 10° off central coronal and central saggital planes. Fast Green dye (0.04%) was used with the virus to verify injections. Pups were carefully put back into the shavings and allowed to be found by the mother.

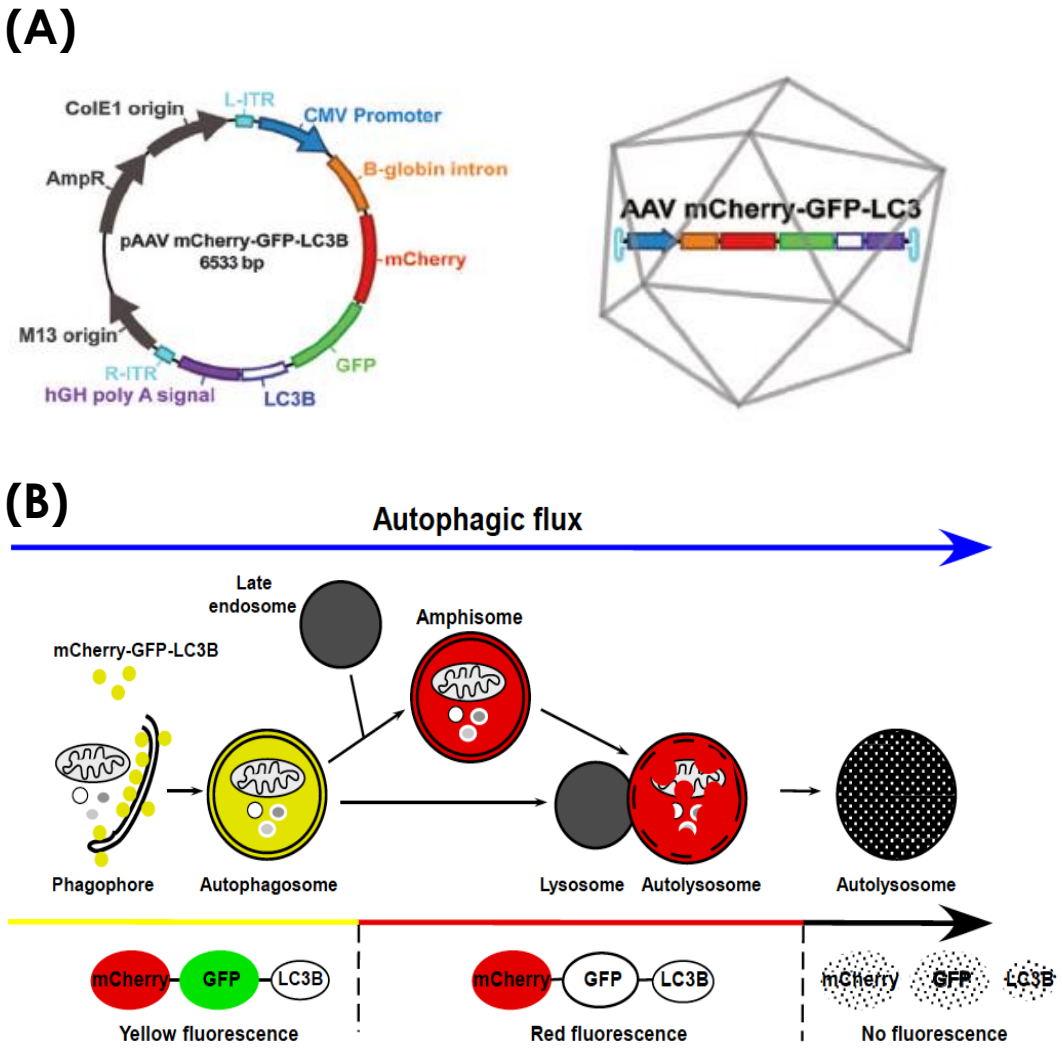


Figure 6. Measuring autophagic flux with mCherry-GFP-LC3 (tLC3). **(A)** tLC3 plasmid and AAV2/2 viral vector. Taken from Castillo *et al*.: Measurement of autophagy flux in the nervous system in vivo. *Cell death & disease* 2013, 4:e917, Creative Commons license. **(B)** Analyzing autophagic flux with tLC3. Taken from Hansen TE, Johansen T: Following autophagy step by step. *BMC Biology* 2011, **9**:39, Creative Commons license.

2.5.3. Measurement of autophagy flux

mCherry-GFP LC3 is a pH-sensitive sensor used to monitor autophagy in live cells. The GFP tag is acid-sensitive while the mCherry tag is acid-insensitive. The double tagged LC3 labels autophagosomes, amphisomes and autolysosomes. In autophagosomes both tags emit fluorescent light resulting in a yellow fluorescence. Fusion of autophagosomes to late endosomes or lysosomes results in acidic amphisomes or autolysosomes where the green fluorescence from GFP is lost. Therefore amphisomes and autolysosomes emit red fluorescence only. Subsequently, the red fluorescence from mCherry is lost when the double tagged protein is degraded. An increase of yellow fluorescent vesicles indicates that autophagic flux is blocked. The increase in red fluorescent vesicles compared with a control of basal autophagy indicates that autophagic flux is normal and that autophagy is increased.

At least sixty cells per treatment were imaged with a Zeiss LSM 710 confocal microscope, and the puncta were manually counted using the ImageJ plug-in Cell Counter. Flux was inferred from the relative proportions of yellow and red puncta per cell, which correspond to early and late autophagic vesicles, respectively. The puncta proportions for each cell were averaged in each group (>60 cells per treatment) and are displayed in histograms.

2.6. Preparation of cells and brain slices for confocal microscopy

N2a cells grown on coverslips were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, and the coverslips were mounted onto glass slides (Fisherbrand, 12-552) with Prolong® Gold Antifade with DAPI (ThermoFisher, P-36931). Mice that underwent ICV injections were euthanized by pentobarbital sodium (Euthanyl, WDDC, Canada) injection at 2 and 4 months of age, and were intracardially perfused with 0.9% saline followed by 4% PFA in 0.1M phosphate buffer. The brains were removed, post-fixed overnight in 4% PFA, and transferred into a sucrose gradient before being flash-frozen in liquid isopentane and mounted with Tissue Tek® Optimal Cutting Temperature (O.C.T.) compound for cryostat microtome sectioning (25 µm; Leica). Saggital slices were mounted onto glass slides as described above. A Zeiss LSM 710 confocal microscope (Cross Cancer Institute, UofA) was used with an argon laser line, and DAPI, Cy3 and FITC3 filters to capture blue, red, and green fluroesence. Images were taken with a plan-apochromat 40x/1.3 Oil DIC M27 objective. Parameters were set with untreated cells.

2.7. Immunoblot analysis

SDS-PAGE with 16% gels and 0.1% SDS was used to separate proteins based on molecular weight. Proteins were transferred to PVDF membranes overnight at 4°C in 25 mM Tris, 192 mM glycine, an 16% methanol buffer. Membranes were blocked in tris-buffered saline with 0.1% tween 20 (TTBS) containing 5% non-fat milk for one hour. Incubations with primary antibodies were performed overnight at 4°C. The membranes were then rinsed as follows: two 5-min rinses with TBS, two 5-min rinses

with TTBS, and two 5-min rinses with TBS. Membranes were then incubated with secondary antibodies (1:2000) for 1 hr at room temperature, followed by the same rinse protocol. The immunoreactivity was detected with Clarity™ Western ECL Substrate (Bio-Rad, 170-5060) and X-Ray films (Super RX-N, 47410). X-ray films were scanned with an Epson V550 Photo scanner, and semiquantification of band density was achieved using UN-SCAN-IT gel 5.3 software.

2.8. Determination of protein prenylation

Two methods were used to determine protein prenylation: capture with Rab-GDI nad extraction with Triton X-114. Rab-GDI capture was performed as previously published (Mohamed et al., 2012; Narita et al., 2005). Rat primary cortical neurons were harvested in GDI-capture buffer containing 75 mM potassium acetate, 30 mM HEPES, 5 mM $MgCl_2$, and cOmplete™ EDTA-free protease-inhibitor cocktail (Roche, 04693159001). The lysates were sonicated on ice, followed by centrifugation at 5000 X *g* for 10 sec to eliminate cellular debris. Each sample was separated into 'input' and 'extract' containing equal amounts of protein. The extracts were diluted fivefold in reaction buffer (75 mM potassium acetate, 30 mM HEPES, 5 mM $MgCl_2$, 100 μM ATP, 500 μM GDP) containing 450 μM GDI-GST, and then incubated at 30°C for 20 min. Prenylated proteins bound to GDI were recovered with Glutathione-sepharose 4B beads (GE Healthcare, 17-0756), and were eluted by boiling with SDS-sample buffer for 5 min, followed by analysis with SDS-PAGE and immunoblot as described above.

Extraction with Triton X-114 was performed as previously published (Coxon et al., 2005; Mohamed et al., 2012). Two-phase mixtures of Triton X-114 were formed

at 37°C so that lipophilic, prenylated Rabs partition into the detergent-rich phase, whereas unprenylated Rabs remain in the aqueous phase. Neurons were harvested at the end of the corresponding treatments in Tris buffer containing Triton X-114 (20 mM Tris-HCl, pH 7.5, containing 150mM NaCl, 1% Triton X-114, and protease inhibitor cocktail) and incubated for 15–20 min at 4°C. Lysates were cleared by centrifugation at 13,000 X *g* for 15 min at 4°C. Protein content was determined in the clear supernatants. Equal amounts of proteins were adjusted to the same volume with Tris buffer containing Triton X-114, were loaded on top of a cushion solution (20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 6% sucrose, 0.06% Triton X-114, and protease inhibitor cocktail) and were incubated at 37°C for 10 min. After centrifugation at 16,000 X *g* for 5 min, the top aqueous clear layer was separated from the detergent-rich, oil-like droplet. Both layers were boiled with SDS sample buffer, and proteins were resolved by SDS-PAGE. Notice that prenylated Rabs run faster than unprenylated Rabs under our experimental SDS-PAGE conditions. The lower band is present in the detergent phase.

2.9. Detection of apoptosis

Apoptotic cell death was identified by nuclear staining with Hoechst 33258. Neurons were fixed with 4% paraformaldehyde in PBS for 20 min and stained with 500 ng/mL Hoescht 33258 for 10-20 min. The percentage of apoptotic neurons was estimated by counting condensed and/or fragmented nuclei versus evenly stained nuclei. The total number of apoptotic neurons included ghost cells characterized by

the absence of chromatin. Nuclei were visualized using a Nikon TE300 inverted fluorescence microscope equipped with a Nikon digital camera DXM-1200. Images were analyzed using Northern Elite V6.0 image capture and analysis software (Emplix Imaging, Missisauga, Ont.). Five hundred to one thousand neurons per treatment were counted by an observer 'blinded' to the neuronal treatment.

2.10. Detection of Rab7 localization

N2a cells cultured on glass slides were co-transfected with DsRed-Rab7 and GFP-LC3 constructs using the Lipofectamine® 3000 kit according to manufacturer's directions. Cells were allowed 24 hr for expression before treatments began. Images were captured using confocal microscopy as described above. The co-localization of LC3-II with Rab7 was quantified by counting the number of yellow puncta (i.e., GFP-LC3 and DsRed-Rab7 positive) compared to the sum of yellow and green puncta (i.e., total number of LC3-positive puncta).

2.11. Quantification

2.11.1. Protein content of lysates

The protein content of harvested cells and homogenized cortex was determined using the bicinchoninic acid (BCA) assay according to manufacurer instructions (Pierce, 23228).

3. Results

3.1. oAβ$_{42}$ impairs autophagic flux

Our laboratory has previously shown that oAβ$_{42}$ inhibits protein prenylation and causes apoptosis of cortical neurons (Mohamed et al., 2012). We extended these findings to a neuronal model, namely N2a cells. N2a cells responded to oAβ$_{42}$ similarly to cortical neurons both in terms of oAβ$_{42}$-induced apoptosis (**Figure 7A**) and oAβ$_{42}$-induced inhibition of prenylation (**Figure 7B**). This validated the use of N2a cells as a model for the studies presented here.

An important consequence of the treatment of cortical neurons with oAβ$_{42}$ is the impairment of intracellular trafficking (Mohamed et al., 2012). Among the cellular processes in which trafficking is pivotal, autophagy is essential for neuronal viability (Hara et al., 2006; Komatsu et al., 2006) and its dysfunction has been linked to AD (Nixon et al., 2005). Furthermore, as previously mentioned, others have shown that Aβ itself alters autophagy function (Cheung et al., 2011; Guglielmotto et al., 2014). In order to investigate the effects of oAβ$_{42}$ on autophagy in cortical neurons and N2a cells we used two approaches namely immunoblot analysis of LC3-II levels, and direct measurement of autophagy flux with the fluorescent reporter mCherry-GFP-LC3 (tLC3). After 24 h of oAβ$_{42}$ treatment, rat cortical neurons and N2a cells had increased levels of LC3-II compared to untreated cells (**Figure 8A**). To determine if the elevation of LC3-II was due to an increase in the formation or a decrease in the degradation of autophagosomes, we tested the effect of oAβ$_{42}$ in cells treated with bafilomycin A1 (bafA1) at a concentration that completely blocks autophagosome

degradation (**Figure 8B**). BafA1 inhibits vacuolar proton ATPase (V-ATPase) inhibiting acidification of lysosomes and blocks LC3-II degradation (Klionsky et al., 2008). In the presence of bafA1, LC3-II levels did not increase by treatment with oAβ_{42} (**Figure 8C**). This suggested that oAβ_{42} did not induce autophagosome synthesis, but rather blocked autophagic flux.

Direct measurement with tLC3 confirmed that oAβ_{42} blocks autophagy flux. First, the total number of cells with LC3 puncta is significantly higher in cells treated with oAβ_{42} (50% vs 20% untreated cells). This value is equivalent to the number of cells with LC3 puncta that result from autophagy induction by starvation or treatment with the mTOR inhibitor torin-1 and from autophagic blockade with bafA1 (**Figure 9A**). Second, when the nature of the LC3 puncta was analyzed we found that oAβ_{42} treatment caused a significant increase of autophagosomes (i.e., yellow puncta) and a decrease of autolysosomes/amphisomes (i.e. red puncta) when compared with autophagy induction by starvation (**Figure 9B, C**). The proportions of autophagic vesicles in cells treated with oAβ_{42} was similar to cells in which autophagy was blocked by treatment with bafA1 or bafA1plus torin-1; and different from cells treated with the autophagy inducer torin-1 alone. Torin-1 caused a significant increase of autolysosomes/amphisomes and decrease of autophagosomes. The high proportion of autophagosomes present in untreated cells (**Figure 9B**) was likely due a selection bias when acquiring images for puncta count. The images correspond to the small population of cells with puncta (**Figure 9A**) and thus, they are not representative of the population.

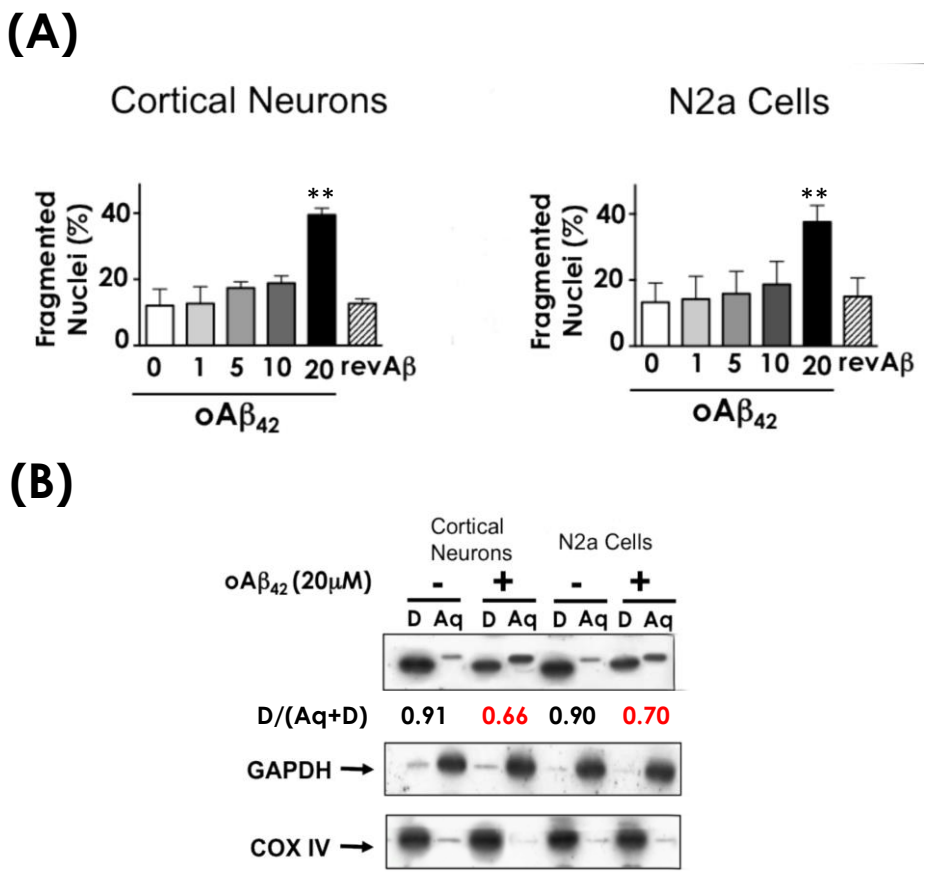


Figure 7: Cortical neurons and N2a cells respond similarly to Aβ challenge. (A) Aβ-induced apoptosis: Cortical neurons and N2a cells were incubated with different concentrations of $oA\beta_{42}$ or with 20μM $revA\beta_{42\text{-}1}$. After 36 h, the percentage of apoptotic nuclei was evaluated by Hoechst 33258 staining. Data are means ± SE of three experiments. Each experiment performed in quintuplicate. 500 –1000 neurons per treatment were counted. **p<0.001, one-way ANOVA. **(B) Aβ inhibits protein prenylation.** Cortical neurons and N2a cells were treated with or without 20μM oAβ42 for 24 h. Analysis of Rab7 prenylation by extraction with Triton X-114. Proteins in detergent (D, prenylated proteins) and aqueous (Aq, unprenylated proteins) phases were analyzed by SDS-PAGE and immunobloting. Ratios between total pixels of Rab7 in detergent/total pixels of Rab7 in detergent plus aqueous were calculated. Values are from the present blot. The efficiency of phase separation was analyzed by the virtual absence of the cytosolic protein GAPDH in the detergent fractions and the absence of the mitochondrial membrane protein COX IV in the aqueous fraction.

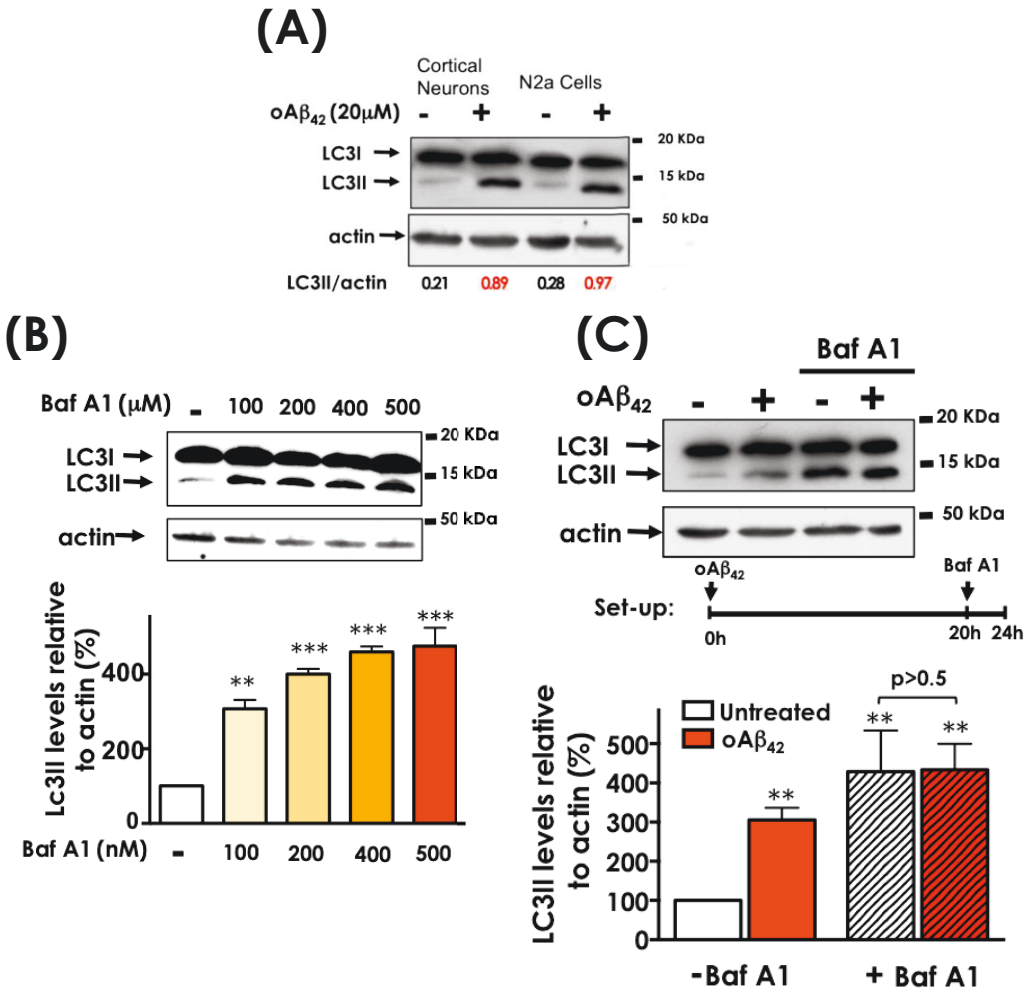


Figure 8: oAβ_{42} inhibits autophagic flux as analyzed by assessing endogenous LC3-II levels with bafilomycin A1. (A) Primary cortical neurons or N2a cells were treated with or without 20μM oAβ_{42} for 24 h. Endogenous LC3-II levels were detected by immunoblotting with anti-LC3 antibody and densitometric analysis relative to actin is expressed **(B)** Cortical neurons were treated with bafA1 at concentration ranging from 100 to 500 nM for 4 h. LC3 levels relative to actin are expressed as percentage of untreated cells. **(C)** Cortical neurons were treated with 20μM oAβ_{42} for 24 h in the presence or absence of 500 nM bafA1in the last 4 h of the oAβ_{42} treatment periods. LC3 levels relative to actin are expressed as percentage of untreated cells. Data are means ±SEM of three experiments combined and analyzed by ANOVA (*** $p<0.001$, ** $p<0.01$ compared to untreated).

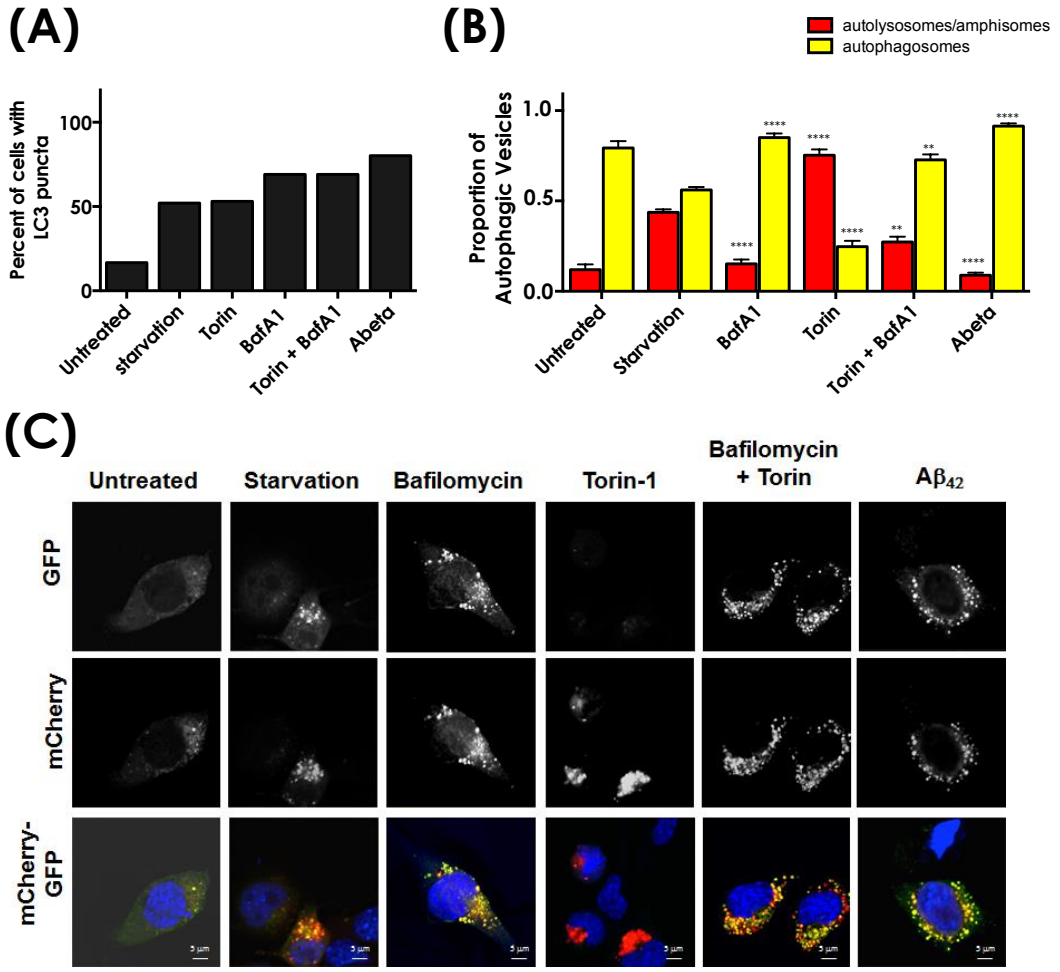


Figure 9: oAβ_{42} inhibits autophagic flux as analyzed by tandem LC3 fluorescence. N2a cells expressing tandem mCherry-GFP-LC3 were left untreated, deprived of aminoacids for 4h (starvation), treated for 4h with 500 nM bafilomycin A1, 150 nM torin or a combination of bafilomycin A1 and torin or treated with 20 µM oAβ_{42} for 24h, after which they were fixed and analyzed by microscopy. **(A)** The number of cells with more than 2 puncta out of 150 cells expressing LC3 were counted at low magnification (10X). **(B)** Using confocal microcopy the number of yellow and red puncta was counted in individual cells. Image analysis was performed using ImageJ software. Data are the average ±SEM of ≥100 cells per treatment. Results are expressed as the proportion of yellow and red puncta per cell analyzed. Significant as compared with cells under starvation was determined by one-way ANOVA (**** $p<0.0001$, ** $p<0.01$). **(C)** GFP and mCherry emit fluorescence in autophagosomes and appear yellow in the merged image. Autolysosomes have mCherry signal only and appear red in the merged image.

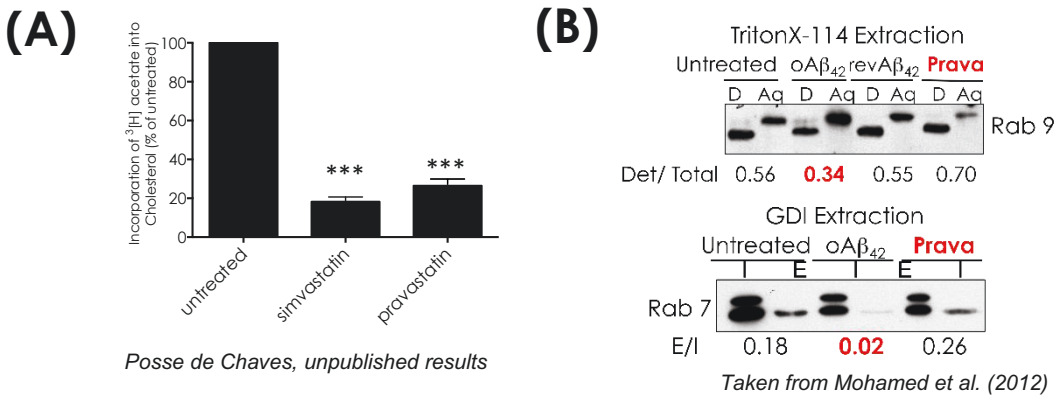
3.2. Inhibition of protein prenylation blocks autophagic flux

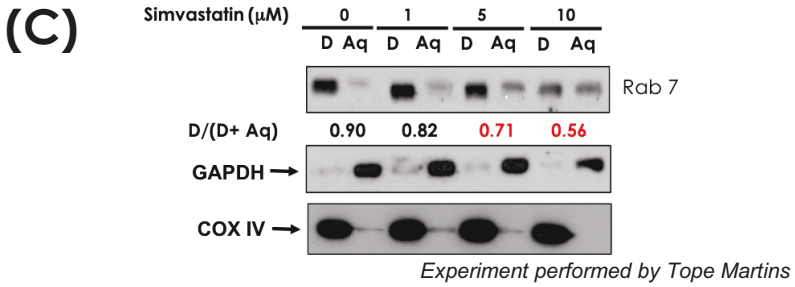
We hypothesized that the block of autophagic flux caused by oAβ_{42} is due to the intracellular trafficking impairment that results from oAβ_{42}-induced inhibition of protein prenylation (Mohamed et al., 2012). If our hypothesis is correct, other agents that cause protein prenylation inhibition should also impair autophagic flux. Our laboratory has previously tested potential inhibitors of protein prenylation. Statins inhibit the mevalonate pathway, but may or may not inhibit isoprenoids synthesis, thus they may or may not decrease protein prenylation (Mohamed et al., 2012; Mohamed et al., 2015). Indeed, under our experimental conditions both simvastatin and pravastatin inhibit cholesterol synthesis (**Figure 10A**), but only simvastatin inhibits protein prenylation (**Figure 10B, C**). We also tested psoromic acid (PA), and confirmed its ability to inhibit prenylation (**Figure 10D**).

oAβ_{42}, simvastatin and PA inhibit protein prenylation by different mechanisms. While the effect of oAβ_{42} and simvastatin is due to decreased isoprenoid synthesis (Mohamed et al., 2012), PA prevents prenylation by catalytically inhibiting the geranylgeranyl transferase-II (GGTase-II) enzyme (Deraeve et al., 2012). Consequently, treatment with the isoprenoid GGPP prevented inhibition of protein prenylation by oAβ_{42} and simvastatin but not by PA (**Figure 11**).

Next, we tested if these treatments affect autophagy, and we found that autophagic flux was blocked when protein prenylation was impaired. Thus, oAβ_{42}, simvastatin, and PA caused an increase of LC3-II levels, whereas pravastatin did not (**Figure 12**). Addition of GGPP prevented the increase of LC3II in oAβ_{42}- and

simvastatin-treated cells, but not in PA-treated cells (**Figure 12**), indicating that indicating that inhibition of protein prenylation causes autophagy dysfunction. In order to determine the nature of the alteration we measured autophagic flux with tLC3 as before. We observed increased numbers of autophagosomes and decreased autolysosomes/amphisomes in cells treated with oAβ_{42}, simvastatin and PA (**Figure 13**), indicating a block in autophagic flux. Compellingly, this effect was prevented with GGPP treatment in cells treated with oAβ_{42} and simvastatin, but not in cells treated with PA. GGPP by itself did not affect autophagic flux. These experiments proved that hypo-prenylation blocks normal autophagic flux.





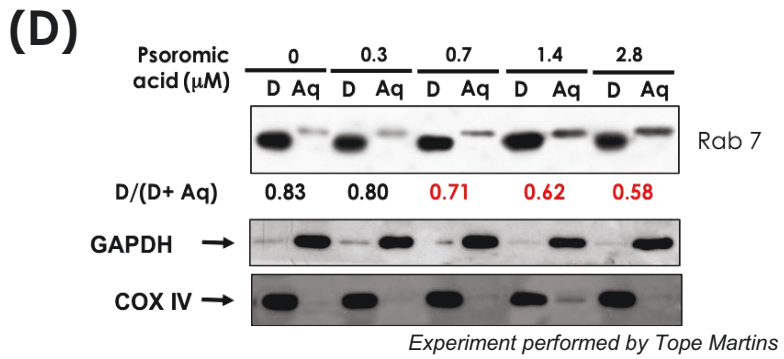


Figure 10. Legend on next page.

Figure 10. (A) Simvastatin and pravastatin inhibit cholesterol synthesis. Cortical neurons were treated with simvastatin (5μM) or pravastatin (50μM) for 24 h. Cholesterol synthesis was examined by measuring incorporation of [^{3}H]acetate into unesterified cholesterol**.** Results are expressed as radioactivity in unesterified cholesterol as percentages relative to untreated neurons. Both statins significantly reduce cholesterol synthesis under these experimental conditions**. (B and C) Simvastatin but not pravastatin inhibits protein prenylation. (B)** Neurons were treated with oAβ42 (20 μM), revAβ (20 μM), or pravastatin 50 μM or **(C)** with different concentrations of simvastatin or for 24 h. Protein prenylation was examined by extraction with Triton X-114 or GST–GDI as indicated. Red values indicate decreased prenylation. Rab prenylation was examined by extraction with Triton-X-114 or GST-GDI capture. Proteins in the detergent (D, prenylated proteins) and aqueous (Aq, unprenylated proteins) phases; and extraction (E, prenylated proteins) and input (I) were analyzed by SDS-PAGE and immunobloting. Ratios between total pixels of Rab7 in detergent/total pixels of Rab 7 in detergent plus aqueous were calculated for Triton extraction. Ratios between total pixels of Rab7 in extract/input were calculated for GST-GDI capture. Values are from the present blots. The efficiency of phases separation was analyzed by the virtual absence of the cytosolic protein GAPDH in the detergent fractions and the absence of the mitochondrial membrane protein COX IV in the aqueous fraction. **Red values indicate decreased prenylation. (D) Psoromic acid inhibits protein prenylation.** Neurons were treated with different concentrations of psoromic acid for 24h. Prenylation was analyzed as described above.

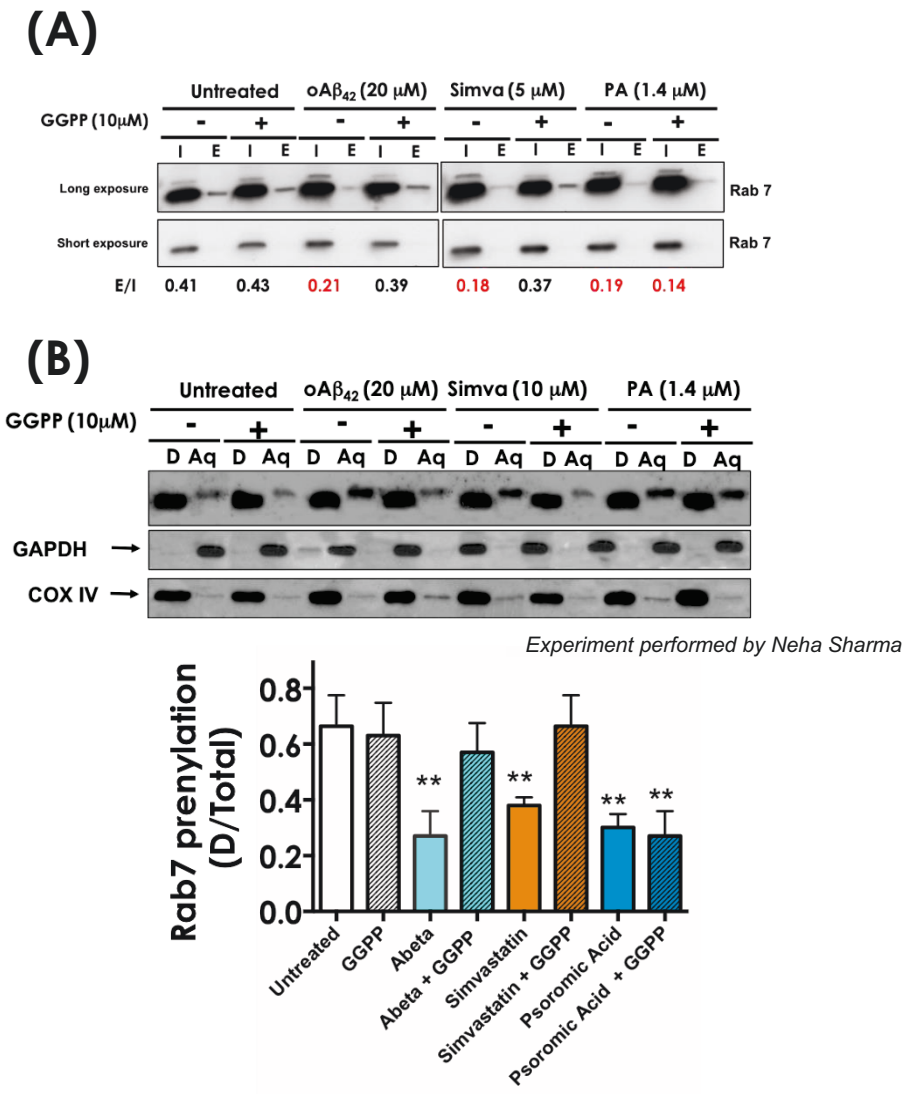


Figure 11: oAβ$_{42}$, simvastatin and PA inhibits protein prenylation by different mechanisms. Neurons were treated with 20 μM oAβ$_{42}$, 5 μM simvastatin, or 1.4 μM psoromic acid with or without GGPP for 24 h. The effect of Aβ and simvastatin but not PA on protein prenylation was reversed by GGPP. Protein prenylation was examined by **(A)** Rab-GDI capture and **(B)** Triton X-114 extraction. Data are the average ±SEM of 5 experiments analyzed by ANOVA (** $p<0.01$).

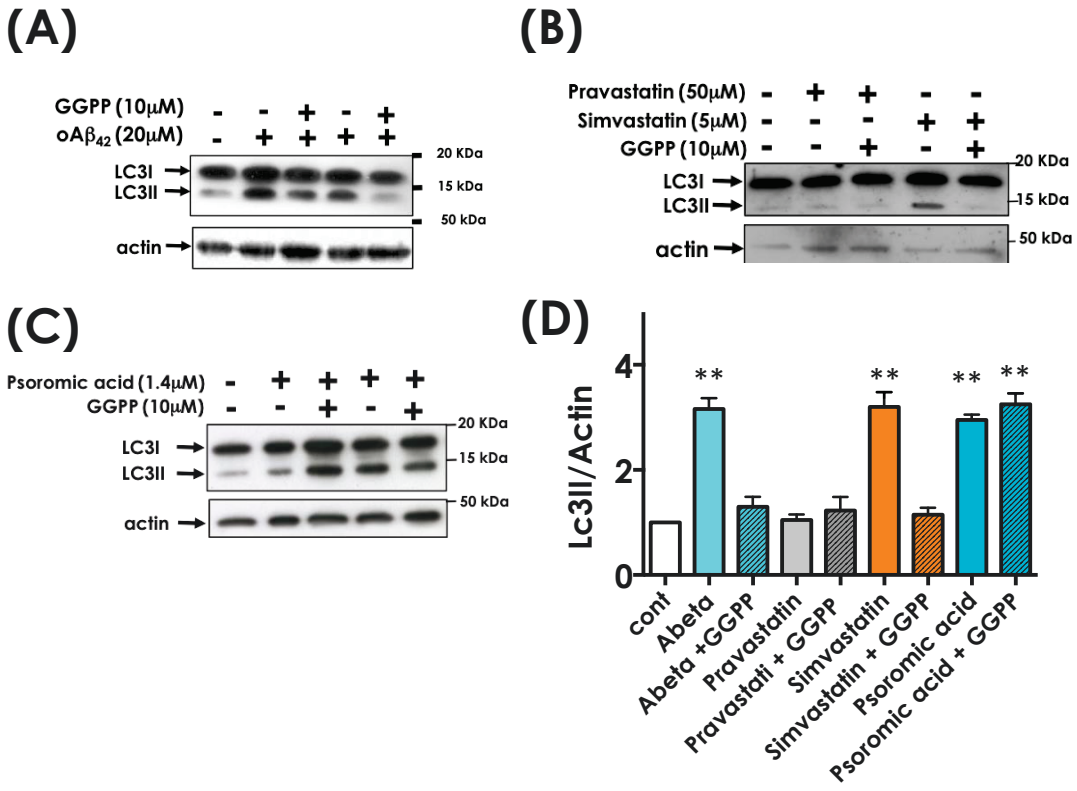


Figure 12: Inhibition of protein prenylation causes autophagy dysfunction. Neurons were treated with: **(A)** oAβ_{42} with or without GGPP, **(B)** statins with or without GGPP or **(C)** psoromic acid with or without GGPP for 24h. The conversion of LC3I to LC3II was examined. **(D)** Quantification of results. Data are the average ±SEM of 5 experiments analyzed by ANOVA (** $p<0.01$ compared to untreated) ($^{\#\#\#}p<0.001$ comparing with and without GGPP).

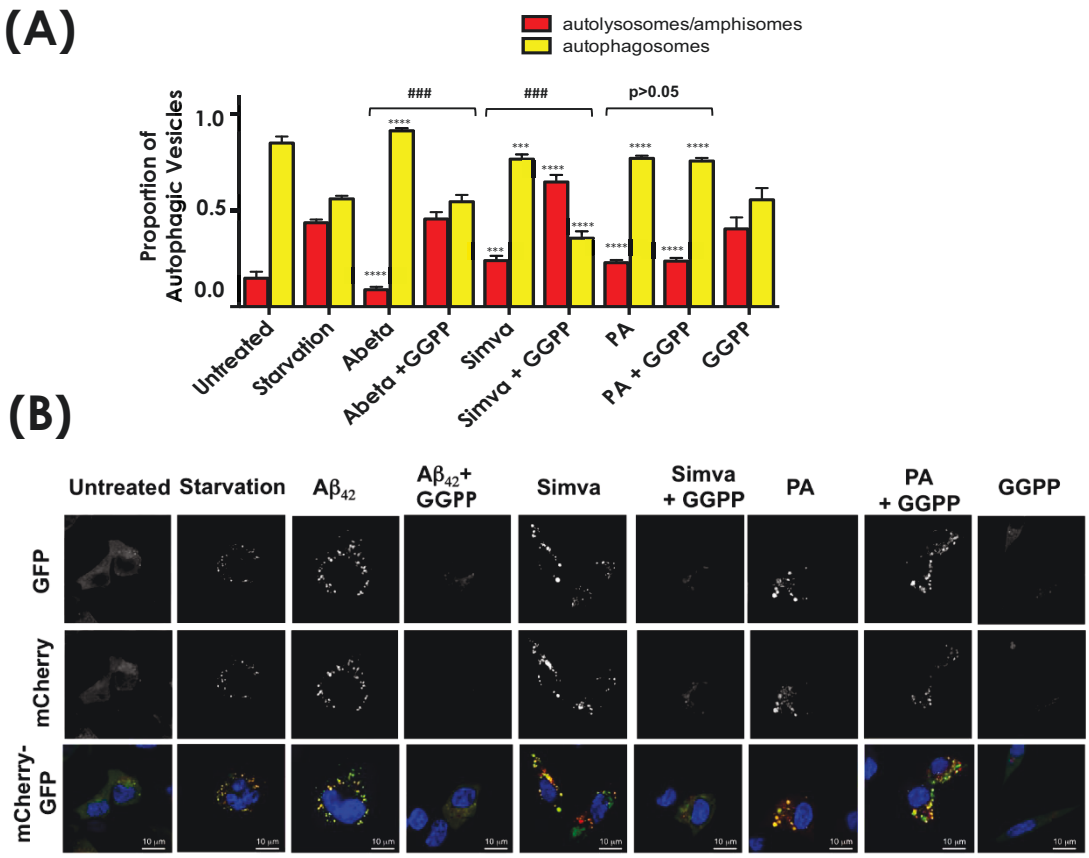


Figure 13: Inhibition of protein prenylation blocks autophagic flux. N2a cells expressing mCherry-GFP-LC3 were left untreated, starved of amino acids for 4h or were treated with 20 μM oAβ_{42}, 10 μM simvastatin, or 1.4 μM PA with or without 10 μM GGPP for 24h at which time they were fixed and analyzed by confocal microscopy. **(A)** The number of yellow and red puncta was counted in individual cells. **(B)** GFP and mCherry emit fluorescence in autophagosomes and appear yellow in the merged image. Autolysosomes have mCherry signal only and appear red in the merged image. Image analysis was performed using ImageJ software. Data are the average ±SEM of ≥100 cells per treatment. Results are expressed as the proportion of yellow and red puncta per cell analyzed. Significance was determined by one-way ANOVA comparing with cells under starvation (**** $p<0.0001$, *** $p<0.001$ and ** $p<0.01$); and comparing with and without GGPP (### $p<0.001$).

3.3. Prenylation is important for role of Rab7 in autophagy

In order to gain insight into how inhibited protein prenylation blocks autophagic flux, we investigated Rab7, which, among prenylated proteins, has a crucial role in autophagosome-lysosome fusion (Gutierrez et al., 2004; Jager et al., 2004). Prenylation is required for membrane localization of Rab proteins (Calero et al., 2003; Gomes et al., 2003), and intracellular localization of Rab7 to autophagic vesicles is pivotal for Rab7 functions in autophagy (Bucci et al., 2000; Feng et al., 1995; Gutierrez et al., 2004; Hyttinen et al., 2013; Jager et al., 2004). Therefore we examined the cellular distribution of Rab7 in N2a cells co-expressing DsRed-Rab7 and GFP-LC3 and treated with prenylation inhibitors. When autophagy was induced by amino acid starvation in untreated N2a cells Rab7 localized to autophagosomes and the majority of LC3 co-localized with Rab7 (**Figure 14**), consistent with the role of Rab7 in regulating autophagosome traffic. On the other hand, in cells treated with oAβ_{42}, simvastatin or PA Rab7 has a less punctate pattern and LC3 co-localization with Rab7 is significantly decreased. The 2.5D images give a unique perspective on the degree of co-localization between LC3-II and Rab7 (**Figure 14A**): green peaks (LC3) stand out from the diffuse red fluorescence (cytosolic Rab7) in hypo-prenylation conditions. The membrane-bound status of Rab7 was restored with the addition of GGPP to cells treated with simvastatin and oAβ_{42}; here the 2.5D images show yellow peaks (LC3 and Rab7). However, as expected GGPP treatment did not restore prenylation in PA-treated cells, and Rab7 remained diffusely cytosolic and the low co-localization of LC3 with Rab7 did not increase. The observations of this experiment

indicate the essential role of protein prenylation for the ability of Rab7 to target to autophagosomes.

3.4. Autophagy dysfunction in AD mice brains

Dysfunctional autophagy is known to occur in brains of the TgCRND8 AD model mice (Yang et al., 2011a; Yang et al., 2014). We hypothesize that the defects are due to hypo-prenylation caused by Aβ, similar to our *in vitro* findings. Western blot analysis of brain cortex homogenates from TgCRND8 mice has provided evidence to support this hypothesis. We confirmed an alteration in autophagy, as revealed by increased endogenous LC3-II levels compared to wild-type mice (**Figure 15A**). Similar to cortical neurons treated with oAβ_{42}, the maturation of SREBP-2 was impaired in TgCRND8 brains (**Figure 15B**), as was the level of protein prenylation (**Figure 15C**). Interestingly, there was selective upregulation of Rab7 in TgCRND8 brains (**Figure 15D**), which is consistent with previous findings from human AD brains (Ginsberg et al., 2011; Ginsberg et al., 2010). To corroborate that these findings provide a mechanistic basis for the defective autophagy in TgCRND8 brains, we use a technique to analyze autophagic flux in the CNS *in vivo*.

Thus far, we have successfully delivered the tLC3 protein into the CNS of wild-type C57BL/6 mice and have observed autophagic vesicles. This was accomplished with ICV injection of the neuronal-specific AAV2/2 viral vector containing tLC3 (**Figure 16A**). Expression was clearly visible in the cerebellar cortex 4 weeks after the injection (**Figure 16B**). Wild-type mice treated with rapamycin, an autophagy

inducer, had increased autophagic flux compared to controls (**Figure 16C**), as suggested by the apparent increase of autolysosomes/amphisomes. Future studies in our laboratory will use this technique to analyze autophagic flux in TgCRND8 mouse brains, which will enable investigations into the effect of protein prenylation-recovery on defective autophagic flux in the brain *in vivo*.

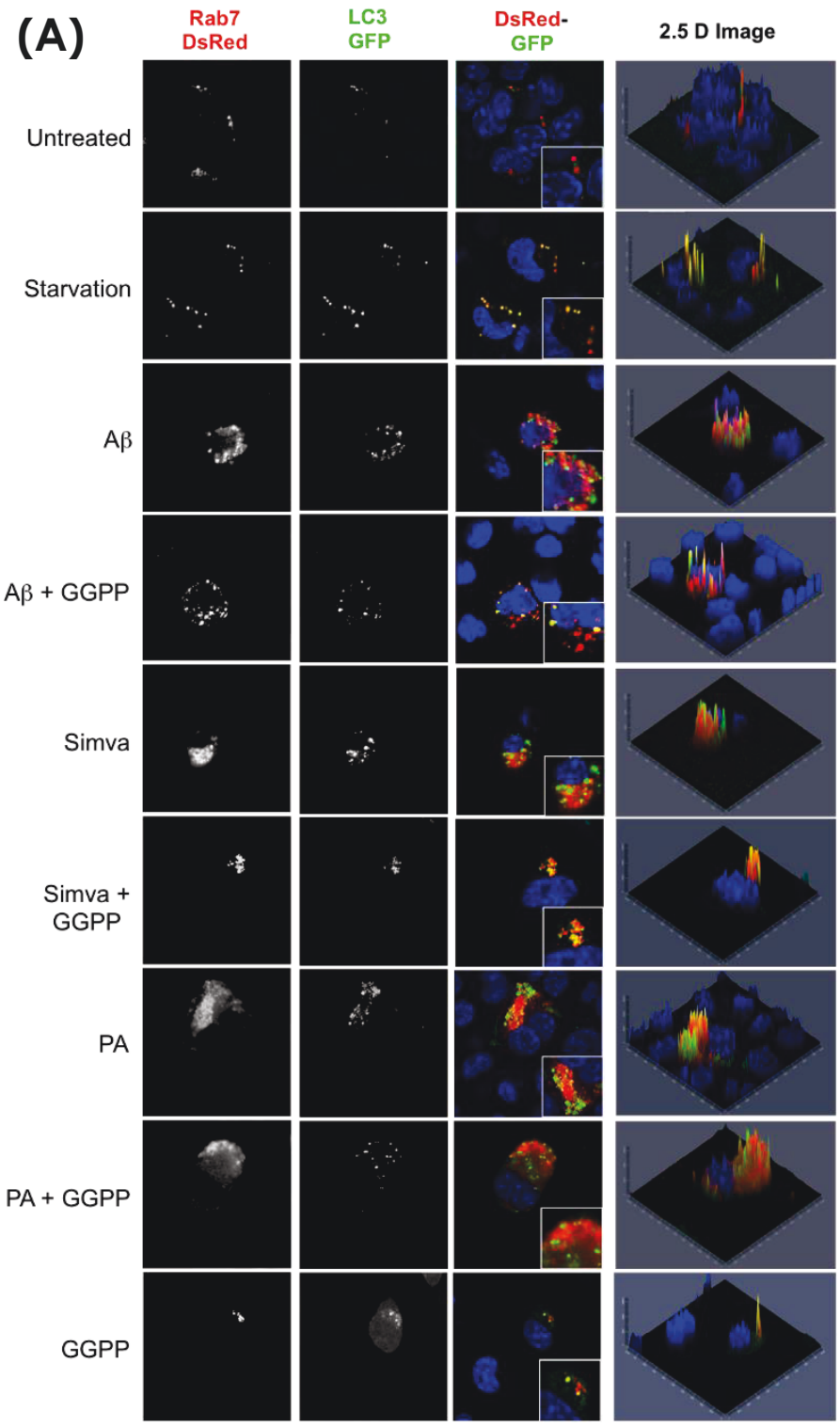


Figure 14: Inhibition of protein prenylation impairs the localization of Rab7 to autophagic vesicles. Legend continued on next page.

(B)

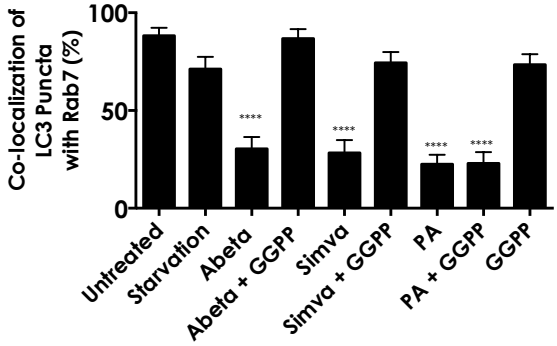
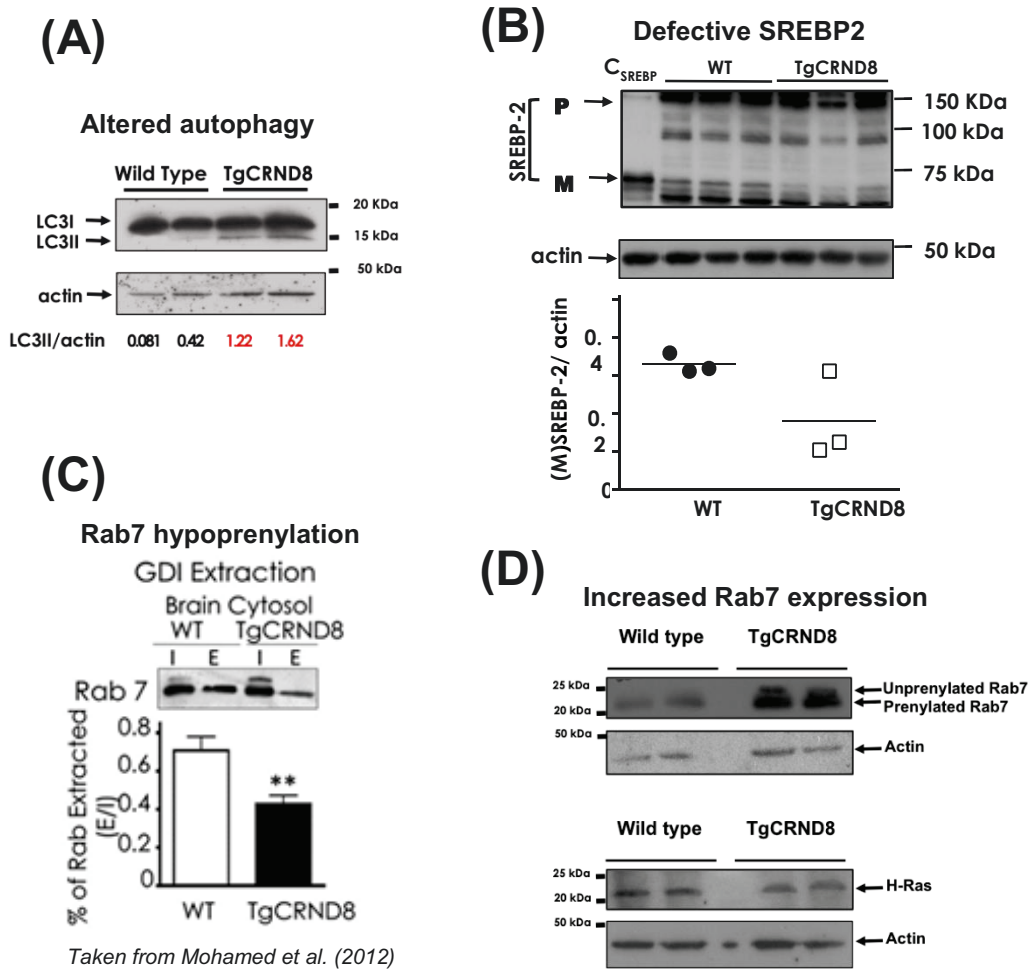


Figure 14: Inhibition of protein prenylation impairs the localization of Rab7 to autophagic vesicles. N2a cells expressing DsRed-Rab7 and GFP-LC3 were left untreated, starved of amino acids for 4h or were treated with 20 μM oAβ_{42}, 10 μM simvastatin, or 1.4 μM psoromic acid (PA) with or without 10 μM GGPP for 24h at which time they were fixed and analyzed by confocal microscopy. **(A)** oAβ_{42}, simvastatin, and PA caused Rab7 to remain diffusely cytosolic and prevented the colocalization of LC3-II with Rab7. GGPP reversed the effect in oAβ_{42} and simvastatin treated cells, but not PA-treated. Zen Blue edition software from Zeiss was used to produce 2.5D images to observe co-localization of Rab7 and LC3-II. **(B)** Co-localization was analyzed by quantifying the number of yellow puncta (LC3-II and Rab7) out of total LC3-positive puncta (green and yellow). Results are expressed as percentage of total LC3 positive puncta that co-localize with Rab7. Data represent the average ±SEM of 30 cells per treatment. Significance determined by one-way ANOVA comparing with untreated cells (**** $p<0.0001$).



Taken from Mohamed et al. (2012)

Figure 15: Mechanisms of autophagy dysfunction activated by Aβ may be present in AD mice brains. Brain cortices from 10 week-old wild type and TgCRND8 mice were analyzed. **(A)** Detection of LC3 by western blotting. LC3-II is elevated in brain cortex of TgCRND8 mice. Values in red indicate increased LC3-II. **(B)** SREBP-2 maturation is reduced in TgCRND8 brain cortex. C_{SREBP} is a control generated by overexpression of (M)SREBP2 in St14A cells and used to confirm the band of (M)SREBP-2. **(C)** Rab7 prenylation is decreased in TgCRND8 brains as determined by Rab-GDI capture. **(D)** Detection of Rab7 and H-Ras by western blotting. Rab7 but not H-Ras is increased in TgCRND8 mouse brain.

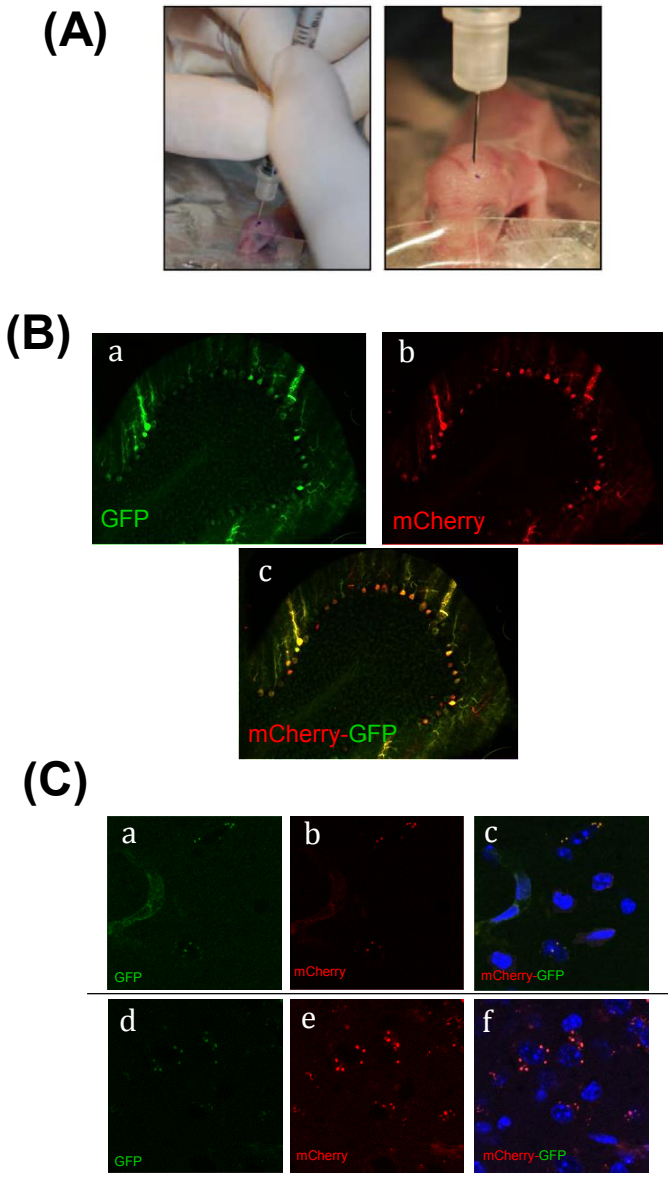


Figure 16: Analyzing autophagic flux *in vivo*. (A) ICV injection of the AAV2/2 vector for tLC3 into newborn C57BL/6 mouse pups **(B)** 20x images of cerebellar cortex from brain slices of 4-week-old C57BL/6 mice expressing tLC3. a) GFP fluorescence b) mCherry fluorescence c) merged. **(C)** 40x images of entorhinal cortical neurons from brain slices of 8-week-old C57BL/6 mice expressing tLC3. **a-c:** untreated animal; a) GFP fluorescence b) mCherry fluorescence c) merged. **d-f:** animal treated with rapamycin (2 μg/g) 3 times a week for 4 weeks starting 4 weeks after viral injection. Rapamycin treatment induced autophagic flux.

4. Discussion

The physiological role of basal autophagy in mammals was shown *in vivo* animal models, and its failure leads to alterations of cavitation during embryogenesis or accumulation of abnormal organelles in several tissues (Madeo et al., 2015). Autophagy induction is organ dependent (Damme et al., 2015), being very active in the brain, a tissue that is particularly susceptible to oxidative and mitochondrial damage. Neurons rely heavily on autophagy to maintain their uniquely large cytoplasm and membrane system of axons and dendrites during their whole life without the aid of cell division. Indeed, autophagy is constitutively active in neurons (Nixon and Yang, 2011), yet the presence of autophagic vesicle intermediates in neurons is uncommon because their clearance is exceptionally efficient (Boland et al., 2008; Nixon, 2007). Brain-specific abrogation of basal autophagy in normal mice by conditional deletion of autophagy-related genes (ATGs) results in neurodegeneration in the absence of any disease-causing mutations, suggesting that impaired autophagy contributes to the etiology of neurodegenerative diseases (Hara et al., 2006; Komatsu et al., 2006).

4.1. oAβ_{42} impairs autophagic flux

Considerable research in the past decade has highlighted the significance of autophagy regulation in AD and other neurodegenerative diseases (reviewed in (Nixon, 2013)). There is no doubt that autophagy is altered in AD (Nixon et al., 2005; Yu et al., 2004). Brains of AD patients and animal models of AD exhibit large numbers

of autophagic vacuoles within dystrophic neurites before extracellular Aβ deposition (Nixon et al., 2005; Suzuki and Terry, 1967; Yu et al., 2005). Yet, the nature of the autophagy dysfunction is unclear. The work presented here elucidates the nature of autophagy dysfunction in a model of $oA\beta_{42}$ toxicity. We tested the hypothesis that Aβ-induced inhibition of protein prenylation is the cause of autophagy impairment.

For our experiments we employed a combination of cultured rat primary cortical neurons and N2a cells. The latter were derived from a mouse neuroblastoma and have been widely used in AD research (Chen et al., 2011; Jeppsson et al., 2012; Seeger et al., 1997). $oA\beta_{42}$ causes N2a cell apoptosis at concentrations similar to those toxic for cortical neurons. Furthermore, $oA\beta_{42}$ inhibits protein prenylation in N2a cells as effectively as in cortical neurons. The advantage of N2a cells is that they are easily transfected with non-viral techniques, which suits the experiments in this thesis.

There have been contradictory reports claiming that autophagy initiation (Pickford et al., 2008; Salminen et al., 2013) or cargo degradation (Funderburk et al., 2010; Harris and Rubinsztein, 2012; Nixon, 2007; Tung et al., 2012) is impaired in AD. The main reason for this ambiguity resides in the methodology employed to assess autophagy. LC3 is the most extensively used marker of autophagy (Klionsky et al., 2012). During autophagy, soluble cytosolic LC3 (LC3-I) is modified via the attachment of phosphatidylethanolamine (PE) and converted into LC3-II, which is recruited to autophagosome membranes (Kabeya et al., 2000; Weidberg et al., 2010). The transition of LC3-I into LC3-II is followed by the appearance of a band of LC3-II in

western blots (Klionsky et al., 2012; Rubinsztein et al., 2009; Winslow et al., 2010). Recruitment of LC3-II to autophagosomes has been examined by expressing GFP-LC3 in cells in culture (Fass et al., 2006; Lin et al., 2015) and in animal models (Katsumata et al., 2010; Mizushima and Kuma, 2008; Mizushima et al., 2004; Pickford et al., 2008; Wang et al., 2006) and detecting LC3-containing vesicles by immunofluorescence. The results obtained with these experimental approaches can be misleading because increased LC3-II levels can also result from impaired autophagosome-lysosome fusion. Furthermore, an enhancement of autophagic flux may be undetected during a concomitant increase in both autophagosome induction and downstream degradation (Klionsky et al., 2012). Hence, it is clearly important to use strategies that enable the quantification of autophagic flux.

Two different approaches were used to examine autophagic flux: 1) we detected changes in LC3-II levels in the presence of bafA1; and 2) we examined autophagic flux directly using the mCherry-GFP-LC3 (tLC3) fluorescent protein. BafA1 prevents lysosomal acidification and inhibits fusion of autophagosomes with lysosomes. Thus BafA1 blocks LC3-II degradation and allows the specific assessment of LC3-II formation rates (Rubinsztein et al., 2009). We found that oAβ_{42} caused a significant increase in the levels of LC3-II, however in the presence of bafA1, oAβ_{42} did not accumulate LC3-II over the increase caused by bafA1 alone suggesting that it does not induce autophagy, but instead blocks autophagic flux. Our studies using the tLC3 definitively confirmed that oAβ_{42} blocks autophagic flux in cortical neurons and N2a cells and causes the accumulation of autophagosomes. This finding is in agreement with previous studies that suggested that autophagic flux is impaired in

AD based on electron microscopic observations of human AD brain tissue (Nixon et al., 2005), and TgCRND8 mouse brains (Lai and McLaurin, 2012). Although electron microscopy is a very powerful technique, it does not provide direct information on autophagic flux. On the other hand our results are in contrast with other work that showed enhanced autophagosome formation in response to oAβ_{42} (Cheung et al., 2011; Fonseca et al., 2013; Hung et al., 2009). In addition, other work reported that Aβ enhances autophagy initiation and concomitantly decreases autophagosome clearance due to blockade of lysosomal degradation (Lipinski et al., 2010). Caveats of these latter studies include the report of ratios of LC3-II/LC3-I levels in immunoblot analyses and the use of DsRed- or GFP-LC3 in microscopic studies. Using the ratio LC3-II/LC3-I can be problematic because LC3-I is unstable (Klionsky et al., 2012). Therefore it is better to report LC3-II levels with respect to actin, as we have done. As stated above, increased GFP-LC3 puncta can result from increased autophagosome formation or inhibition of autophagosome clearance. The problems of using DsRed- or GFP-LC3 apply both to studies in cultured cells (Cheung et al., 2011; Fass et al., 2006) as well as studies on transgenic mice expressing GFP-LC3 (Katsumata et al., 2010; Mizushima and Kuma, 2008; Pickford et al., 2008). Conversely the method using tLC3 that we employed here enables the differentiation between induced autophagosome production and impaired autophagosome clearance and, crucially, provides information on autophagic flux (Castillo et al., 2013; Kimura et al., 2007). This, coupled with the bafA1 experiment, allowed us to convincingly show that oAβ_{42} impaired autophagosome clearance. A previous study that examined the role of Aβ_{42} in autophagic flux using the tLC3 has found that Aβ monomers blocked autophagic

flux but Aβ oligomers did not, and instead they induced apoptosis (Guglielmotto et al., 2014). Inconsistencies in the results between the two laboratories might be explained simply by experimental conditions since our treatments with oAβ_{42} (20 μM for 24h) are set to trigger inhibition of protein prenylation but only mild induction of apoptosis (~20%) as determined before (Mohamed et al., 2012; Song et al., 2006). In conclusion, our work supports the evidence that Aβ directly affects autophagy.

In addition others have reported that Aβ may be generated in autophagic vesicles (Mizushima, 2005; Yu et al., 2005; Yu et al., 2004), suggesting a role for autophagy in Aβ metabolism although evidence against the involvement of autophagy in APP cleavage has also been published (Boland et al., 2010). Thus, Aβ might be part of a loop in which it is both the substrate of altered autophagy and the cause of autophagy abnormalities.

Precise knowledge of whether Aβ regulates autophagy *in vivo* and how autophagy is affected in AD is crucial for developing autophagy-targeted therapies (Nixon, 2013). Our evidence that oAβ_{42} blocked autophagic flux supports the rationale for a therapeutic strategy that restores autophagosome-lysosome fusion (Nixon and Yang, 2011). However, deeper insight into the mechanism triggered by oAβ_{42} that causes defective autophagic flux is required for any possible therapeutic intervention. Since oAβ_{42}-induced inhibition of protein prenylation caused intracellular trafficking defects (Mohamed et al., 2012), we next investigated if the defects in autophagic flux were due to decreased prenylation.

4.2. Inhibition of protein prenylation blocks autophagic flux

The most important finding of this work is that inhibition of protein prenylation causes autophagic flux blockade and that oAβ_{42} triggers this mechanism. We have used simvastatin and PA, two known inhibitors of protein prenylation (Deraeve et al., 2012; Mohamed et al., 2012). Simvastatin and PA inhibit protein prenylation by different mechanisms. Simvastatin inhibits HMGR, the rate-limiting enzyme of the mevalonate pathway causing a shortage of the prenylation substrate GGPP (and FPP) (reviewed in (Mohamed et al., 2015)). PA is an inhibitor of the enzyme geranylgeranyl transferase II (Deraeve et al., 2012). Both simvastatin and PA significantly inhibited autophagic flux. On the other hand, pravastatin, which also inhibits HMGR and causes a decrease of cholesterol synthesis did not inhibit protein prenylation and did not alter autophagic flux, strongly suggesting that the effect of simvastatin was due to the decrease of isoprenoids and not to the inhibition of cholesterol synthesis. Moreover, the rescue of autophagic flux with the isoprenoid GGPP in simvastatin-, but not PA-treated cells, was a confirmation that the effect is due to the impairment of protein prenylation. There is extensive evidence of the ability of GGPP to enter cultured cells (Kukar et al., 2005), and to counteract some effects of inhibitors of the mevalonate pathway (Meske et al., 2003; Ostrowski et al., 2007; Schulz et al., 2004). Using [^{3}H]GGPP our laboratory confirmed that neurons take up GGPP added to the medium and incorporate it in cellular proteins (Mohamed et al., 2012). Moreover, GGPP recovers protein prenylation *in vivo* in animal models of mevalonate kinase deficiency (Marcuzzi et al., 2011; Marcuzzi et al., 2008) and in

sertoli cells in which altered protein prenylation is associated with adult infertility resulting from childhood mumps infection (Wang et al., 2013).

Important to AD, the effect of oAβ_{42} was similar to that of simvastatin: GGPP prevented the oAβ_{42}-induced autophagy dysfunction because it prevented the inhibition of protein prenylation. This finding is consistent with previous discoveries from our laboratory that showed that GGPP prevented the impairment of intracellular trafficking, and induction of apoptosis induced by Aβ (Mohamed et al., 2012). Altogether the data presented here strongly indicates a cause-effect relationship between inhibition of protein prenylation and autophagy dysfunction.

Protein prenylation is crucial to the function of many proteins that regulate diverse cellular functions (McTaggart, 2006). Three hundred proteins in the human genome contain the C-terminal prenylation motif and, hypothetically, could be prenylated (Sebti, 2005); nuclear lamins, heterotrimeric G protein subunits, and small GTPases are confirmed to be prenylated (McTaggart, 2006).

We focused on proteins in the small GTPase family because their role in regulating autophagic flux is well established (reviewed in (Bento et al., 2013)). For example, the Rho GTPases help to coordinate cytoskeletal organization, and RhoA has been shown to positively regulate starvation-induced autophagy by an actin-dependent mechanism, while Rac1 was a negative regulator (Aguilera et al., 2012). The Rab GTPases constitute another crucial group of proteins in autophagy. Each Rab has a signature localization, which is inherently linked with their physiological function and ability to coordinate the different steps of intracellular membrane

trafficking including vesicle formation, microtubule-dependent transport, targeting to the acceptor membrane, and fusion of the vesicle with the target membrane (Bento et al., 2013; Kelly et al., 2012). Autophagic flux heavily relies on efficient intracellular membrane trafficking. Several Rabs have been implicated in autophagosome formation and expansion, and in the fusion of autophagosomes with late endosomes and lysosomes, among other processes. For example, Rab1 helps coordinate phagophore assembly and autophagosome formation by regulating the location of Atg9, a factor that putatively recruits membranes to the phagophore assembly site (PAS) from intermediate compartments (i.e., vesicles that mediate ER to Golgi transport). At recycling endosomes, Rab11 helps to regulate ULK1, the serine/threonine kinase that induces autophagy through mTOR. Relatedly, the early endosomal Rab5 has a role in regulating the activity of the lipid kinase Vps34, which recruits Atg components necessary for autophagosome formation (reviewed in (Ao et al., 2014; Bento et al., 2013)). These examples highlight an interesting aspect of autophagy: the autophagy machinery makes use of various intracellular membrane reservoirs to generate autophagosomes throughout the cytoplasm, which are then transported toward the perinuclear MTOC where lysosomes are abundant and fusion is more likely to occur (Kimura et al., 2008). Rab7, Rab8B, and Rab24 have important roles in both the trafficking of autophagosomes and their fusion with lysosomes (Ao et al., 2014; Bento et al., 2013; Gutierrez et al., 2004). For instance, Gutierrez *et al.* showed that the inactive mutant Rab7 (i.e., Rab7-T22N) prevented the normal progression of autophagic flux, which led to enlarged autophagosomes (Gutierrez et al., 2004).

Although studying the role of small GTPases in autophagy is a "scientific hotspot and may provide answers to various crucial questions currently debated in the autophagy field" (Bento et al., 2013), there is no data on the importance of their prenylation status for their functional roles in autophagy. To our knowledge, the studies in this thesis provide the first evidence that implicates protein prenylation as a crucial factor for the proper function of autophagic flux.

Autophagy is a potential target for disease treatment in AD (Nixon, 2013), and studies in animal models have shown the therapeutic value of autophagy regulation. For example, in TgCRND8 mice, the accumulation of autophagic substrates such as lipids, ubiquitinated proteins, and Aβ was cleared by genetically deleting cystatin B, the lysosomal protease inhibitor (Yang et al., 2011a; Yang et al., 2014). However, until our discovery, the mechanisms of autophagy dysfunction in AD were unclear, which has prevented the development of autophagy-directed therapies with disease-modifying value. Only a few molecular mechanisms for autophagy dysfunction have been proposed. It has been suggested that beclin-1 deficiency, potentially caused by its sequestration in protein aggregates (Ma et al., 2010), its cleavage by caspases (Rohn et al., 2011), or its binding to the neurovirulent ICP34.5 protein from HSV1 virus (Orvedahl et al., 2007) results in the autophagy dysfunction present in AD (reviewed in (Salminen et al., 2013)). This deficiency would impair the formation of autophagosomes. This disagrees with the finding of extensive accumulation of autophagic vesicles in brains of AD patients (Nixon et al., 2005). Our evidence also indicated that oAβ_{42} did not impair autophagosome formation, and, instead, blocked autophagosome-lysosome fusion. There is controversial evidence for the cause of

lysosomal dysfunction in AD. The decrease of lysosomal acidification was identified as another anomaly in AD. Insufficient delivery of the V-ATPase proton pump was proposed to cause defective lysosomal acidification in presenilin-1 deficient neurons (Lee et al., 2010; Wolfe et al., 2013). In contrast, it was shown that presenilin-1 deficiency did not affect lysosomal acidification, but instead altered lysosomal calcium storage/release, which impaired lysosome fusion capacity (Coen et al., 2012). ROS seem to be important in induction of autophagy in response to Aβ; however, lysosomal blockage, also caused by Aβ is independent of ROS (Lipinski et al., 2010). Here we clearly show that inhibition of protein prenylation is a mechanism of defective autophagosome-lysosome fusion. Crucially, our discovery may allow for the development of autophagy-targeted therapies specific for AD that enhance the clearance of autophagosomes.

4.3. Role of Rab7 prenylation in autophagy

Although it is well established that prenylation is necessary for biological functions of prenylated proteins (Gomes et al., 2003; McTaggart, 2006), there is evidence that unprenylated Rho proteins have physiological functions (Allal et al., 2000; Lebowitz et al., 1997) and may localize to membranes in a GTP-bound state (Khan et al., 2011). Furthermore, the concept that protein prenylation is constitutive and that proteins are prenylated as soon as they are synthesized has been challenged by evidence that prenylation of small GTPases is altered by phosphorylation of their unprenylated forms upon physiological activation of cell surface receptors (Williams,

2013). In addition, prenyltransferases are regulated by extracellular signals in very important physiological processes such as synapse formation (Li et al., 2013; Luo et al., 2003), dendritic morphogenesis (Wu et al., 2010b) and the insulin pathway (Goalstone et al., 2010). Therefore, protein prenylation is a highly regulated process and it is possible that not all functions of small GTPases require prenylation.

The inhibition of protein prenylation caused by oAβ_{42} affects several proteins and, therefore, recovery studies with GGPP identified the general requirement of protein prenylation for normal autophagy. One of the challenges facing the field of prenylation is the complexity of prenylated proteins. Interestingly, recent studies indicated that Rab GTPases display different rates of *in vivo* prenylation of up to two orders of magnitude (Kohnke et al., 2013) indicating that not all prenylated proteins will be equally affected by an isoprenoid shortage. A good strategy used in most studies is to focus on the prenylation of individual proteins, although this provides a partial view of the overall defect. A long-term objective will be to characterize global changes in protein prenylation in AD using methods recently described (Nguyen et al., 2009) and to link them to autophagy and other biological processes. These approaches will enable a quantitative proteome-wide analysis of the dysregulation of protein prenylation during disease.

We focused our attention on Rab 7 because: 1) the intracellular trafficking defects elicited by Aβ and associated with inhibited prenylation resembled endosomal abnormalities (e.g., cholesterol sequestration) (Mohamed et al., 2012), indicating defects in microtubule-dependent trafficking, which Rab7 has a role in

regulating (reviewed in (Bento et al., 2013)); 2) Rab7 is upregulated in AD and its levels are correlated with disease severity (Ginsberg et al., 2011; Ginsberg et al., 2010); and 3) Rab7 is essential for normal autophagic flux (Gutierrez et al., 2004; Hyttinen et al., 2013).

We found that oAβ_{42}-induced inhibition of protein prenylation caused a reduction of the co-localization of autophagosomes (i.e., LC3-positive puncta) with Rab7 due to the more cytosolic, diffuse, localization of Rab7. Importantly, we were able restore the membrane-bound status of Rab7 and its co-localization with autophagosomes with the isoprenoid GGPP. This evidence supports our hypothesis that prenylation *per se* is necessary for the crucial roles of Rab7 in autophagic flux (reviewed in (Ao et al., 2014; Bento et al., 2013; Hyttinen et al., 2013).

Rab7 is found in late endosomes, autophagosomes, and lysosomes. Rab7 participates in the regulation of transport from early to late endosomes and from late endosomes to lysosomes (Feng et al., 1995; Press et al., 1998; Vanlandingham and Ceresa, 2009). By controlling aggregation and fusion of late endocytic structures to lysosomes, Rab7 is essential for maintenance of the perinuclear lysosome compartment (Bucci et al., 2000). Expression of mutant Rab7T22N, a GDP-bound dominant negative form of Rab7, caused the accumulation of lysosomal hydrolases such as cathepsin-D in abnormal endosomes suggesting a role in transport from early to late endosomes (Press et al., 1998). Importantly, enlarged endosomes containing abnormally high levels of lysosomal hydrolases are characteristic of early sporadic AD (Cataldo et al., 1997), indicating possible Rab7 dysfunction in AD. During

autophagy, Rab7 is recruited to autophagosomes to allow their subsequent fusion with late endosomes and/or lysosomes. It was demonstrated that Rab7 is targeted to autophagosome membranes upon autophagy induction irrespective to its activation state (i.e., GDP- or GTP-bound) and independent of fusion events with endosomes, MVBs, and lysosomes (Gutierrez et al., 2004; Jager et al., 2004). Our data suggest that the ability of Rab7 to localize to autophagosomes depends on its prenylation status. Rab prenylation enables the membrane targeting and recycling of Rabs. Indeed, the proteins Rab Escort Protein (REP)-1, which delivers Rabs to membranes after prenylation, and GDP-Dissociation Inhibitors (GDIs), which extract Rabs from membranes and allow their recycling to other vesicles (Gavriljuk et al., 2013; Pfeffer et al., 1995), have a preference for prenylated Rabs. Moreover, our data is consistent with studies in both yeast and mammalian cells which showed that dual-geranylgeranylation of Rabs is necessary for their specific membrane locations (Calero et al., 2003; Gomes et al., 2003). Interestingly, mutant Rab7T22N caused the accumulation and enlargement of autophagosomes (Bains et al., 2011; Gutierrez et al., 2004), further supporting a role for normal Rab7 function in autophagic flux. It is unknown if Rab7T22N becomes prenylated and it is still controversial if it localizes to autophagosomes (Bucci et al., 2000; Saxena et al., 2005) although there is good evidence that Rab7T22N does not localize to lysosomes and does not mediate autophagosome-lysosome fusion (Bucci et al., 2000; Gutierrez et al., 2004; Jager et al., 2004; Kimura et al., 2007; Wang et al., 2011). Downregulation of Rab7 (siRNA) also blocks fusion of autophagosomes with lysosomes (Zhou et al., 2013). In addition, Rab7 plays an essential role in the movement of autophagosomes, which relies on a

precise balance between dynein- and kinesin-dependent mechanisms. In neurites of PC12 cells the bidirectional transport of autolysosomes depends on kinesin/dynein mechanisms (Yang et al., 2011b). These same mechanisms may mediate the bi-directional movement of autophagosomes at the tip of the axons (Maday et al., 2012), and dynein-mediated mechanisms operate for the retrograde transport of autophagosomes and amphisomes (Katsumata et al., 2010; Lee et al., 2011). In live mouse cortical neurons, LC3-positive autophagosomes forming in axons rapidly acquired Rab7 and LAMP1 and underwent exclusive retrograde movement (Lee et al., 2011). Rab7 is present in neuronal soma and in endosomes along axons (Saxena et al., 2005). Rab7 controls the retrograde trafficking of neurotrophins (NGF, BDNF) and their receptors TrkA and TrkB (Cai et al., 2010; Deinhardt et al., 2006; Saxena et al., 2005; Zhou et al., 2012), although there is also evidence against the retrograde transport of Rab7 (Delcroix et al., 2003; Kaasinen et al., 2008).

Two hypothetical explanations may account for blocked autophagic flux caused by hypoprenylation of Rab7. Firstly, Rab7 mislocalization caused by impaired prenylation may prevent its interactions with downstream effectors on the autophagosome membrane such as FYCO (FYVE (Fab1-YotB-Vac1p-EEA1) and coiled-coil domain containing protein) and RILP (Rab-interacting lysosomal protein), which help recruit the kinesin and dynein/dynactin microtubule-motor complexes, respectively (Cantalupo et al., 2001; Johansson et al., 2007; Jordens et al., 2001; Wu et al., 2005; Zhang et al., 2013). The dynein/dynactin motor complex mediates the minus-end-directed transport of autophagosomes, bringing them to the MTOC where lysosomes are abundant and, thus, promotes autophagosome-lysosome fusion

(Kimura et al., 2008). An observation from our studies was that oAβ_{42} caused an increase in the number of autophagosomes; often, they were located peripherally throughout the cytoplasm compared to the perinuclear autolysosomes/amphisomes observed in torin-1 treated cells and in cells under starvation conditions. A speculation, and the topic of future studies in our laboratory, is that unprenylated Rab7 was unable to engage RILP at the autophagosome membrane and, thus, impaired the recruitment of the dynein/dynactin motor complex, causing defective trafficking of autophagosomes and reduced the opportunity to fuse with lysosomes. Supporting this idea, Rab effectors have preference for membrane-bound GTP-Rabs (Cantalupo et al., 2001; Pfeffer, 2013). In addition, studies of Rab7 interaction *in vitro* indirectly suggested that Rab7 prenylation is important for its interaction with RILP (Cantalupo et al., 2001). Secondly, in addition to its involvement in autophagosome transport, Rab7 has a direct role in mediating autophagosome-lysosome fusion by interacting with the membrane tethering complex named HOPS (homotypic fusion and vacuolar protein sorting; also known as C-Vps) (reviewed in (Bento et al., 2013; Ganley, 2013)). Tethering complexes mediate membrane-specific docking of vesicles, and help to initiate signaling cascades that culminate in soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE)-mediated membrane fusion (Pfeffer, 1999). HOPS has guanidine exchange factor (GEF) activity toward Rab7, thus during its interaction with membrane-bound Rab7 it further activates more Rab7, creating a positive-feedback that induces autophagosome-lysosome fusion (reviewed in (Bento et al., 2013; Ganley, 2013)). Therefore it is plausible that Rab7 would be

unable to properly engage this cascade while being restricted to the cytosol, which we observed because of oAβ_{42}-induced inhibition of Rab7 prenylation.

Decreased Rab7 prenylation will alter not only autophagy but other vesicular trafficking events in which Rab7 is crucial. Rab7 regulates the maturation of early endosomes into late endosomes. This process relies on the HOPS complex and its activation of Rab5-to-Rab7 conversion (Rink et al., 2005). Late endosome formation is crucial for the delivery of proteases from the plasma membrane and trans-Golgi network (TGN) to lysosomes mediated by insulin-like growth factor-II/mannose-6-phosphate (M6P) (IGFII/M6P) receptor, a type-I transmembrane protein widely expressed in the brain (Hille-Rehfeld, 1995; Kar et al., 2006). oAβ_{42}-induced hypo-prenylation of Rab7 could render it unable to function in endosome maturation, impairing late endosome formation and, furthermore, the transport of lysosomal proteases from late endosomes to lysosomes. However, an important note is that thapsigargin, an ER stressor, inhibited Rab7 localization to autophagosomes and impaired autophagosome-lysosome fusion, without affecting endocytosis-mediated cargo degradation, which confirmed a specific mechanistic role for Rab7 in autophagic flux compared to general endocytic fusion (Ganley et al., 2011).

The importance of Rab7 in AD has been recently highlighted. Expression profiling studies performed to identify mechanisms that underlie selective vulnerability of specific neurons during the progression of AD showed that in cholinergic basal forebrain neurons microdissected from postmortem brains of individuals with mild cognitive impairment (MCI) and AD there is selective

upregulation of rab4, rab5, rab7, and rab27 genes (Ginsberg et al., 2011). Importantly, this upregulation correlated with cognitive decline and neuropathological criteria for AD. Rab7 and Rab5 protein levels were upregulated within basal forebrain, frontal cortex, and hippocampus but not in less vulnerable cerebellum and striatum in MCI and AD (Ginsberg et al., 2010). The increase of Rab7 and Rab5 in AD brains has been interpreted as overactivation of the endosomal pathway. In addition, increased levels of Rab7 and LC3 have been found in cerebrospinal fluid (CSF) from AD patients and may represent a novel CSF biomarker for AD (Armstrong et al., 2014). We have also found increased levels of Rab7 in the cortex of TgCRND8 mice and in neurons treated with Aβ. We foresee that Rab7 expression may be regulated by its prenylation status, based on the evidence that inhibition of the mevalonate pathway by statins upregulated expression of Rab7 and Rab5 (Bifulco, 2005; Laezza et al., 1998). Bifulco proposed that the levels of Rabs at the membrane (which are regulated by prenylation) could serve as an intracellular signal for Rab expression regulation (Bifulco, 2005).

Because membrane trafficking is highly dynamic, it is plausible that subtle alterations to its regulation could have pathological consequences. This would be especially true in neurons, which have extensive membranes and cytoplasm, and rely on intracellular trafficking for axonal transport of neurotrophins (Levi-Montalcini, 1987; Senger and Campenot, 1997) and for highly efficient basal autophagy (Boland et al., 2008; Kimura et al., 2008). However, investigating subtle alterations over an extended time period is not suitable *in vitro* where cells are typically cultured for less than a month. Hence, the need to determine if autophagy is regulated by protein

prenylation *in vivo*. Measuring brain autophagic flux *in vivo* may provide the temporal resolution to observe the precise role of protein prenylation in the defective autophagy-lysosomal system in AD. This would help confirm if restoring prenylation is a viable disease-modifying strategy against AD pathology. Additionally, such studies may identify a critical point where autophagic flux may shift from a protective mechanism against $A\beta_{42}$ to a harmful sponsor of its toxicity (Ling and Salvaterra, 2011).

Our laboratory showed that prenylation was impaired in the TgCRND8 mouse model of AD (Mohamed et al., 2012). We also revealed an upregulation of Rab7 expression in TgCRND8 mice, comparable to human AD brains (Ginsberg et al., 2011; Ginsberg et al., 2010). Importantly, this evidence suggests that similar pathogenic mechanisms caused by $oA\beta_{42}$ were occurring *in vivo*. Herein, we have established protein prenylation as a crucial factor for normal autophagic flux and, thus, it is plausible that the autophagy impairment in TgCRND8 mice (Yang et al., 2011a; Yang et al., 2014) is due to impaired prenylation. We have confirmed an alteration of autophagy in TgCRND8 mouse brain cortex by the detection of endogenous LC3; however, this evidence does not differentiate the induced formation from defective clearance of autophagosomes. Examining autophagy in the nervous system *in vivo* has been a major challenge in the study of autophagy regulation within neuropathological contexts (Castillo et al., 2013; Klionsky et al., 2012). To determine the nature of autophagy dysfunction in TgCRND8 mice, we have adopted a novel strategy to measure autophagic flux within the central nervous system *in vivo* (Castillo et al., 2013). Specifically, we deliver the mCherry-GFP-LC3 (tLC3) in an AAV2/2 viral vector

by ICV injection into newborn mice (Castillo et al., 2013). We have had success using this method on wild-type mice, and have observed widespread expression of tLC3 in brain areas including the hippocampus, frontal cortex, and entorhinal cortex. We are planning to use this technique in TgCRND8 mice to gain insight on autophagy dysfunction in this AD model, and to test if ventricular infusion of GGPP can restore autophagy.

4.4. Conclusion

The growing importance of autophagy is clearly shown by the exponentially increasing number of publications appearing on a daily basis in PubMed. The amount of data concerning the involvement of Rabs in the regulation of autophagy has also grown exponentially during the past several years. In spite of this, the role of protein prenylation as a mechanism of autophagy regulation has been overlooked. On the other hand, the interest in understanding the regulation of isoprenoid production and protein prenylation in the brain has increased considerably in the past few years due to the importance of protein prenylation in several cellular processes such as cell growth, cytoskeletal organization and remodeling, and vesicle trafficking; and to the fact that some of the beneficial effects of statins in neurodegenerative diseases have been attributed to changes of protein prenylation (Butterfield et al., 2011; Cole and Vassar, 2006; Hooff et al., 2010b; Li et al., 2012; Liao, 2002; van der Most et al., 2009; Wood et al., 2010). Non-sterol isoprenoids and protein prenyltransferases have emerged as attractive therapeutic targets for several diseases (Holstein and Hohl,

2012; Li et al., 2012) but we still need a deeper understanding of their roles in the brain in order to determine their value for treating neurodegeneration in general, and AD in particular. This thesis demonstrated that autophagy is dependent on normal Rab7 prenylation and, therefore, the isoprenoid GGPP. Thus, we have bridged a gap between the role of prenylation in autophagy, and the possibility of targeting isoprenoids for AD-relevant therapeutic strategies.

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