University of Alberta

Amyloid beta peptide, Cholesterol and Isoprenoids in Alzheimer's disease

by

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Dedication

To Halim, Walid, my mom and my dad.

Abstract

Alzheimer's disease (AD) is the most common form of dementia in the elderly. The major pathological features of AD are extracellular amyloid plaques, intracellular neurofibrillary tangles and neuronal loss. Amyloid beta (Aβ) peptide, the main component of the amyloid plaques, has been proposed to be the initiator of most of the pathological changes that occur in AD (Amyloid Hypothesis). Mounting genetic, epidemiological and biochemical evidence indicate the involvement of cholesterol in AD pathology. Cholesterol accumulation has been confirmed in AD brain and has demonstrated to play a role in AD pathology, however, the origin of this cholesterol accumulation has not been identified yet. In this thesis we examined the regulation of cholesterol homeostasis by oligomers of $A\beta_{42}$ ($oA\beta_{42}$) in primary neurons. We demonstrated for the first time that $oA\beta_{42}$ causes intracellular cholesterol sequestration at the late endosome/lysosome, the Golgi and the plasma membrane. Subsequently, we investigated the mechanism underlying $oA\beta_{42}$-induced cholesterol sequestration. We discovered that $oA\beta_{42}$ inhibits the maturation of the transcription factor; sterol regulatory element binding protein-2 (SREBP-2), which is crucial for the synthesis of isoprenoids. As a result we demonstrated for the first time that protein prenylation of small GTPases is reduced in $oA\beta_{42}$-treated neurons as well as in cortices of TgCRND8, an AD transgenic mouse model. Hence, exogenous supply of geranylgeranyl pyrophosphate (GGPP) was able to restore normal protein prenylation, prevent cholesterol sequestration and reduce neurotoxicity in $oA\beta_{42}$-treated neurons. Our work revealed that $oA\beta_{42}$-induced inhibition of protein prenylation is a novel mechanism of $oA\beta_{42}$-induced cholesterol sequestration and neurotoxicity. Furthermore, we illustrated that $oA\beta_{42}$-induced reduction of phosphorylated Akt leads to reduced SREBP-2 transport from the endoplasmic reticulum to the Golgi leading to reduced SREBP-2 processing. Our work provided an evidence that cholesterol sequestration and impaired cholesterol trafficking in AD is induced by $oA\beta_{42}$. Moreover, we identified SREBP-2 and protein

prenylation as new targets of oAβ_{42}, which opens a new area of research investigating the role of oAβ_{42}-induced inhibition of SREBP-2 processing and consequent inhibition of protein prenylation in the development of many pathological changes observed in AD.

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List of Abbreviations

2HP-β-CD	2-hydroxy propyl-β-cyclodextrin
ABC transporters	Adenosine triphosphate-binding cassette transporters
AD	Alzheimer's disease
ACAT	Acyl coA-Cholesterol Acyltransferase
Akt	Serine/threonine protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
Aβ	Amyloid beta peptide
BBB	Blood brain barrier
BFA	Brefeldin A
CNS	Central nervous system
COPII	Coat protein complex II
CSF	Cerebrospinal fluid
CYP46A1	Cholesterol-24-hydroxylase
DS	Down Syndrome
ER	Endoplasmic Reticulum
FPP	Farnesyl pyrophosphate
GGPP	Geranyl geranyl pyrophosphate
GSK-3β	Glycogen synthase kinase 3β
HMGR	Hydroxy methyl glutaryl coA reductase
IDE	Insulin degrading enzyme
Insig-1 or -2	Insulin-Induced gene-1 or -2
IR	Insulin Receptor

LDLR	Low Denisty Lipoprotein Receptor
LRP	LDLR-related protein
LTP	Long term potentiation
LXRs	Liver-X-Receptors
MβCD	Methyl-β-cyclodextrin
(M)SREBP-2	Mature SREBP-2
MMP	Matrix metalloproteinases
MVBs	Multivesicular bodies
NGF	Nerve growth factor
NEP	Neprilysin
NMDA	N-Methyl-D-Aspartate
NPC	Niemann Pick type C
NPC1	Niemann Pick type C protein-1
NPC2	Niemann Pick type C protein-2
$oA\beta_{42}$	Oligomers of $A\beta_{42}$
pAkt	Phosphorylated Akt
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide-3-Kinase
PS1	Presenilin-1
(P)SREBP-2	Precursor SREBP-2
Rab-GDI	Rab GDP Dissociating Inhibitor
S1P	Site-1-Protease
S2P	Site-2-Protease
SCAP	SREBP Cleavage-Activating Protein
Seladin-1	SELective Alzheimer's Disease INdicator-1
SREBP-2	Sterol Regulatory Element Binding Protein-2

TLC	Thin Layer Chromatography
TrkA	Tropomyosin-receptor-kinase A

Chapter 1: General Introduction and Literature Review

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1.1. Alzheimer's disease (AD)

Alzheimer's disease (AD) is the most devastating and common form of dementia in the elderly. Five hundred thousand people in Canada and 25 to 30 million people worldwide are currently suffering from AD. Five to eight percent of Canadians over the age of 65, and 30 to 50 percent over the age of 85 have the sporadic form of the disease. The most important risk factor for AD is age, therefore the increased average age of the world population results in a significant increase of the number of people afflicted with AD. The incidence of AD has been predicted to approximately triple by 2040 if more effective therapeutic strategies are not made available (Alzheimer-Society, 2010; Minati et al., 2009).

AD is characterized clinically by progressive cognitive deterioration and memory decline. Memory decline starts with loss of episodic memory; a subcategory of declarative memory, which leads to reduced recollection of recent events. Understanding the molecular mechanism that initiates the memory decline and cognitive deterioration in AD is urgently required (Chopra et al., 2011).

The two main pathological hallmarks of AD brains were first described by Alois Alzheimer in 1907 (Alzheimer, 1907; LaFerla et al., 2007) and their detection in post-mortem examination is still required for AD diagnosis. These pathological features of AD include; 1) extracellular amyloid/senile plaques, that Alzheimer called "peculiar substance" (Figure 1.1-A). The amyloid plaques were found later to consist mainly of amyloid beta peptide (Aβ) (Glenner and Wong, 1984b; Masters et al., 1985). In addition, amyloid plaques have been shown to contain cholesterol and apolipoproteinE (apoE) among other components (Burns et al., 2003b; Mori et al., 2001; Namba et al., 1991; Panchal et al., 2010; Uchihara et al., 1996). 2)

Intracellular neurofibrillary tangles (Figure 1.1-B), which are composed of hyperphosphorylated tau protein (Goedert et al., 1988; Grundke-Iqbal et al., 1986). Although Aβ-containing senile plaques and phospho-tau-containing neurofibrillary tangles are hallmark lesions of AD, neither is specific of the disease. Other pathological changes important in AD brains are synaptic dysfunction; severe synaptic loss manifested by reduced presynaptic, postsynaptic markers as well as decreased number and density of synapses in cortex and hippocampus of AD brains (Selkoe, 2002; Shankar and Walsh, 2009) and the loss of neurons, especially cholinergic neurons from the basal forebrain (Davies and Maloney, 1976; Whitehouse et al., 1982) (Figure 1.1-C). In fact, from a biochemical point of view the most consistent finding in AD is a deficit in the cholinergic system, decreased level of choline acetyltransferase, and other cholinergic markers. The degree of AD dementia correlates with the death of basal forebrain cholinergic neurons (Davies and Maloney, 1976; Ladner and Lee, 1998). Due to the severe neuronal degeneration, the brains of AD patients may weigh about one third of the brains of age-matched non-demented persons (Selkoe, 2001).

Only 5% of AD cases are familial and result from genetic mutations in amyloid beta peptide precursor protein (APP) or in presenilins (PS1& PS2) genes (Chartier-Harlin et al., 1991; Cruts et al., 1998; De Strooper, 2007; Goate et al., 1991; Hardy, 2007; Murrell et al., 1991; Naruse et al., 1991; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006; Yoshioka et al., 1991). The majority of AD cases are non-familial or sporadic and share the same clinical and pathological features with familial AD, but they differ in the age of onset. Familial AD appears in the fourth to fifth decade of life, while sporadic AD is usually manifested in the seventh or eighth decade of life (Pimplikar, 2009).

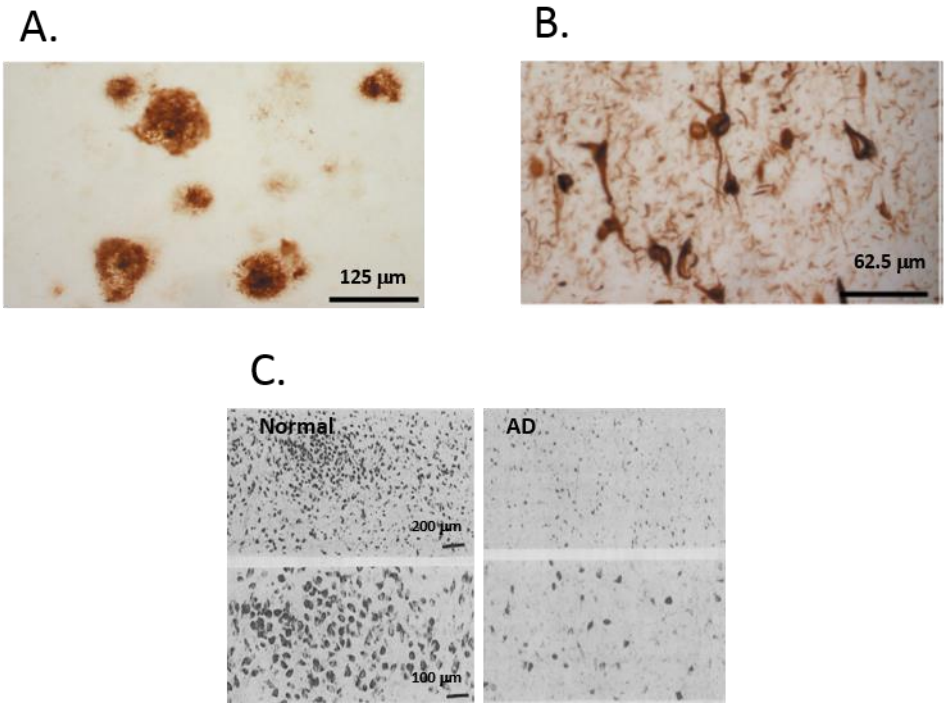

Figure 1.1 Plaques, tangles and neuronal loss are the major pathological features in the AD brain. (A) A representative microphotograph of amyloid plaques in the AD brain. Amyloid plaques were visualized by immunostaining with an anti-Aβ42 specific antibody. Scale bar: 125 µm. (B) A representative microphotograph of neurofibrillary tangles. Tangles were visualized by immunostaining with an anti-PHF1 specific antibody. Scale bar: 62.5 µm. A and B were acquired from (LaFerla and Oddo, 2005) Copy Right Licence number 3257701442293 (C) Photomicrographs from the midportion of the nucleus basalis of Meynert (Basal forebrain neurons) of an age-matched control (Normal) and a patient with Alzheimer's disease (AD) show the normal number and density of neurons in the control case and profound loss of neurons in the AD patient. Scale bar: 200 µm and 100 µm. C is adapted from (Whitehouse et al., 1982) Copy Right Licence number 3257700617062.

Due to the complexity of AD pathology, several hypotheses have been postulated to explain the development and progression of the neuropathological changes observed in AD. The three main hypotheses are the cholinergic hypothesis, the tau hypothesis and the amyloid hypothesis.

The reduced cholinergic transmission observed in AD (Davies and Maloney, 1976; Perry et al., 1977) and the role of acetylcholine in memory and learning (Drachman and Leavitt, 1974) led to the development of the cholinergic hypothesis, which stated that the degeneration of cholinergic neurons in the basal forebrain and the associated loss of cholinergic transmission in the cortex and other brain areas play the major role in the development of cognitive deteriorations observed in AD (Bartus et al., 1982; Coyle et al., 1983; Cummings and Back, 1998). This was supported by the strong correlation observed between reduced acetylcholine synthesis and the degree of cognitive impairment in AD patients (Perry et al., 1978; Wilcock et al., 1982). Based on the cholinergic hypothesis, the cholinomimetic therapy is the best developed approach for symptomatic treatment of AD. This therapy includes cholinesterase inhibitors, which potentiate central cholinergic transmission and consequently improve cognition in AD (Chopra et al., 2011; Francis et al., 1999). However, cholinergic hypothesis have many weaknesses including; the failure of vigorous cholinergic therapy to normalize cognition in patients with dementia, failure of cholinergic denervation to produce cortical plaques, the absence of cholinergic lesions in early AD, and recent studies demonstrating that loss of cholinergic transmission in AD may not be as prominent as loss of other neurotransmitters (reviewed in (Mesulam, 2004)).

Normal tau protein binds to and stabilizes microtubules maintaining the microtubules network required for normal axonal transport in neurons (Drechsel et al., 1992; Forman et al., 2004; Hirokawa et al., 1988). It has been identified that the building subunit of the neurofibrillary tangles in AD is the abnormally phosphorylated tau (Lee et al., 1991). Phosphorylation of tau negatively regulates the binding of tau to microtubules (Bramblett et al., 1993; Bramblett et al., 1992). Therefore, conversion of normal tau into a functionally impaired phosphorylated form that cannot bind to microtubules will lead to destabilization and depolymerization of microtubules and consequently impaired microtubule-based axonal transport that compromises neuronal function and viability and finally leads to neurodegerenation (Lee and Trojanowski, 1992). Based on this, the tau hypothesis of AD proposed that abnormally phosphorylated tau mediates neurodegeneration in AD via impaired intraneuronal transport (Lee and Trojanowski, 1992).

The amyloid hypothesis will be discussed in details below. Several other hypotheses have been recently postulated including; Tau-axis hypothesis linking Aβ and tau hypotheses together (Ittner and Gotz, 2011) and calpain-cathepsin hypothesis proposing that age-dependent oxidative stress could impair autophagic-lysosomal system leading to neuronal cell death in AD (Yamashima, 2013).

1.2. Amyloid hypothesis:

More than two decades ago, Hardy and Selkoe proposed that the initial pathological events that lead to development and progression of AD are APP aberrant metabolism and Aβ deposition into plaques in the brain (Amyloid

hypothesis) (Hardy and Allsop, 1991; Selkoe, 1991). The amyloid hypothesis was based on two major findings. First, the discovery that the APP gene exists in chromosome 21 (Goldgaber et al., 1987) that followed earlier observations that people with Down syndrome (DS) (Trisomy 21) develop neuropathological features that are indistinguishable from those of AD (Glenner and Wong, 1984a; Olson and Shaw, 1969). Moreover, the study of the temporal sequence of events in DS demonstrated that plaque formation is the initial event, followed by the appearance of neurofibrillary tangles, and later in life neuronal loss (Rumble et al., 1989). Second, APP gene mutations were discovered in many cases of familial AD (Chartier-Harlin et al., 1991; Goate et al., 1991; Murrell et al., 1991; Naruse et al., 1991; Yoshioka et al., 1991).

Later on it was found that some of APP mutations are located at or close to the site of proteolytic cleavage of APP and they lead to increased Aβ production (Cai et al., 1993; Citron et al., 1992). Other mutations that exist in the middle of the Aβ sequence lead to enhanced Aβ aggregation (Wisniewski et al., 1991). Moreover, mutations in presenilin proteins observed in familial AD increases APP amyloidogenic processing (discussed below) and Aβ production (Scheuner et al., 1996). Collectively, these studies support the amyloid hypothesis.

Other studies supporting the amyloid hypothesis argued against the possibility that tau hyperphosphorylation would be the initial event in AD pathology. Gene mutation in the gene encoding tau protein leads to frontotemporal dementia that is associated with severe deposition of neurofibrillary tangles but no amyloid plaques (Hutton et al., 1998). In addition, transgenic mice overexpressing mutant human APP and mutant human tau genes have increased number of neurofibrillary

tangles compared to mice overexpressing mutant human tau alone but they have the same structure and number of amyloid plaques compared to mice overexpressing only mutant human APP (Lewis et al., 2001).

Although the amyloid hypothesis received many revisions and critiques (Hardy, 2009; Hardy and Selkoe, 2002; Pimplikar, 2009), there is no doubt that Aβ is a main player in AD pathogenesis.

1.3. Amyloid beta peptide (Aβ):

1.3.1. Aβ generation from APP

Aβ derives from the ubiquitously expressed transmembrane protein APP by sequential endoproteolytic cleavage by β-secretase and γ-secretase (Selkoe, 2001; Thinakaran and Koo, 2008) (Figure 1.2). The γ-secretase activity corresponds to a complex of proteins that include presenilin, nicastrin, anterior pharynx defective-1 (APH1) and presenilin enhancer-2 (PEN2) (Vetrivel and Thinakaran, 2006). Under physiological conditions APP is predominantly cleaved by α-secretase and γ-secretase generating soluble APP (sAPPα) and P3 and preventing Aβ formation (non-amyloidogenic processing). However, the amyloidogenic pathway of APP cleavage by β-secretase and γ-secretase also occurs under normal conditions in all cells (Haass et al., 1992). Aβ peptides of different length are formed by this pathway. Aβ peptides are continuously secreted and are present in normal cerebrospinal fluid (CSF) and human plasma (Seubert et al., 1992). The most secreted Aβ is $A\beta_{40}$, but a small percentage (5-15%) is $A\beta_{42}$ (Asami-Odaka et al., 1995; Selkoe, 2000). APP amyloidogenic proteolytic processing takes place predominantly in post-Golgi secretory and endocytic compartments while non-amyloidogenic processing of APP takes place mainly at

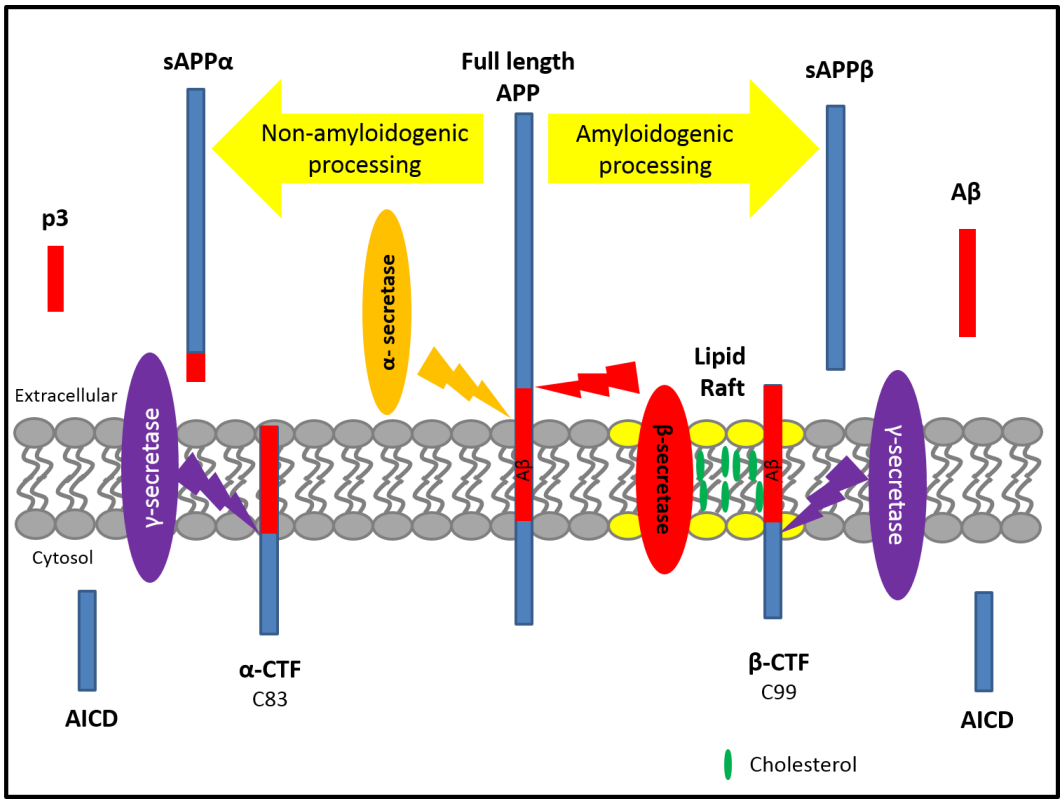

Figure 1.2 APP Cleavage. The amyloid Aβ peptide is derived via proteolysis from a larger precursor molecule called the amyloid precursor protein (APP), a type 1 transmembrane protein. APP undergoes proteolytic processing by two pathways. The first enzymatic cleavage (non-amyloidogenic processing) is mediated by α-secretase. Cleavage by α-secretase occurs within the Aβ domain, thereby preventing the generation and release of Aβ peptides. Two fragments are released, the larger ectodomain (sAPPα) and the smaller carboxy-terminal fragment α-CTF(C83). Furthermore, α-CTF undergoes an additional cleavage mediated by γ-secretase to generate P3 and APP intracellular domain (AICD). APP molecules that are not cleaved by α-secretase become a substrate for β-secretase in lipid rafts (amyloidogenic processing) releasing an ectodomain (sAPPβ), and retaining the last 99 amino acids of APP (known as β-CTF (C99)) within the membrane. The first amino acid of β-CTF is the first amino acid of Aβ. β-CTF is subsequently cleaved 38–43 amino acids from the amino terminus to release Aβ, by the γ-secretase complex. This cleavage predominantly produces Aβ and AICD.

the plasma membrane. It is still unclear what organelles represent the site of Aβ production in neurons (reviewed by (Thinakaran and Koo, 2008)) (Figure1.2).

While in the familial form of AD Aβ accumulation correlates directly with increased production from APP, in the non-familial forms it results from intricate interactions of factors that affect Aβ clearance and aggregation (Minati et al., 2009).

1.3.2. Aβ structure:

Aβ peptides are 39 to 43 amino acids long and have high propensity to form stable cross-β aggregates. The C-terminal amino acid sequence of Aβ is composed mainly of hydrophobic residues, which play an important role in peptide insolubility and in the initial steps of aggregation. The N-terminal region of the Aβ peptide is amphipathic and participates in the binding of Aβ to apoE (Strittmatter et al., 1993b), cholesterol (Yao and Papadopoulos, 2002), glycosaminoglycans (Buee et al., 1993; Narindrasorasak et al., 1991), and metal ions (Adlard and Bush, 2006) . The 19-25 region of Aβ peptide has been recognized as an essential region in the aggregation process (El-Agnaf et al., 1998). The relevance of this central region in Aβ aggregation has been underscored by enhanced Aβ aggregation and deposition induced by Aβ mutants linked to familial forms of AD, including Flemish (A21G) (Hendriks et al., 1992), Dutch (E22Q) (Clements et al., 1993; Levy et al., 1990; Miravalle et al., 2000; Van Broeckhoven et al., 1990; Wisniewski et al., 1991), Arctic (E22G) (Nilsberth et al., 2001), and Iowa (D23N) (Grabowski et al., 2001; Van Nostrand et al., 2001).

1.3.3. Aβ peptides present *in vivo*

Aβ peptides generated from APP under normal metabolic conditions are

the subunits of amyloid fibrils that deposit in brain parenchyma and within the walls of brain blood vessels in AD patients (Selkoe, 2001). Healthy aged non-demented humans may contain Aβ deposits that occur primarily as diffuse amyloid deposition (Selkoe, 2001). Several different soluble forms of Aβ are also present in brains of normal individuals and AD patients (Selkoe, 2008). The process of Aβ oligomerization and fibrillization has been extensively studied and the roles of fibrillar deposits and soluble oligomeric Aβ forms in AD have been examined considerably.

The original amyloid cascade hypothesis had proposed that the key event in AD development is the extracellular accumulation of insoluble, fibrillar Aβ (Hardy and Allsop, 1991; Hardy and Higgins, 1992; Selkoe, 1991). This "extracellular insoluble Aβ toxicity" hypothesis was later modified to acknowledge the role of extracellular soluble Aβ oligomers as pathogenic agents. Only more recently the importance of intraneuronal Aβ accumulation in the pathogenesis of AD has been recognized, despite the original reports showing Aβ accumulation inside neurons are dated more than 20 years ago. The "intraneuronal Aβ hypothesis" does not argue against a role for extracellular Aβ but complements the traditional amyloid cascade hypothesis (Cuello, 2005; Gouras et al., 2005; Wirths et al., 2004).

1.3.3.1. Insoluble Assemblies of Aβ peptides:

Senile plaques from brains of AD patients and animal models of AD consist of various hydrophobic Aβ peptides, which might contain up to 43 amino acids. Studies using end-specific antibodies against Aβ have shown that in AD brain, all senile plaques were $Aβ_{42(43)}$ positive, while only some of them were positive for $Aβ_{40}$ (Gravina et al., 1995; Iwatsubo et al., 1994). Diffuse plaques represent the

earliest stage of Aβ deposition. They are positive for $Aβ_{42(43)}$ but completely negative for $Aβ_{40}$ (Guntert et al., 2006; Iwatsubo et al., 1994). These data indicate that the Aβ alloform that is initially deposited in AD is $Aβ_{42}$ and that $Aβ_{40}$ is deposited at a later stage of plaque maturation (Selkoe, 2001). $Aβ_{42}$ is believed to be the main component of the plaque core, whereas less hydrophobic $Aβ_{40}$ seems to be present predominantly at the outer rim of the plaque (Hartig et al., 2010). $Aβ_{40}$ is the predominant component of cerebrovascular amyloid fibrils (Joachim et al., 1988; Roher et al., 1991; Suzuki et al., 1994), even if the peptide that deposits initially on the vessel is $Aβ_{42}$ (Selkoe, 2001).

Despite $Aβ_{40}$ and $Aβ_{42}$ are the most abundant products of APP cleavage, endogenous Aβ peptides present in brain and CSF are significantly more heterogeneous in length (Mori et al., 1992; Saido et al., 1995; Seubert et al., 1992). Aβ peptides isolated from senile plaques (Miller et al., 1993) and from cerebrovascular amyloid fibrils (Joachim et al., 1988; Miller et al., 1993) as well as water-soluble Aβ peptides from patients with AD or with DS can be truncated at the N-terminus (Miller et al., 1993; Sergeant et al., 2003; Tekirian et al., 1998). Aβ peptides truncated at glutamate residues in positions 3 or 11 undergo intramolecular dehydration forming Aβ species that begin with pyroglutamate (pE). A significant proportion of Aβ in plaques and vessels from AD brains exists as $Aβ_{3pE-X}$ (Harigaya et al., 2000). $Aβ_{3pE-40/42}$ peptides may play a pivotal role in the development of AD since they are more resistant to extracellular aminopeptidases (Kuo et al., 1998; Schilling et al., 2008), are more prone to aggregate (D'Arrigo et al., 2009; He and Barrow, 1999), and might represent the seed that starts Aβ oligomerization (Frost et al., 2012; Schilling et al., 2006). $Aβ_{3pE-40/42}$ exhibited significant toxicity in neuronal and glial cultures (Hosoda et al., 1998; Russo et al.,

2002) and triggered neurodegeneration and lethal neurological deficits in a transgenic mouse model (Wirths et al., 2009).

1.3.3.2. Soluble Aβ aggregates:

During the nineties several studies proposed that non-fibrillar $A\beta_{42}$ is the first species to accumulate extracellularly in the brain and vessels of AD and DS patients (McLean et al., 1999; Russo et al., 1997; Tabaton et al., 1994), and to correlate better than plaque load with AD symptoms (Gouras et al., 2000; Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000). The three major species of soluble Aβ identified in brains as well as in CSF from AD and DS patients are the full-length form $A\beta_{42}$, and the two N-terminal truncated pyroglutamate peptides discussed above, $A\beta_{3pE-42}$ and $A\beta_{11pE-40/42}$ with relative molecular masses of 4.5 kDa, 4.0 kDa, and 3.3 kDa, respectively (Russo et al., 1997; Tabaton et al., 1994). Oligomeric Aβ peptides (mostly $A\beta_{42}$) associate and co-localize with both diffuse and neuritic plaques and with cerebrovascular deposits of Aβ in human brain (van Helmond et al., 2010). AD brains contain ~ 6-fold more water-soluble Aβ than control brains (Kuo et al., 1996). The majority of soluble Aβ peptides in AD brains end in amino acid 42 ($A\beta_{N-42}$) (Kuo et al., 1996; Russo et al., 1997; Tabaton and Gambetti, 2006) and their levels are about 50 times greater than the levels of soluble $A\beta_{N-42}$ found in the CSF of AD patients (Kuo et al., 1996). On the other hand, the predominant forms of soluble Aβ in normal brains were $A\beta_{N-40}$ (Kuo et al., 1996).

Both the full-length Aβ and pyroglutamate-truncated Aβ peptides form stable aggregates that are water-soluble (Russo et al., 1997). Different soluble Aβ peptide assemblies have been isolated from AD brains. Early work in AD tissue

using purification by size-exclusion chromatography detected the presence of Aβ monomers, dimers and trimers (Roher et al., 1991). Further studies found monomers and oligomers ranging from less than 10 kDa to greater than 200 kDa (Tabaton and Piccini, 2005). SDS-stable dimers of Aβ were detected in CSF of subjects with AD (Walsh et al., 2000) and the formic soluble fraction obtained from amyloid cores of AD patients also contains SDS-stable dimers together with trimers (Enya et al., 1999; Funato et al., 1999; Roher et al., 1996). Additionally, water soluble SDS-resistant tetramers were isolated from the frontal cortex and temporal cortex from AD brain (Lambert et al., 2001). Higher molecular weight (HMW) oligomers, identified in water-soluble extracts of frontal cortex of AD brain, have a MW of 56 kDa and correspond to Aβ 12-mer assemblies (Gong et al., 2003). Similar molecular weight $A\beta_{42}$- dodecamers were detected in AD brain neocortex homogenates (van Helmond et al., 2009). Oligomers ranging from 10-100 kDa have been collectively designated Aβ-Derived Diffusible Ligands (ADDLs) and isolated from human AD brain and CSF (Cleary et al., 2005). Recently, ELISA assays have been developed to detect higher molecular weight (HMW) Aβ oligomers (between 40 kDa and 200 kDa) in CSF (Fukumoto et al., 2010). The levels of these 10-20 mers oligomers were significantly higher in AD patients, and a negative correlation between the levels of HMW oligomers and Mini-mental state examination was demonstrated suggesting a crucial role of these oligomers in the pathogenesis of AD (Fukumoto et al., 2010).

The low amounts of oligomers that can be isolated from brain and CSF of patients with AD and the complex population of Aβ species present *in vivo*, together with changes in the proportion and structure of Aβ assemblies during isolation make their study extremely difficult by most of the classical biophysical

techniques. For this reason, the synthetic Aβ peptide has been used to generate a wide range of assemblies of different size, shapes and toxicity including soluble oligomers and fibrils. LaDu's group reported detailed protocols (Dahlgren et al., 2002; Stine et al., 2003) for preparation of SDS-stable Low Molecular Weight (LMW) oligomers. LMW oligomers represent a mixture of Aβ assemblies ranging from 2-mers to 8-mers that are in equilibrium with monomers (Lazo et al., 2005). LMW oligomers prepared from $A\beta_{42}$ peptide under these controlled conditions were resolved as monomers, trimers and tetramers by SDS-PAGE whereas those prepared from $A\beta_{40}$ remained predominantly as monomers and tetramers (Chen and Glabe, 2006; Dahlgren et al., 2002) . The different size distribution between $A\beta_{42}$ and $A\beta_{40}$ highlights the importance of the two extra C-terminal amino acids in the formation of Aβ assemblies. **For all experiments with cultured neurons treated with $oA\beta_{42}$ presented in this thesis, $oA\beta_{42}$ was prepared according to LaDu's protocol (Dahlgren et al., 2002).**

There is a growing body of evidence suggesting that soluble Aβ aggregates, rather than Aβ fibrils and plaques are the toxic entities in AD. Aβ oligomers are present in the CSF (Lue et al., 1999; Pitschke et al., 1998), are significantly increased in brains of AD patients (Gong et al., 2003; Kuo et al., 1996), and their content in human brain is better correlated with the severity of the disease than are the classical amyloid plaques containing insoluble Aβ deposits (Gouras et al., 2000; Lue et al., 1999; McLean et al., 1999; Naslund et al., 2000). Moreover, transgenic mice Tg2576 produce Aβ oligomers as well as amyloid, but the onset of behavioral abnormalities occurs around the time of oligomeric Aβ accumulation (Kawarabayashi et al., 2001). In addition, newly developed APP transgenic mice expressing the E693Δ mutation, which causes AD by enhanced Aβ oligomerization

without fibrillization displayed impaired hippocampal synaptic plasticity and memory (Tomiyama et al., 2010). Interestingly, these mice showed abnormal tau phosphorylation, microglia and astrocyte activation and neuronal loss suggesting that Aβ oligomers cause not only synaptic alteration but also other features of AD pathology. In summary, it is likely that Aβ toxic effects observed in humans and animal models result from the activity of more than one Aβ assembly. In agreement, studies in J20 mice demonstrated the presence of different Aβ assemblies before, coincident with, and after the onset of detectable synapto-dendritic compromise (Shankar et al., 2009).

The first studies that proposed that soluble assemblies of Aβ rather than Aβ fibrils were responsible for the neuropathological features of AD were performed with a mixture of $A\beta_{42}$ and small quantities of clusterin (apoJ) (Oda et al., 1995). In many other studies including work from our laboratory, it was shown that Aβ oligomers are more toxic than fibrilar Aβ aggregates (Dahlgren et al., 2002; Song et al., 2006; Walsh et al., 2002). Aβ oligomers localize preferentially to axons and axon termini in Tg2576 mice (Kokubo et al., 2005b) and humans with AD (Kokubo et al., 2005a) and to dendrites (Lacor et al., 2004). This particular localization of Aβ oligomers has been interpreted as an indication that Aβ oligomers are responsible for the synaptic dysfunction in AD brain. Moreover, work from our laboratory demonstrated that Aβ oligomers are preferentially internalized by axons leading to axonal degeneration and neuronal apoptosis (Saavedra et al., 2007; Song et al., 2006). Interestingly, it was reported that amyloid plaques are surrounded by a halo of oligomeric Aβ that is responsible for the synaptic loss which supports the idea that oligomers are the true toxic moieties and could explain the synaptic loss that occurs around plaques (Koffie et al., 2009).

1.3.3.3. Intraneuronal Aβ

1.3.3.3.1. Accumulation of Aβ inside brain neurons in AD

Evidence from several immunohistochemical studies suggested the accumulation of Aβ inside neurons in AD. Yet, the acceptance of this concept was hampered by the fact that many of the earlier studies were performed using antibodies that could not distinguish between Aβ and APP. This problem and other experimental issues that precluded the acknowledgment of the intracellular Aβ presence have been analyzed in detail in several reviews (Bayer and Wirths, 2011; Gouras et al., 2010; LaFerla et al., 2007; Wirths and Bayer, 2012). Despite these initial technical complications, more recent work using antibodies specific for $A\beta_{40}$ and $A\beta_{42}$ have confirmed the presence of intraneuronal Aβ and suggested a pathophysiological role for this Aβ pool (Bayer and Wirths, 2011; D'Andrea et al., 2001; Gouras et al., 2000; Youmans et al., 2012). In the past few years several excellent reviews have discussed the evidence available on accumulation of intracellular Aβ in brains of AD patients and animal models of AD, and its impacts on pathogenesis of AD, synaptic impairment and neuronal loss (Bayer and Wirths, 2011; Christensen et al., 2010; Gouras et al., 2012; LaFerla et al., 2007; Wirths and Bayer, 2012).

Intraneuronal accumulation of Aβ is one of the earliest pathological events in humans and in animal models of AD. Intraneuronal $A\beta_{42}$ immunoreactivity precedes both neurofibrillary tangles and amyloid plaque deposition (D'Andrea et al., 2001; Gouras et al., 2000). In the triple transgenic mouse model, Long Term Potentiation (LTP) abnormalities and cognitive dysfunctions correlate with the appearance of intraneuronal Aβ, prior to the occurrence of plaques or tangles

(Billings et al., 2005; Oddo et al., 2003). Moreover, when Aβ is removed by immunotherapy, the intracellular pool of Aβ reappears before tau pathology (Oddo et al., 2004). In a novel gene transfer animal model created using lentiviral $A\beta_{42}$ that results in intracellular but not extracellular Aβ accumulation, intraneuronal $A\beta_{42}$ led to neuronal loss and tau phosphorylation (Rebeck et al., 2010). Importantly, Aβ accumulation within neurons precedes neurodegeneration in nearly all the animal models in which intracellular Aβ and neuronal loss have been reported, and all models in which intracellular accumulation of Aβ was examined showed synaptic dysfunction (Eimer and Vassar, 2013; Jawhar et al., 2012; Takahashi et al., 2013; Wirths and Bayer, 2010; Youmans et al., 2012). Studies in cultured cells also showed accumulation of intracellular Aβ (Nagele et al., 2002; Saavedra et al., 2007; Song et al., 2011; Yu et al., 2010).

Accumulation of Aβ within neurons has been causally linked to neuronal death (see below). There is also mounting evidence that intracellular Aβ accumulation is associated with neuritic and synaptic pathology (Meyer-Luehmann et al., 2008; Takahashi et al., 2004; Takahashi et al., 2002), and with alterations of synaptic proteins (Almeida et al., 2005; Takahashi et al., 2013). Besides, the internalization of Aβ antibodies reduced intraneuronal Aβ and protected synapses (Tampellini et al., 2007) as well as reversed cognitive impairment (Bayer and Wirths, 2011; Billings et al., 2005).

With respect to the specific form of Aβ that accumulates intracellularly, the use of C-terminal specific antibodies against $A\beta_{40}$ and $A\beta_{42}$ in immunocytochemical studies of human brains with AD pathology, indicated that it is $A\beta_{42}$ the peptide present within neurons (D'Andrea et al., 2001; Gouras et al., 2000; Ohyagi et al.,

2007; Tabira et al., 2002). Furthermore, using laser capture microdissection of pyramidal neurons in AD brains, Aoki and collaborators showed increased $A\beta_{42}$ levels and elevated $A\beta_{42}/A\beta_{40}$ ratio in neurons from sporadic as well as from familial cases of AD, whereas $A\beta_{40}$ levels remained unchanged (Aoki et al., 2008).

An interesting development of the “intracellular Aβ” cascade model is the possibility that Aβ plaques would originate from death and destruction of neurons that contained elevated amounts of Aβ (Bahr et al., 1998; D'Andrea et al., 2001; Glabe, 2001). In AD brains, intraneuronal accumulation of Aβ occurs early in the pathology, Aβ starts to aggregate inside the neurons, diffuse plaques are located directly outside of the neurons and intraneuronal Aβ level is negatively associated with the plaque deposition (Gouras et al., 2000). This suggested that the release of Aβ from intracellular stores by dying cells to be responsible for the reduction or loss of intraneuronal $A\beta_{42}$ immunoreactivity in areas of plaque formation (Gouras et al., 2000). Recently, a model was presented in which internalized Aβ starts fibrillization in the multivesicular bodies (MVBs) upon spontaneous nucleation or in the presence of fibril seeds, thus penetrating the vesicular membrane causing cell death and releasing amyloid structures into the extracellular space (Friedrich et al., 2010).

The contribution of intracellular Aβ to formation of neurofibrillary tangles has also been proposed. The intracellular pool of Aβ associates with tangles (Murphy et al., 1994); and intracellular Aβ may disrupt the cytoskeleton and initiate the formation of aggregated intracellular Tau protein (Gouras et al., 2000). Contrary to the concept that intracellular Aβ is linked to neurofibrillary tangles, one report found that intracellular Aβ is not a predictor of extracellular Aβ deposition or

neurofibrillary degeneration, although in this study mostly an N-truncated form of Aβ was examined (Wegiel et al., 2007).

1.3.3.3.2. Origin of intracellular Aβ

The intraneuronal pool of Aβ has a double origin: slow production from APP inside the neurons and uptake from the extracellular space. These two mechanisms are quite distinct and are regulated differently. Hence, understanding which pathway is more relevant to AD pathogenesis may help in the identification of potential targets to treat the disease. There is extensive evidence supporting the production of $A\beta_{42}$ from APP "*in situ*" inside the neurons (Martin et al., 1995; Nathalie and Jean-Noel, 2008; Pierrot et al., 2004; Turner et al., 1996; Xia et al., 1998). This mechanism of intracellular Aβ accumulation has been reviewed recently (Bayer and Wirths, 2010; LaFerla et al., 2007).

Several studies favour a mechanism that involves uptake of Aβ from the extracellular pool (D'Andrea et al., 2001; LaFerla et al., 1997; Nagele et al., 2002; Ohyagi et al., 2007). This mechanism of internalization occurs selectively in neurons at risk in AD as demonstrated using organotypic hippocampal slice cultures in which $A\beta_{42}$ gradually accumulates and is retained intact by field CA1, but not by other subdivisions (Bahr et al., 1998; Bi et al., 2002). Moreover, under conditions in which the blood brain barrier (BBB) is compromised, Aβ enters the brain from the periphery and in that case it accumulates in neurons but not in glia (Clifford et al., 2007). Recent work also favours a mechanism of Aβ uptake from the extracellular pool as the main origin of intracellular Aβ based on the fact that the appearance of intracellular Aβ was always accompanied by an increase of extracellular Aβ, while in subjects without increase of extracellular Aβ there was

no detection of intracellular Aβ (Aho et al., 2010).

Aβ uptake from the extracellular space and Aβ generation from APP inside neurons have been linked in what can be considered an autocatalytic vicious cycle or loop. According to this concept intracellular accumulation of $A\beta_{42}$ causes pronounced up-regulation of $A\beta_{42}$ newly generated within neurons. Glabe's group has shown that internalization of exogenous $A\beta_{42}$ by HEK-293 cells over expressing APP resulted in accumulation of amyloidogenic β-secretase carboxy-terminal fragments of APP (Yang et al., 1995). The effect was specific since the amount of non-amyloidogenic α-secretase carboxy-terminal fragments was only slightly affected. The accumulation of the amyloidogenic fragments did not result from an increase in APP synthesis but instead it was due to specific enhancement of peptides stability, possibly by interaction of the fragments with stable Aβ aggregates causing evasion of the normal degradation pathway leading to enhanced Aβ production and deposition, supporting the hypothesis that amyloid accumulation is a process mechanistically related to prion replication (Glabe, 2001; Yang et al., 1999). Exogenous $A\beta_{42}$ might initiate the cycle in the MVBs or lysosomes, where $A\beta_{42}$ accumulates (Bahr et al., 1998; Mohamed and Posse de Chaves, 2011; Yang et al., 1995). The increased production of amyloidogenic APP fragments by $A\beta_{42}$ was also documented in the field CA1 of hippocampal slices (Bahr et al., 1998) and the accumulation of intracellular Aβ upon $A\beta_{42}$ uptake was demonstrated in dendrites of primary neurons (Tampellini et al., 2009). Importantly, the Aβ-induced synaptic alterations demonstrated in this last study required amyloidogenic processing of APP. Indeed, the decrease in synaptic proteins caused by extracellular Aβ (Almeida et al., 2005; Snyder et al., 2005) is reversed when Aβ is provided together with a γ-secretase inhibitor, or given to APP-knock

out neurons (Tampellini et al., 2009).

1.3.3.3.3. Consequences of intraneuronal accumulation of Aβ

The cellular uptake and degradation of Aβ have been originally considered as mechanisms that reduce the concentration of Aβ in interstitial fluids. However, $Aβ_{42}$ is degraded poorly and its accumulation inside neurons has dramatic consequences. Intraneuronal Aβ accumulates within the endosomal/lysosomal system, in vesicles sometimes identified as lysosomes (Burdick et al., 1997; Hu et al., 2009; Zerbinatti et al., 2006) and some others as late endosomes/ MVBs (Almeida et al., 2006; Langui et al., 2004; Takahashi et al., 2002). $Aβ_{42}$ internalized from the extracellular milieu is quite resistant to degradation possibly due to formation of protease resistant aggregates; but shorter Aβ peptides are degraded and do not accumulate after endocytosis (Ditaranto et al., 2001; Knauer et al., 1992; Yang et al., 1995). In one study $Aβ_{42}$ was shown to be cleared rapidly after delivery to lysosomes, although it previously concentrated and aggregated within the cells, possibly serving as a seed for further Aβ aggregation (Hu et al., 2009).

Aβ accumulation in lysosomes may cause loss of lysosomal membrane impermeability and leakage of lysosomal content (proteases and cathepsins) causing apoptosis and necrosis (D'Andrea et al., 2001; Ditaranto et al., 2001; Liu et al., 2010b; Nixon, 2005; Song et al., 2011; Umeda et al., 2011). The release of lysosomal contents into the cytoplasmic compartments has been considered one of earliest events in intracellular Aβ-mediated neurotoxicity *in vitro* (Ditaranto et al., 2001); and inhibition of lysosomal acidification protect against Aβ toxicity in cultured cells (Kane et al., 1999). Electron microscopy studies using immunogold suggested that the disruption of MVBs could release enough $Aβ_{42}$ to induce

neurotoxicity (Takahashi et al., 2002). An increase in cathepsin D levels secondary to Aβ internalization has been reported in hippocampal slices (Bi et al., 2002; Hoffman et al., 1998) and cultured cortical neurons (Song et al., 2011). Elevation of cathepsin D levels is a characteristic of AD brains (Callahan et al., 1999; Cataldo et al., 1995; Ginsberg et al., 2000; Yamashima, 2013).

Endosomal dysfunction occurs early in AD, before amyloid deposition. In AD, early endosomes have increased size and number which is associated with abnormally increased endocytic activity leading to endosomal dysfunction (reviewed in (Cataldo et al., 2000; Nixon, 2005)). Endosomal dysfunction might not necessarily involve lysosomal leakage in all cases but could involve defects in intracellular trafficking. MVBs are considered late endosomes, which form by fusion of early endosomes with signaling endosomes and serve as vehicle for the transport of receptors and signaling molecules (Weible and Hendry, 2004). MVBs are important vesicles in retrograde transport, and accumulation of Aβ within MVBs would impair their degradative and trafficking functions. MVBs contain inner vesicles with lower pH in the lumen. Aβ interacts with, and partitions into negatively charged membranes (Gibson Wood et al., 2003) and there is evidence that $A\beta_{42}$ is localized to the outer membrane of the MVBs in brains of patients with AD (Takahashi et al., 2002), and is inserted in the membrane of lysosomes in cultured cells that internalized Aβ (Liu et al., 2010b). The MVBs represent a good location for Aβ aggregation because MVBs are rich in membranes and have low pH (Takahashi et al., 2002). In addition, Aβ accumulation in MVBs membranes will likely disrupt intracellular trafficking as mentioned above.

In neurons, axonal retrograde transport is essential for neuronal life since

it secures the delivery of growth factors and/or their survival signals to the soma. This requires the normal function of the endosomal system in axons (Deinhardt et al., 2006; Delcroix et al., 2004) and will likely be affected by Aβ accumulation in axonal MVBs. We demonstrated that axons are entry points of Aβ and apoE (de Chaves et al., 1997; Saavedra et al., 2007) suggesting that accumulation of Aβ in axonal MVBs could impair retrograde transport. The impairment of retrograde transport has been proposed to play an important role in degeneration of basal forebrain cholinergic neurons in AD (Salehi et al., 2003; Salehi et al., 2004). Recent work has shown impairment of brain derived neurotropic factor (BDNF)-mediated TrkB retrograde transport in Tg2576 axons and in cultured neurons treated with Aβ (Poon et al., 2011).

Protein sorting into MVBs is a highly regulated event. One of the mechanisms of MVB sorting is the ubiquitin proteasome system (UPS) (Katzmann et al., 2002). Aβ inhibits the proteasome (Gregori et al., 1995; Oh et al., 2005; Tseng et al., 2008). Part of Aβ internalized by neurons appears in the cytosol, where it could get in contact with the proteasome (Oh et al., 2005). LaFerla's group demonstrated an age-dependent proteasome inhibition in the triple transgenic mice model of AD (Tseng et al., 2008). This inhibition was responsible for tau phosphorylation and was reversed by Aβ immunotherapy. Inhibition of the ubiquitin proteasome system (UPS) system was responsible for impairment of the MVB sorting pathway in cultured Tg2576 neurons (Almeida et al., 2006).

1.3.3.3.4. Intraneuronal Aβ toxicity

The role of Aβ in neuronal death and dysfunction has been investigated extensively. The attention has focused mainly on how extracellular Aβ causes

neuronal death. On the other hand, whether the intracellular accumulation of Aβ is a cause of neuronal death has been a matter of debate. Some groups consider that intracellular accumulation of Aβ is not responsible for neuronal loss. For instance, the appearance of Aβ immunoreactivity in neurons in infants and during late childhood, adulthood, and normal aging, suggests that this is part of the normal neuronal metabolism (Wegiel et al., 2007). Moreover, Aβ did not produce clear signs of cell death upon infusion in hippocampal slices (Bahr et al., 1998) although in combination with transforming growth factor-β (TGF-β) it induced neuronal degeneration in field CA1 (Harris-White et al., 1998). On the other hand, evidence that supports the importance of intracellular Aβ in cell death includes the observations that different mice models of AD show dramatic intraneuronal Aβ accumulation and neuronal cell death that correlates with intraneuronal Aβ accumulation and precedes Aβ deposition (Bayer and Wirths, 2011; Casas et al., 2004; Chui et al., 2001; Cuello and Canneva, 2008; Gouras et al., 2010; LaFerla et al., 2007; Tampellini and Gouras, 2010; Wirths and Bayer, 2012). Moreover, the abnormalities and cognitive dysfunctions in several models of AD correlate with the appearance of intraneuronal Aβ, before the appearance of plaques or tangles (Billings et al., 2005; Oddo et al., 2003); markers of apoptosis are present in the subset of neurons that accumulate Aβ in brains of DS and AD patients (Busciglio et al., 2002; Chui et al., 2001) and microinjections of $A\beta_{42}$ or a cDNA-expressing cytosolic $A\beta_{42}$ rapidly induces cell death of primary human neurons (Zhang et al., 2002). Generation of transgenic mice harboring constructs that target Aβ intracellularly has developed neurodegeneration (LaFerla et al., 1995). Furthermore, quadruple-mutant mouse (mouse overexpresses APP with K670N/M671L (Swedish mutation), V717I (London mutation) and carrying the

M233T and L235P mutations into the endogenous PS1) and quintuple-mutant mouse (mouse overexpresses APP with K670N/M671L (Swedish mutation), I716V (Florida mutation), and V717I (London mutation) and PS1 with M146L and L286V mutations) have shown neuronal loss in association with intracellular accumulation of Aβ (Casas et al., 2004; Eimer and Vassar, 2013; Jawhar et al., 2012). In addition, treatment of cultured neurons or neuron-like cells with $A\beta_{42}$ causes Aβ internalization and death (Ditaranto et al., 2001; Nagele et al., 2002; Picone et al., 2009; Saavedra et al., 2007; Song et al., 2011; Song et al., 2008; Song et al., 2006; Yu et al., 2010). Inhibition of Aβ endocytosis in N2A cells (Yu et al., 2010), primary cortical neurons (Song et al., 2011) and sympathetic neurons (Saavedra and Posse de Chaves, unpublished observations) resulted in significantly reduced intracellular Aβ accumulation and reduced Aβ toxicity. Besides, the selective toxicity of Aβ oligomers versus Aβ fibrils has been explained by the preferential uptake of oligomeric Aβ by receptor-mediated endocytosis (Chafekar et al., 2008).

Several mechanisms have been proposed to explain the toxicity of intracellular Aβ (reviewed recently in (Gotz et al., 2011; Penke et al., 2012; Sakono and Zako, 2010)). We will briefly mention the major mechanisms of intracellular Aβ-induced neuronal loss identified so far; namely lysosomal leakage, mitochondrial dysfunction and endoplasmic reticulum (ER) stress (Umeda et al., 2011). Aβ accumulating in the lysosomes increases lysosomal permeability leading to leakage of lysosomal enzymes in the cytoplasm, which induces cell death (D'Andrea et al., 2001; Ditaranto et al., 2001; Liu et al., 2010b; Nixon, 2005; Song et al., 2011; Umeda et al., 2011). Moreover, some studies indicated that Aβ accumulates in mitochondria in brain of transgenic AD models (Gillardon et al., 2007; Umeda et al., 2011). Aβ accumulation in mitochondria reduces mitochondrial

membrane potential in cortical neurons (Eckert et al., 2008). Aβ directly interacts and potently inhibits mitochondrial cytochrome c oxidase (Crouch et al., 2005; Hernandez-Zimbron et al., 2012) leading to inhibition of respiratory chain, reduction of ATP production, increased production of reactive oxygen species. Besides, Aβ causes cytochrome c release and consequently apoptotic cell death (Aleardi et al., 2005; Umeda et al., 2011). In addition, Aβ induces p53-dependent neuronal apoptosis via direct activation of p53 promoter (Ohyagi et al., 2005). Furthermore, Aβ induces ER stress (Ferreiro et al., 2006; Kong and Ba, 2012; Umeda et al., 2011) and ER collapse (Lai et al., 2009) that are involved in Aβ-induced cell death. Since the ER plays a vital role in protein folding, ER stress results in accumulation of unfolded proteins that activates unfolded protein response. The unfolded protein response primarily works to compensate for damage, but it can cause cell death if ER stress is severe or prolonged (Xu et al., 2005). Moreover, Aβ-induced ER stress results in ER-calcium release, which activates glycogen synthase kinase 3β (GSK3β), which phosphorylates tau (Resende et al., 2008). Primary hippocampal neurons prepared form tau-knockout mouse are resistant to Aβ toxic effect and APP-transgenic mice lacking tau gene have higher learning and memory performance than APP transgenic mouse indicating that hyperphosphorylation of tau plays an essential role in Aβ toxicity (Rapoport et al., 2002; Roberson et al., 2007). Furthermore, Aβ inhibits proteosomal degradation leading to Aβ and tau accumulation (Tseng et al., 2008) and cell death (Agholme et al., 2012). In this thesis, we present a novel mechanism of neuronal loss induced by intracellular Aβ. This new mechanism involves the dysregulation of the mevalonate pathway.

1.4. The mevalonate Pathway

The Nobel Prize for physiology or medicine for 1964 was awarded to Konrad Bloch at Chicago University and Feodor Lynen at Munich University for their discovery of the cholesterol synthetic pathway/the mevalonate pathway (Zetterstrom, 2009). Mammalian cells can synthesize cholesterol through the mevalonate pathway starting from acetyl coenzyme A (acetyl-CoA) by a series of reactions that are catalyzed by more than 20 enzymes and require energy and molecular oxygen (Gaylor, 2002). Hydroxy methyl glutaryl CoA reductase (HMGR), the rate limiting enzyme (Reinhart et al., 1987; Rodwell et al., 1976). Some of the enzymes involved in the mevalonate pathway exist in the ER; including HMGR (Gaylor, 2002; Goldstein and Brown, 1990), others exist at the peroxisomes and some are cytosolic enzymes (Kovacs et al., 2002; Wiemer et al., 2011). After lanosterol production, cholesterol biosynthesis follows two alternative routes: 1) Bolch pathway in which lanosterol is converted into desmosterol that is reduced to cholesterol by the enzyme desmosterol reductase/ 3-β-hydroxysterol delta-24-reductase (DHCR24)/ SELective Alzheimer's Disease INdicator-1 (seladin-1). 2) Kandutsch-Russell pathway in which lanosterol is converted into 7-dehydrocholesterol, which is reduced to cholesterol by the action of 7-dehydrocholesterol reductase (Goldstein and Brown, 1990) (Figure 1.3). The majority of newly synthesized cholesterol moves from the ER to the plasma membrane (Baumann et al., 2005). Some of this newly synthesized cholesterol is taken up into the endocytic compartments (Chang et al., 2006; Cruz and Chang, 2000). In addition to cholesterol, the mevalonate pathway produces several other important lipid molecules called isoprenoids (discussed later).

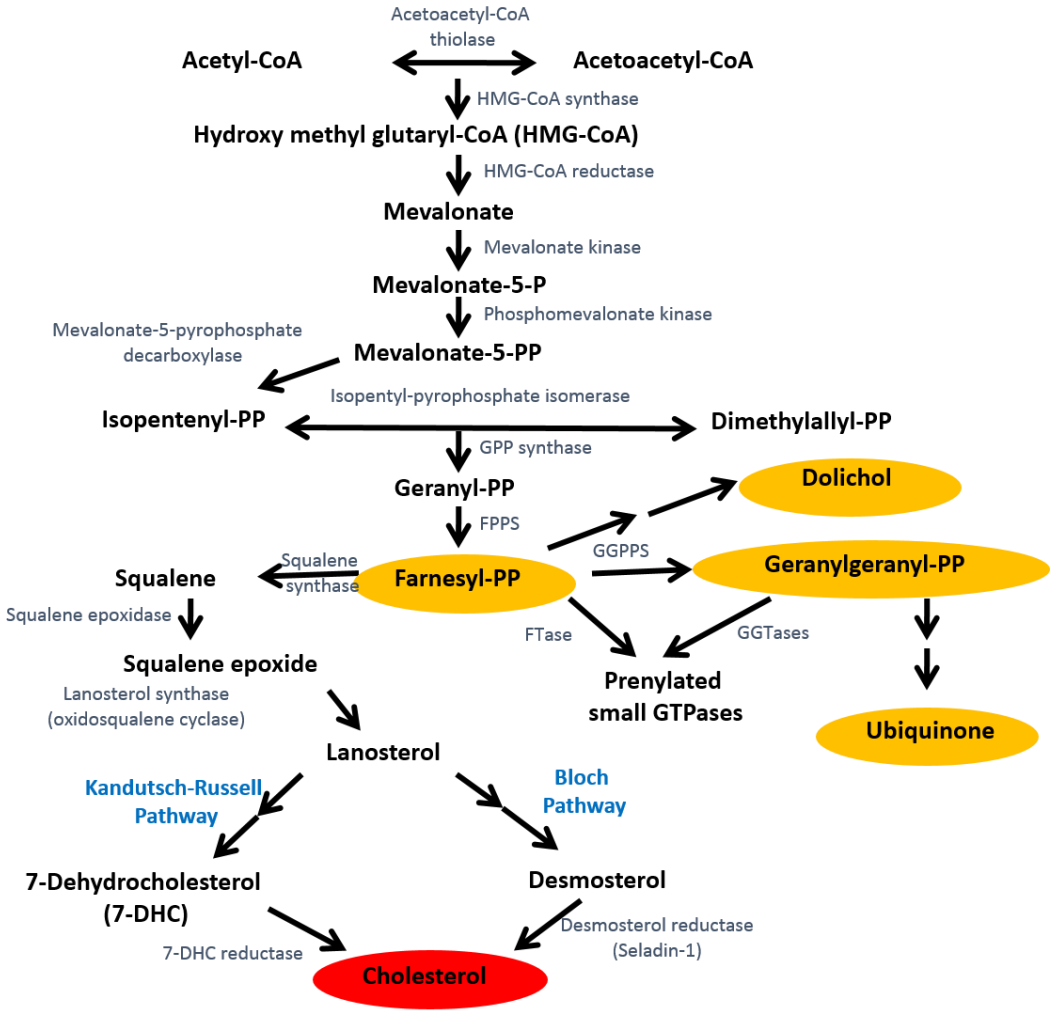

Figure 1.3 Schematic presentation of the cholesterol synthetic pathway (the mevalonate pathway). The mevalonate pathway is a crucial metabolic pathway, which converts mevalonate into cholesterol. Acetyl coA is the initial precursor in cholesterol synthesis. HMGR is the rate limiting enzyme in the mevalonate pathway. The post-lanosterol steps of cholesterol biosynthesis have been divided into Bloch and Kandutsch–Russell pathways. In addition to cholesterol, the mevalonate pathway provides the cell with isoprenoids; such as farnesyl pyrophosphate (FPP), geranylgeranylpyrophosphate (GGPP), dolichol, and ubiquinone. Major enzymes are shown in blue. FPPS is farnesylpyrophosphate synthase; GGPPS is geranylgeranylpyrophosphate synthase; FTase is farnesyltransferase; GGTases are geranylgeranyltransferases.

1.5. Cholesterol

Cholesterol, a 27 carbon four ring structure, is a very important lipid in the cellular membranes. It controls the membrane physical properties, ionic homeostasis, endocytosis and signaling mechanisms (Ikonen, 2006; Maxfield and van Meer, 2010). Moreover, cellular cholesterol serves as a precursor for steroid hormones and vitamin D synthesis (Ikonen, 2006). In the nervous system, cholesterol plays essential roles in synapse development (Goritz et al., 2002; Goritz et al., 2005; Mauch et al., 2001), synaptic vesicle formation (Thiele et al., 2000), dendrite differentiation (Goritz et al., 2005), LTP (Koudinov and Koudinova, 2002), neurotransmitter release (Zamir and Charlton, 2006) and axonal elongation (de Chaves et al., 1997; Posse De Chaves et al., 2000).

The plasma membrane and the endocytic compartments are the organelles richest in cholesterol in the cell (Mesmin and Maxfield, 2009). 50-90% of cellular cholesterol resides in the plasma membrane (Lange et al., 1989). Cholesterol is highly enriched in lipid rafts. Lipid rafts, ordered dynamic subcompartments of the membrane, are composed of closely packed sphingolipids and cholesterol clusters and are involved in membrane signaling and trafficking (Simons and Ikonen, 1997; Simons and Sampaio, 2011).

1.5.1. Cholesterol in the Brain

The human brain, which represents 2% of the total body mass, contains 25% of the total body cholesterol (Dietschy and Turley, 2004). The majority of central nervous system (CNS) cholesterol exists in its unesterified form in the myelin sheath and the cellular membranes (Dietschy and Turley, 2001).

The peripheral pool of cholesterol is separated from the CNS by the BBB (Dietschy and Turley, 2004). Cholesterol in the brain derives almost exclusively from *de novo* biosynthesis since plasma lipoproteins cannot cross the BBB (Dietschy and Turley, 2001; Dietschy and Turley, 2004; Quan et al., 2003). Cholesterol synthesis *in situ* in the brain is sufficient to meet the demands during development and in adult life, this local synthesis decreases with age (Dietschy, 2009; Dietschy and Turley, 2004; Thelen et al., 2006; Yanagisawa, 2002). The importance of endogenous cholesterol synthesis for normal brain function is highlighted by the fact that genetic defects in enzymes involved in cholesterol synthesis cause severe neurological abnormalities (Bjorkhem et al., 2010; Waterham, 2006).

In situ hybridization demonstrated that the distribution of the transcript levels of many enzymes involved in cholesterol synthesis retain neuron specific distribution pattern in pyramidal and granular layers of mouse hippocampus (Valdez et al., 2010). However, neurons have been shown to have a lower rate of cholesterol synthesis than astrocytes (Nieweg et al., 2009). Therefore, neurons outsource the majority of their cholesterol need to form and maintain their axons, dendrites and synapse (Hayashi et al., 2004; Mauch et al., 2001; Pfrieger and Ungerer, 2011; Ullian et al., 2004) from astrocyte-derived lipoproteins. The major astrocyte-secreted lipoprotein is apoE containing lipoproteins (Boyles et al., 1985), whose lipidation is mediated via adenosine triphosphate-binding cassette (ABC) transporters mainly ABCA1 (Wahrle et al., 2004). Neuronal uptake of astrocyte-derived lipoproteins is mediated by low density lipoprotein receptor (LDLR) family mainly LDLR related protein (LRP) (Holtzman et al., 1995; Ishiguro et al., 1995; Pfrieger and Ungerer, 2011). Lipoprotein-derived cholesterol delivered to

endosomes is transported out of the endosomal-lysosomal system to be distributed to other organelles by the action of Niemann Pick type C protein-1 (NPC1) and Niemann Pick type C protein-2 (NPC2). Mutation of either NPC1 protein (in 95% cases) or NPC2 protein (in 5% cases) genes leads to a neurodegenerative disease called Niemann Pick type C disease (NPC). This illustrates the importance of lipoprotein-derived cholesterol for normal neuronal functions (Carstea et al., 1997; Ikonen, 2008; Karten et al., 2009; Pfrieger and Ungerer, 2011) (Figure 1.4).

1.5.2. Cellular cholesterol homeostasis

Cells have developed a very sophisticated mechanism to sense and control cholesterol levels. Despite most of the cellular cholesterol is located at the plasma membrane; the cellular machinery that senses cellular cholesterol levels is present in the ER. Normally the ER contains a small pool of cholesterol that represents around 1% of the total cellular cholesterol, which makes the ER very sensitive to any change in cellular cholesterol (Lange, 1991). The ER regulates cellular cholesterol homeostasis by regulating cholesterol synthesis, uptake, esterification and hydroxylation (Figure1.5). ER regulates cholesterol synthesis and uptake via sterol regulatory element binding protein-2 (SREBP-2) discussed below.

Cholesterol esterification is catalyzed by the enzyme Acyl coA-Cholesterol Acyltransferase (ACAT), which exists in two forms ACAT1 and ACAT2 (Buhman et al., 2000). ACAT1 is the major functional enzyme in mouse brain (Bryleva et al., 2010). Cholesterol esters represent only 1% of the total cholesterol content in brains of human (Johnson and Shah, 1978) and mice (Bryleva et al., 2010).

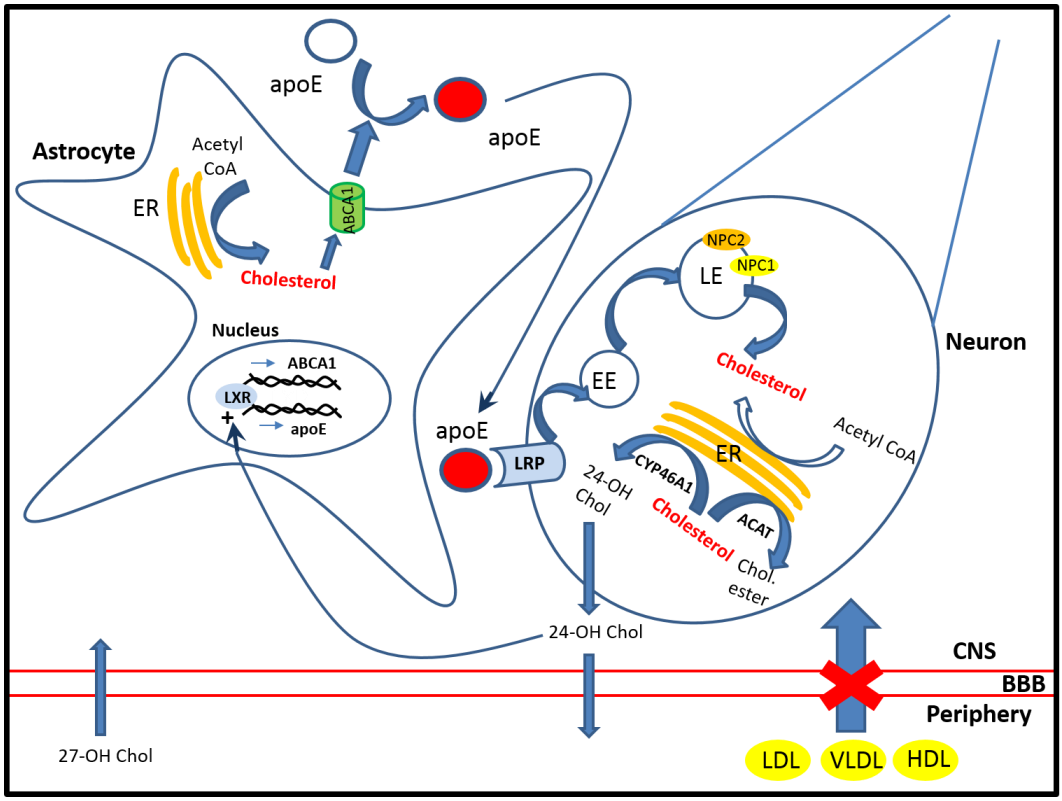

Figure 1.4 Cholesterol in the brain. In the brain, cholesterol mainly derives from *de novo* cholesterol synthesis as peripheral lipoproteins (HDL, LDL, VLDL) are not able to cross the BBB. The majority of cholesterol in the brain is synthesized by astrocytes. In astrocytes, cholesterol is transported to apoE molecule by the action of ABC transporters, especially ABCA1. Lipidated apoE is taken up by neurons via members of LDLR family, mainly LRP. Internalized lipidated apoE is delivered to early endosome (EE) and then to late endosome (LE). In the LE, lipoproteins are hydrolyzed and free cholesterol is transported out of the LE by the action of NPC1 and NPC2. In addition to using exogenously supplied cholesterol, neurons can synthesize cholesterol but at a lower rate than astrocytes. Excess cholesterol is converted into cholesterol esters by the action of ACAT at the ER. Moreover, cholesterol can be converted into 24S-hydroxycholesterol (24-OH Chol) by the action of CYP24A1, specially expressed by neurons. 24-OH Chol can freely cross the BBB to be excreted in the bile. Peripherally produced 27-hydroxy cholesterol (27-OH Chol) can cross the BBB into the brain. 24-OH Chol regulates apoE lipoprotein production in astrocytes via LXR mediated mechanism.

The level of cholesterol esters and the number of ACAT transcripts increase with age in rat brain (Mulas et al., 2005). Immunohistochemical staining demonstrated the presence of ACAT1 at the ER in neurons but not in astrocytes (Sakashita et al., 2000), however, astrocytes have been shown to esterify cholesterol only if they lack apoE and they were challenged with high amount of exogenous cholesterol (Karten et al., 2006).

Cholesterol hydroxylation is catalyzed by cholesterol hydroxylases, which convert cholesterol into its hydroxylated forms (oxysterols) (Ikonen, 2008). In the CNS, cholesterol is converted to 24(S)-hydroxycholesterol by cholesterol-24-hydroxylase (CYP46A1) selectively expressed in the brain (Diczfalusy et al., 1996; Lund et al., 1999). Immunohistochemical staining revealed that CYP46A1 is highly expressed in pyramidal neurons of hippocampus, cortex and in Purkinje cells in cerebellum but not in astrocytes (Ramirez et al., 2008). CYP46A1 is localized to the ER (Ramirez et al., 2008). Although oxysterols are more hydrophilic than cholesterol, oxysterols have higher membrane permeability than cholesterol (Ikonen, 2008). 24(S)-hydroxycholesterol crosses the BBB freely and is cleared by the liver (Lund et al., 2003; Martin et al., 2010). Brains of CYP46A1 knockout mice have 40% reduction in the rate of cholesterol synthesis compared to wild type suggesting that the conversion of cholesterol into 24(S)-hydroxycholesterol represents the major mechanism of cholesterol metabolism in the brain (Lund et al., 2003). Outside of the brain, cholesterol is hydroxylated mainly into 27-hydroxycholesterol, which is detected in the brain because of its daily influx into the brain from the periphery (Bjorkhem et al., 2009; Heverin et al., 2005).

Oxysterols are active lipids that play a role in regulating cellular cholesterol through the enhancement of HMGR degradation (DeBose-Boyd, 2008). Moreover, oxysterols are natural ligands to Liver X Receptors (LXRs) (Janowski et al., 1996). LXRs are nuclear receptors that upon activation enhance expression of apoE and ABC transporters, which are involved in cholesterol efflux and lipoprotein lipidation (Eckert et al., 2007; Martin et al., 2010; Pfrieger and Ungerer, 2011; Whitney et al., 2002). Interestingly, 24(S)-hydroxycholesterol stimulates LXR-mediated expression of apoE and different ABC transporters and consequently apoE-mediated cholesterol efflux in astrocytes but not in neurons (Abildayeva et al., 2006). This suggests that neuron-derived 24(S)-hydroxycholesterol could penetrate into astrocytes to regulate lipoproteins production via LXR-mediated mechanism (Figure 1.4).

1.5.2.1. Sterol Regulatory Element Binding Protein-2 (SREBP-2)

SREBP-2 belongs to a family of membrane-bound transcription factors that regulate cholesterol and fatty acid homeostasis. Studies in knockout and transgenic mice demonstrated that cholesterol synthesis is preferentially regulated by SREBP-2 (Brown and Goldstein, 1997; Horton, 2002; Horton et al., 2003). SREBP-2 is synthesized and inserted in the ER as an inactive precursor (P)SREBP-2 (Brown and Goldstein, 1997). (P)SREBP-2 has two transmembrane helices with the N- and C- terminals projecting into the cytosol (Brown and Goldstein, 1997). The C-terminus of (P)SREBP-2 interacts with C-terminal of SREBP cleavage-activating protein (SCAP), a sterol-regulated escort protein (Brown and Goldstein, 1997; Rawson, 2003). SCAP has eight transmembrane helices, of which transmembrane helices 2-6 are defined as a sterol sensing domain (Brown and Goldstein, 1997; Rawson, 2003). (P)SREBP-2-SCAP complex

has to be transported into coat protein complex II (COPII) vesicles that bud from the ER and travel to the Golgi complex (Horton et al., 2003). At the Golgi, sequential proteolytic cleavage of (P)SREBP-2 by Site-1-protease (S1P) (Espenshade et al., 1999) and Site-2-protease (S2P) (Zelenski et al., 1999) releases the N-terminal /mature/nuclear SREBP-2 ((M)SREBP-2) that enters the nucleus to regulate gene transcription (Brown and Goldstein, 1997; Horton, 2002; McPherson and Gauthier, 2004). In the nucleus, (M)SREBP-2 binds to sterol regulatory elements (SREs) in the promoter of target genes in order to regulate gene expression (Brown and Goldstein, 1997) (Figure 1.5).

The level of cholesterol at the ER controls the proteolytic processing of SREBP-2 and therefore its transcriptional activity. When ER cholesterol falls below 5% of total ER lipids (molar basis), SCAP-(P)SREBP-2 complex is transported to the Golgi, where (M)SREBP-2 is released (Radhakrishnan et al., 2008). (M)SREBP-2 increases the expression of LDLR involved in exogenous cholesterol uptake and the expression of many enzymes involved in the mevalonate pathway (including HMGR gene) (Horton et al., 2003) in order to compensate for the reduction in cellular cholesterol level.

On the other hand, when cholesterol accumulates at the ER, cholesterol binds to SCAP inducing a conformational change in SCAP that promotes it's binding to ER integral membrane proteins called Insulin-Induced gene-1 or 2 (Insig-1 and Insig- 2)(Brown et al., 2002; Radhakrishnan et al., 2004). Once bound to Insig, SCAP is unable to bind to COPII and the complex SCAP-(P)SREBP-2 is not transported to the Golgi (McPherson and Gauthier, 2004; Radhakrishnan et al., 2008; Sun et al., 2007; Yabe et al., 2002; Yang et al., 2002). Oxysterols induce

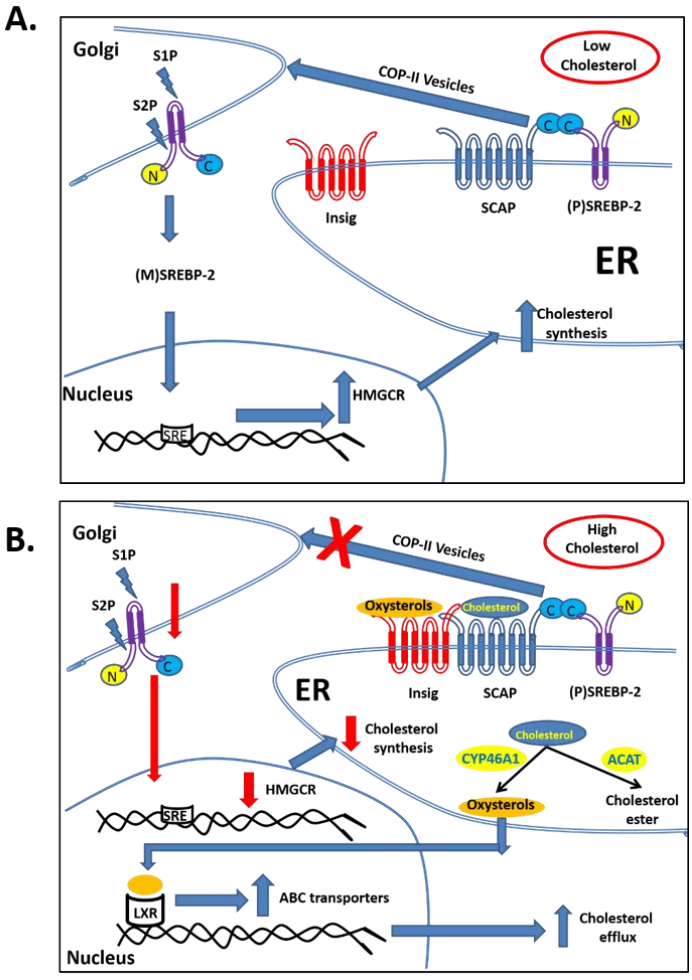

Figure 1.5 Cellular cholesterol Homeostasis. A) Under low cholesterol, cholesterol synthesis is transcriptionally upregulated by SREBP-2. (P)SREBP-2 exists at ER membrane associates with SCAP. SCAP-(P)SREBP-2 complex is transported to the Golgi apparatus by COP-II vesicles. In the Golgi, (P)SREBP-2 is processed sequentially by two proteases, S1P and S2P releasing (M)SREBP-2, which enters the nucleus where it binds to the SRE sequence in the target genes leading to increased expression of these genes. One of the major target genes of SREBP-2 is HMGR, the rate limiting enzyme of the cholesterol synthesis. **B)** Under high cellular cholesterol level, (P)SREBP-2-SCAP complex is retained at the ER by binding of SCAP to Insig. The interaction between SCAP and Insig are stimulated by cholesterol binding to SCAP or oxysterols binding to Insig. Inhibition of (P)SREBP-2-SCAP complex transport to the Golgi reduces the level of (M)SREBP-2 leading to decreased transcriptional level of HMGR and consequently reduced cholesterol synthesis. Excess cholesterol can be converted at the ER into cholesterol esters by the action of ACAT. Moreover, cholesterol can be converted into oxysterols by cholesterol hydroxylases, the most abundant one in CNS is CYP46A1. Oxysterols activate LXR leading to upregulation of the expression of ABC transporters, which mediate cholesterol efflux. Red arrows represent reduction or reduced level.

the same retention of SCAP-(P)SREBP-2 complex at the ER via oxysterols direct binding with Insig proteins (Radhakrishnan et al., 2007). Considering that Golgi processing is essential for SREBP-2 activation, the accumulation of cholesterol at ER results in the reduction of transcription of genes required for cholesterol biosynthesis and uptake, thereby lowering membrane cholesterol and restoring cholesterol balance (Goldstein et al., 2006; Sun et al., 2007).

1.5.3. Cholesterol in AD pathology

The role of cholesterol in neurodegeneration is still controversial, however in the case of AD, extensive evidence indicates that cholesterol homeostasis is perturbed, possibly in more than one way (reviewed in (Di Paolo and Kim, 2011; Liu et al., 2010a; Martins et al., 2009; Orth and Bellosta, 2012).

Alois Alzheimer's described an increased "adipose inclusions" or "lipoid granules" in AD brain indicating impaired lipid metabolism (Di Paolo and Kim, 2011). There is no agreement on whether total brain cholesterol is increased or decreased in brains of AD patients (Cossec et al., 2010a; Martin et al., 2010; Wood et al., 2002). One study showed no difference between cholesterol level in AD brain and control (Eckert et al., 2009), and a different study reported increase of cholesterol mass in non-affected area and reduced in affected ones in AD brain (Kagedal et al., 2010). Some studies demonstrated increased level of cholesterol in AD brains compared to age-matched non-demented controls (Cutler et al., 2004; Xiong et al., 2008). The accumulation of cholesterol is local and occurs in the areas with high levels of Aβ deposition and neurofibrillary tangle including frontal cortex (Sparks, 1997) but not the unaffected cerebellum (Cutler et al., 2004). Amyloid plaques but not diffuse plaques have cholesterol (Burns et al., 2003b; Mori et al.,

2001), which could explain the increased cholesterol level observed in those areas. Increased cholesterol levels have also been shown in the middle frontal gyrus of AD brain (Bandaru et al., 2009). Recent study in brains of AD patients demonstrated an overload of cholesterol in cerebral cortex (Lazar et al., 2013).

In addition, there is evidence that membrane cholesterol increases with the progress of the disease (Cutler et al., 2004). Moreover, cholesterol accumulation has been observed in Aβ immunopositive neurons in brains of AD patients (Gomez-Ramos and Asuncion Moran, 2007; Ohm et al., 2003; Xiong et al., 2008). Similarly, brain cholesterol accumulation was detected in APP/PS1 mouse model of AD (Fernandez et al., 2009). Cholesterol accumulation in intracellular compartment (herein called cholesterol sequestration) in the brain is the main feature of NPC, which shares many similarities with AD (Vance et al., 2005). The similarities between AD and NPC include accumulation of Aβ, formation of neurofibrillary tangles, endosomal abnormalities and the influence of apoE genotype (Nixon, 2004; Saito et al., 2002). Furthermore, impaired intracellular cholesterol trafficking in NPC has been shown to enhance amyloidogenic processing of APP and consequently increase Aβ production (discussed in detail bellow) (Burns et al., 2003a; Jin et al., 2004; Kosicek et al., 2010; Mattsson et al., 2011; Runz et al., 2002).

The existence of a link between AD and cholesterol was first and foremost highlighted by the discovery that the allele ε4 of apoE gene is the most important risk factor for the development of non-familial/sporadic AD (Corder et al., 1993; Poirier et al., 1993; Strittmatter et al., 1993a). Afterwards, mounting genetic,

epidemiological and biochemical evidence revealed other aspects of the involvement of cholesterol in AD pathology.

1.5.3.1. Genetic evidence of cholesterol involvement in AD pathology

ApoE, a 34 KDa protein, is the major apolipoprotein in CNS and it plays a major role in cholesterol homeostasis in CNS as a cholesterol transport protein (as discussed above). In human, three alleles of apoE are found, apoE2, apoE3 and apoE4 (Mahley, 1988). The inheritance of ε4 allele of apoE gene increases the risk of developing late familial (Corder et al., 1993; Strittmatter et al., 1993a) and sporadic AD (Poirier et al., 1993) as well as certain early-onset forms of the disease (Chartier-Harlin et al., 1994). The number of ε4 alleles inherited determine the expected age of AD onset to be less than 70 years for people with two ε4 allele, 80 years for people with one ε4 allele and 90 years for people with no ε4 allele (Corder et al., 1993).

The role of apoE4 in AD pathogenesis has been studied extensively and several articles have reviewed this topic comprehensively (Huang, 2010; Leduc et al., 2010; Liu et al., 2013; Mahley and Huang, 2012; Verghese et al., 2011). Here I just mention some significant aspects.

In brains of both AD patients and AD transgenic mouse model expressing mutant APP and different isoforms of human apoE, the level of Aβ deposition was lower in apoE2 than apoE3 and apoE3 was lower than apoE4 (Roses, 1996; Schmechel et al., 1993). In a cohort of AD patients, apoE4 was associated with reduced level of soluble Aβ in CSF and increased levels of insoluble Aβ in the brain (Prince et al., 2004). These studies suggested that apoE4 could enhance Aβ production and aggregation. In support of this hypothesis, level of cholesterol in

the exofacial leaflet of the membrane was double in apoE4 knock-in mice compared to apoE3 knock-in mice (Hayashi et al., 2002). This increased membrane cholesterol would increase Aβ production and aggregation (Chauhan, 2003; Kakio et al., 2001; Yanagisawa et al., 1995) (discussed below). Furthermore, apoE may play a role in Aβ internalization, since clathrin-mediated endocytosis of Aβ involves a receptor that belongs to LDLR family which binds to apoE (reviewed in (Mohamed and Posse de Chaves, 2011). In support to the role of apoE in Aβ uptake, some studies correlated the intracellular accumulation of Aβ with apoE uptake (Gouras et al., 2000; LaFerla et al., 1997). Brains of APP transgenic mouse model of AD lacking apoE have reduced intraneuronal Aβ (Zerbinatti et al., 2006), while the presence of apoE4 allelle was associated with increased intraneuronal Aβ level (Levi et al., 2007). This could be explained in part by the fact that apoE4 in its lipid free form has a larger space than apoE3 for Aβ binding (Wisniewski et al., 1994). Moreover, apoE has been shown to play an important role in Aβ removal and degradation (DeMattos et al., 2004; Fan et al., 2009; Jiang et al., 2008; Koistinaho et al., 2004). ApoE4 is the least effective in enhancing Aβ clearance across the BBB (Deane et al., 2008). Collectively, increased Aβ production, internalization and reduced degradation in the presence of apoE4 allele could explain the higher ability of apoE4 compared to apoE3 to enhance Aβ neurotoxicity in cell culture (Manelli et al., 2007). Another proposed mechanism for the increased risk of AD in apoE4 carriers is based on the poor ability of apoE4 to transport cholesterol compared to apoE3 and apoE2 (Rapp et al., 2006). The reduced efficiency of apoE4 to transport cholesterol could explain the reduced LTP observed in apoE4 transgenic mice compared to apoE3 mice (Trommer et al., 2004).

In addition to apoE, several other genes encoding proteins involved in cholesterol homeostasis such as CYP46A1, ACAT, the cholesterol efflux transporter ABCA1 and the lipoprotein receptor-related protein (LRP) have been linked to the risk, development or progression of AD (Di Paolo and Kim, 2011; Schreurs, 2010; Shobab et al., 2005).The mechanisms through which these proteins could affect AD pathogenesis are still not well understood.

1.5.3.2. Epidemiological evidence of cholesterol involvement in AD pathology

Epidemiological studies examining the association between increased plasma cholesterol level and the risk of AD have controversial results (reviewed in (Shepardson et al., 2011a)). Some studies demonstrated a direct correlation between high plasma cholesterol level and the risk of developing AD and the rate of cognitive decline (Helzner et al., 2009; Kivipelto et al., 2002; Kivipelto et al., 2001; Notkola et al., 1998; Solomon et al., 2007; Whitmer et al., 2005; Zambon et al., 2010). However, others showed no correlation or even a negative correlation (Mielke et al., 2005; Reitz et al., 2004; Reitz et al., 2008). In order to understand the correlation between the serum cholesterol level and the risk of AD, several studies have examined the effect of increased peripheral cholesterol level on Aβ production in different animal models (reviewed (Maulik et al., 2013)). Increased peripheral cholesterol in different transgenic AD mouse models and in rabbits fed high cholesterol diet has been shown to increase Aβ production and accumulation (Ghribi et al., 2006; Hooijmans et al., 2007; Hooijmans et al., 2009; Jaya Prasanthi et al., 2008; Levin-Allerhand et al., 2002; Li et al., 2003; Refolo et al., 2000; Shie et al., 2002; Sparks et al., 1994; Thirumangalakudi et al., 2008; Umeda et al., 2012).

Although, peripheral cholesterol cannot cross the healthy BBB, the peripheral pool of cholesterol could regulate Aβ production in the brain via 27-hydroxycholesterol (Leoni and Caccia, 2011). 27-hydroxycholesterol is an oxysterol produced predominantly in peripheral organs and tissues that closely correlates with the peripheral cholesterol level (Babiker et al., 2005). 27-hydroxycholesterol accumulates in AD brains (Heverin et al., 2004; Shafaati et al., 2011). Interestingly, 27-hydroxycholesterol enhances Aβ production in human neuroblastoma cells (Prasanthi et al., 2009) and in rabbit neurons (Ghribi, 2008).

Alternatively, elevated levels of circulating cholesterol could lead to vascular inflammation and compromise the integrity of the BBB resulting in the passage of plasma lipoproteins into the brain as shown in rabbits fed with a high cholesterol diet (Sparks et al., 2000).

Statins are inhibitors of cholesterol synthesis that have been used widely for treatment of cardiovascular disease. Retrospective studies suggested that statins reduce the risk of developing AD, which is in agreement with the notion that an increase in brain cholesterol is detrimental for AD (Jick et al., 2000; Wolozin et al., 2000; Wolozin et al., 2007). Other prospective clinical studies however, have failed to prove the beneficial effect of statins in AD (Arvanitakis et al., 2008; Feldman et al., 2010). All clinical studies aimed at evaluating the effectiveness of the use of statins to protect against or to slow down the progress of AD have been reviewed recently (Shepardson et al., 2011b). The controversy between the results of these studies has been correlated to:1) the difference in lipophilicity of the chosen statins and consequently its ability to cross the BBB, 2) the dose and duration of statin treatment, 3) the time point of statin treatment in relation to the

onset and severity of AD, 4) the cholesterol-independent pleiotropic effects of statins including anti-inflammatory effect, 5) the lack of proper classification of dementia in some studies, and 6) the absence or inaccurate monitoring of compliance to statin treatment. Therefore, better designed studies are needed to accurately examine the effectiveness of statins in AD (Shepardson et al., 2011b).

1.5.3.3. Biochemical evidence of cholesterol involvement in AD pathology

A large amount of studies *in vivo* and *in vitro* have tried to identify the role of cholesterol in AD pathogenesis. As discussed below, cholesterol has been shown to regulate Aβ production, aggregation, internalization, degradation and toxicity.

1.5.3.3.1. Regulation of Aβ production by cholesterol

Cholesterol regulates APP processing and Aβ production through several mechanisms (Burns and Rebeck, 2010; Rushworth and Hooper, 2010; Vetrivel and Thinakaran, 2010). Plasma membrane cholesterol controls APP endocytosis, which is required for Aβ production (Cirrito et al., 2008; Koo and Squazzo, 1994) since the proteolytic processing of APP takes place predominantly in post-Golgi secretory and in endocytic compartments, although a portion of Aβ may be produced at the plasma membrane (Vetrivel and Thinakaran, 2010). High cholesterol level at the plasma membrane enhances APP endocytosis and consequently increases Aβ production by increasing the level of APP in the endosomes where amyloidogenic APP processing is performed by β- and γ-secretases (Cossec et al., 2010b; Marquer et al., 2011). On the other hand, cholesterol depletion by methyl-β cyclodextrin (MβCD), the most efficient cholesterol acceptor (Leventis and Silvius, 2001; Zidovetzki and Levitan, 2007),

reduces APP endocytosis and increases the level of APP at the plasma membrane, where APP is cleaved predominantly by α-secretase (Kojro et al., 2001).

Membrane cholesterol also controls the localization of APP to lipid rafts, possibly by direct interaction with APP (Beel et al., 2010). Under normal conditions, the majority of APP is present in non-raft regions of membranes (Rushworth and Hooper, 2010), while β-secretase (BACE1) is located predominately in the raft portions (Ehehalt et al., 2003; Riddell et al., 2001). The amyloidogenic processing of APP occurs in the lipid rafts of post Golgi network or endosomes, and the non-amyloidogenic processing takes place in the non-raft segments of the plasma membrane (Rushworth and Hooper, 2010; Vetrivel and Thinakaran, 2010) (Figure 1.2). Therefore, enhanced APP amyloidogenic processing and Aβ production observed in NPC cell models and in cells treated with the pharmacological agent U18666A that causes intracellular cholesterol sequestration and mimics the NPC phenotype (Burns et al., 2003a; Runz et al., 2002) could be explained by the ability of sequestered cholesterol in NPC models to shift APP into lipid rafts, which enhances the interaction between APP and β-secretase and consequently increases Aβ production (Kosicek et al., 2010).

Mild reduction of membrane cholesterol by combined treatment with statins and MβCD enhances Aβ production (Abad-Rodriguez et al., 2004). This effect was explained by the disorganization of the lipid raft structure under mild depletion of membrane cholesterol which could facilitate the interaction between β-secretase and APP (Kaether and Haass, 2004). Similarly, a stronger interaction between APP and β-secretase and consequently increased Aβ production upon

disorganization of lipid rafts is induced by genetic deletion seladin-1 (Crameri et al., 2006). Seladin-1 is an enzyme that converts desmosterol into cholesterol and is down regulated in brains of AD patients (Greeve et al., 2000; Iivonen et al., 2002). Other studies however, showed that strong reduction of membrane cholesterol inhibits β-secretase activity by reducing its association with the lipid rafts leading to reduced Aβ production (Ehehalt et al., 2003; Fassbender et al., 2001; Simons et al., 1998). Moreover, cholesterol depletion enhances α-secretase activity and non-amyloidogenic processing of APP by reducing the membrane fluidity (Kojro et al., 2001). Collectively, the strength of membrane cholesterol depeltion would decide the effect on APP processing and Aβ production depending on the effect on lipid raft structure, and trafficking in and out the raft.

Furthermore, membrane cholesterol regulates APP processing by regulating the proteolytic activity of the cleavage enzymes directly. Cholesterol increases β-secretase (BACE1) activity in cell-free assays (Kalvodova et al., 2005). Moreover, LXR activation *in vivo* and *in vitro* causes ABCA1-mediated reduction of membrane cholesterol level that reduces the expression and activity of β-secretase and consequently reduces Aβ production (Cui et al., 2011). High membrane cholesterol level inhibits α-secretase activity by direct binding to APP at the α-secretase cleavage site (Yao and Papadopoulos, 2002). The effect of membrane cholesterol on γ-secretase activity is controversial; some studies demonstrated that high membrane cholesterol increases γ-secretase activity (Osenkowski et al., 2008; Wahrle et al., 2002), while others did not find any effect of membrane cholesterol on γ-secretase activity (Wada et al., 2003).

1.5.3.3.2. Regulation of cellular uptake of Aβ by cholesterol

Several mechanisms that mediate Aβ internalization by neurons and glia have been identified (reviewed in detail in (Mohamed and Posse de Chaves, 2011)). Two of these mechanisms are affected by cellular cholesterol levels. Aβ is taken up in a complex with apoE-containing lipoproteins through receptors of the LDLR family, by clathrin-mediated endocytosis. Lipidation of apoE influences its isoform-specific interaction with Aβ (Tokuda et al., 2000); therefore brain cholesterol levels would affect apoE-dependent Aβ internalization. In addition, work from our laboratory demonstrated that the mechanism of Aβ internalization in the absence of apoE is mediated via lipid rafts, independently of caveolae and is reduced significantly by inhibiting cholesterol and sphingolipids synthesis (Saavedra et al., 2007). A reduction of cholesterol and sphingolipids disorganizes lipid rafts structure, which may cause relocation of a putative receptor involved in Aβ internalization. Furthermore, membrane cholesterol regulates Aβ binding to the cell surface, which may affect Aβ internalization. While some studies demonstrated that MβCD-induced membrane cholesterol depletion reduces Aβ binding to cell surface (Wakabayashi and Matsuzaki, 2007; Wakabayashi et al., 2005), others demonstrated an inverse correlation between membrane cholesterol level and Aβ binding (Cecchi et al., 2009; Cecchi et al., 2008; Yip et al., 2001). The regulation of Aβ binding to the cell surface by cholesterol would not only affect Aβ internalization, but also its toxicity (discussed below).

1.5.3.3.3. Regulation of Aβ degradation by cholesterol.

Several proteases, including insulin-degrading enzyme (IDE), neprilysin (NEP), endothelin converting enzyme (ECE), plasmin and matrix metalloproteinases (MMP), have been reported to degrade Aβ and consequently

regulate its physiological level in the brain (Eckman and Eckman, 2005; Farris et al., 2003; Iwata et al., 2000; Miller et al., 2003; Miners et al., 2008). The level and activity of many of these enzymes have been shown to be altered in AD (Miners et al., 2008).

Some studies demonstrated that cellular cholesterol level regulate the expression and the activity of Aβ degrading enzymes. High cholesterol reduces expression level and enzymatic activity of NEP, IDE and MPP-9 (Jaya Prasanthi et al., 2008; Kim et al., 2007; Wang et al., 2012). On the other hand, reduced cellular cholesterol level secondary to MβCD treatment or to genetic deficiency of Seladin-1 results in mis-localization of IDE and plasmin out of the lipid rafts/detergent resistant membranes (DRMs) that reduces their enzymatic activity (Bulloj et al., 2008; Crameri et al., 2006).

Recently, cellular cholesterol level has been shown to regulate Aβ degradation via regulating intracellular trafficking of Aβ to the lysosomes for degradation without affecting the activity of Aβ degrading enzymes (Lee et al., 2012). In this study, apoE-induced cholesterol efflux and consequent reduction of cellular cholesterol levels speed up Aβ trafficking to lysosomes for degradation, on the other hand, intracellular cholesterol sequestration upon U18666A treatment slows down Aβ intracellular trafficking and degradation (Lee et al., 2012).

1.5.3.3.4. Regulation of Aβ toxicity by cholesterol.

Studies examining the regulation of Aβ toxicity by cholesterol have controversial results. Some studies indicated that high membrane cholesterol content protects against Aβ toxicity. An increase in cellular membrane cholesterol reduced Aβ toxicity in PC12, neuroblastoma SHSY5Y cells as well as primary

cortical neurons, while depleting membrane cholesterol increased the susceptibility of these cells to the toxic effect of Aβ (Arispe and Doh, 2002; Cecchi et al., 2009; Cecchi et al., 2008; Sponne et al., 2004; Yip et al., 2001; Zhou and Richardson, 1996). The protective effect of high membrane cholesterol against Aβ toxicity might be due to the ability of cholesterol to modulate the membrane physicochemical properties, therefore, an increase in cholesterol would reduce Aβ interaction with and its insertion into the membrane. Arispe and Doh proposed a model in which Aβ interaction and insertion into the plasma membrane creates open channels that permit calcium flow inside the cell causing toxicity (Arispe and Doh, 2002). This model, which has been supported by others (Peri et al., 2011; Peri and Serio, 2008), foresees that high membrane cholesterol would reduce Aβ-induced membrane channel opening, reduce calcium influx and inhibit cell death. Further evidence for this model showed that the rise in intracellular calcium in response to Aβ challenge diminishes when cell cholesterol increases but becomes more pronounced upon cholesterol depletion, reducing and enhancing Aβ cytotoxicity, respectively (Cecchi et al., 2009; Cecchi et al., 2008). The increase in cellular membrane cholesterol has been shown to mediate the neuroprotective action of seladin-1 against Aβ toxicity (Cecchi et al., 2008; Peri et al., 2011).

Contrary to the evidence presented above, several studies found a positive correlation between cellular cholesterol level and Aβ toxicity. First, cortical neurons isolated from SREBP-2 overexpressing mice and NPC1 deficient transgenic mice exhibit mitochondrial cholesterol accumulation, which increases the susceptibility of these cortical neurons to the toxic effect of Aβ (Fernandez et al., 2009). Second, mature hippocampal neurons contain more cholesterol than young hippocampal neurons and are more sensitive than young neurons to Aβ toxic effects (Nicholson

and Ferreira, 2009). The difference is due to cholesterol content since depletion of membrane cholesterol by MβCD decreases the susceptibility of mature neurons to Aβ induced cell death. Third, increased membrane cholesterol potentiates Aβ-induced rise in cytosolic calcium leading to death of neurons and astrocytes (Abramov et al., 2011). This suggests that Aβ incorporation/insertion into membranes is facilitated by cholesterol, perhaps due to the ability of cholesterol to directly interact with Aβ (Avdulov et al., 1997; Chochina et al., 2001; Igbavboa et al., 2003). Fourth, enhanced Aβ toxicity by increased cellular cholesterol level was associated with increased production of reactive oxygen species and elevated lipoperoxidation (Ferrera et al., 2008). In support to the detrimental effect of high cholesterol in Aβ neurotoxicity, membrane cholesterol level is increased in vulnerable brain regions of AD patients (Cutler et al., 2004). Moreover, the two main risk factors of AD, aging and apoE4, are associated with increased cholesterol level in the exofacial surface of the plasma membrane (Hayashi et al., 2002; Igbavboa et al., 1996).

1.5.3.4. Regulation of cholesterol homeostasis by Aβ

Contrary to the large body of information on the regulation of Aβ production, internalization, degradation and toxicity by cholesterol, very little is known about the regulation of cholesterol homeostasis by Aβ. There are some indications that Aβ alters intracellular vesicle trafficking and impairs cholesterol homeostasis. Studies in brains of AD patients demonstrated that cholesterol accumulates in Aβ immunopositive neurons (Gomez-Ramos and Asuncion Moran, 2007; Ohm et al., 2003; Xiong et al., 2008). Similarly, in the APP/PS1 transgenic mouse model of AD cholesterol accumulated in granules, either associated with individual amyloid deposits or spread over entire regions undergoing amyloidogenesis (Fernandez et

al., 2009), and in the brain of the Tg2576 mouse, cholesterol localized to hippocampal regions undergoing amyloid-beta deposition (Sole-Domenech et al., 2013). Furthermore, only affected areas of AD brains have increased NPC1 gene and protein expression, which might reflect the existence of a compensatory mechanism to an impairment of cholesterol trafficking (Kagedal et al., 2010). Aβ was identified as a main player in impairing cholesterol trafficking and homeostasis in AD based on an earlier study that demonstrated the ability of $A\beta_{40}$ to increase free cholesterol in hippocampal neurons and to impair vesicle trafficking (Liu et al., 1998). Moreover, cholesterol accumulation observed in APP/PS1 transgenic mice was preceded by Aβ accumulation (Fernandez et al., 2009). All together these data indicate that intracellular Aβ could impair cholesterol trafficking leading to cholesterol accumulation.

De novo cholesterol synthesis is the main supply of more than 95% of cholesterol demands in the brain since plasma lipoproteins cannot cross normal BBB (Dietschy and Turley, 2001; Dietschy and Turley, 2004; Quan et al., 2003). Little is known about the status of cholesterol synthesis in AD brain. One study showed that the transcript level of HMGR was not different between AD and age-matched control (Yasojima et al., 2001). However, seladin-1 is highly reduced in AD (Greeve et al., 2000; Iivonen et al., 2002) suggesting an overall inhibition of cholesterol synthesis. Moreover, reduced level of different cholesterol precursors in CSF of AD patients compared to non-demented persons supports the notion that *de novo* cholesterol synthesis could be reduced in AD brain (Kolsch et al., 2010). $A\beta_{40}$ but not $A\beta_{42}$ has been shown to inhibit cholesterol synthesis (Gong et al., 2002; Grimm et al., 2005; Koudinova et al., 1996). Mevalonate, the product of HMGR, restored the normal cholesterol synthesis in $A\beta_{40}$ treated cells pinpointing

the HMGR as the target of $A\beta_{40}$ to inhibit cholesterol synthesis (Grimm et al., 2005). Not all cell types in the brain will be affected equally by the inhibition of cholesterol synthesis. Astrocytes and oligodendrocytes synthesize the large amounts of cholesterol required for brain lipoprotein production and myelin formation respectively. Therefore, inhibition of cholesterol synthesis will significantly impair the functions of these cells (Posse de Chaves and Narayanaswami, 2008; Saher et al., 2005). Mature brain neurons rely mainly on the uptake of astrocyte-derived lipoproteins as a source of cholesterol (Aqul et al., 2011; Funfschilling et al., 2007; Hayashi et al., 2004; Mauch et al., 2001; Pfrieger and Ungerer, 2011; Ullian et al., 2004). Therefore, neurons might be less sensitive to inhibition of cholesterol synthesis than glia, yet normal cholesterol synthesis in neurons is required for normal neuronal functions. Down regulation of 7-dehydrocholesterol reductase (Dhcr7), the last enzyme in the cholesterol synthesis pathway, reduces the expression of several genes involved in lipid biosynthesis in Neuro2a cell. The presence of exogenous source of cholesterol could not protect against this change in gene expression (Korade et al., 2009). Furthermore, inhibition of cholesterol synthesis in hippocampal neurons by lovastatin reduces synapse density and impairs release of the synaptic vesicles. Exogenous supply of cholesterol successfully restores the normal level of cellular cholesterol but fails to restore the normal synaptic vesicle release (Mailman et al., 2011).

1.6. Isoprenoids:

1.6.1. Isoprenoids and protein prenylation

In addition to cholesterol, the mevalonate pathway also produces other important lipids called isoprenoids (Figure-1.3.). Isoprenoids include short chain

isoprenoids, namely farnesyl pyrophosphate (FPP) and geranyl geranylpyrophosphate (GGPP), and long chain isoprenoids such as dolicol and ubiquinone. FPP, a 15 carbon lipid, is at the branching point in the mevalonate pathway which then continues to either cholesterol synthesis or GGPP, dolicol and ubiquinone synthesis (Holstein and Hohl, 2004). Ubiquinone plays a role in the respiratory chain in the mitochondria (Lenaz and Genova, 2009). Dolichol interacts with phospholipids in the cell membranes increasing the membrane fluidity and acts as an antioxidant (Vigo et al., 1984). In addition, free and phosphorylated dolichol act as sugar carriers in protein glycosylation (Holstein and Hohl, 2004). Both FPP and GGPP are substrates for farnesyl transferase and geranyl geranyl transferases respectively that catalyze post-translational protein prenylation (Casey and Seabra, 1996; Leung et al., 2006).

Protein prenylation is a post-translational lipid modification that involves a covalent thioester formation between cysteine residue in the target protein and farnesyl (farnesylation) or geranylgeranyl (geranylgeranylation) group. Prenylation requires a cysteine residue at the carboxyl terminal of the target protein to exist in a defined consensus motif. Farnesylation or geranylgeranylation of a cysteine residue in a CaaX motif is catalyzed by farnesyl transferase or geranylgeranyl transferase I, respectively. A cysteine residue in a CC or CXC motif is geranylgeranylated by geranyl geranyltransferase II (Casey and Seabra, 1996; Crowell and Huizinga, 2009; Leung et al., 2006; Schafer and Rine, 1992; Zhang and Casey, 1996). Protein prenylation is essential for protein-membrane interaction and sometimes in protein-protein interaction (Crowell, 2000; Crowell and Huizinga, 2009). Proteins that undergo posttranslational prenylation belong to one of four protein families; small GTPases, trimeric G proteins, nuclear lamins

and fungal lipopeptide pheromones (Konstantinopoulos et al., 2007; Schafer and Rine, 1992).

Small GTPases are guanine nucleotide binding proteins. They bind to guanosine triphosphate (GTP) when activated and to guanosine diphosphate (GDP) when inactive. Small GTPases are involved in several signaling cascades regulating various cellular activities including vesicle transport, vesicle trafficking, endocytosis, cell adhesion, cell cycle progression, receptor signaling and cytoskeleton organization (Takai et al., 2001). Small GTPases superfamily is classified into five main families, including; Ras, Rho, Rab, Ran, and Sar1/Arf families (Cole and Vassar, 2006; Takai et al., 2001). Prenylation increases small GTPases lipophilicity allowing their insertion in the cellular membranes, which is required for their proper function (McTaggart, 2006).

1.6.2. Isoprenoids in AD

There is only limited information on the levels of isoprenoids in AD (Hooff et al., 2010). In one study dolichol level was reduced in all brain areas of AD patients, while dolicyl phosphate level was increased (Soderberg et al., 1992). This might indicate the increased level of protein glycosylation in AD as dolichoyl phosphate acts as a sugar carrier. Ubiquinone level was significantly elevated in AD brain (Soderberg et al., 1992), which could be a defense mechanism to protect against neuronal damage by oxidative stress (Yang et al., 2008). However, the exact roles of dolichol and ubiquinone in AD have not been identified yet. FPP and GGPP levels were found to be significantly higher in brain tissues of AD patients compared to control samples providing that only thirteen cases of each group were analysed in this study (Eckert et al., 2009). GGPP may be required for LTP in

hippocampal slices (Kotti et al., 2008; Kotti et al., 2006; Matthies et al., 1997). In addition, GGPP enhances γ-secretase activity leading to increased Aβ production (Zhou et al., 2008).

1.6.3. Small GTPases in AD:

Different small GTPases have been shown to be altered and involved in AD pathology. Some studies demonstrated increased Ras expression in AD brains (Gartner et al., 1999; Gartner et al., 1995). Other studies found decreased Ras level in AD brains (Shimohama et al., 1999). Reduced Ras activation has been linked to reduced expression of genes involved in learning and memory (Fernandez-Medarde et al., 2007). In addition, Ras induced cell cycle re-entry of post-mitotic cortical neurons has explained tau phosphorylation and neurodegeneration observed in AD (McShea et al., 2007).

Total RhoA level is reduced in hippocampus of AD brains (Huesa et al., 2010). Reduced RhoA prenylation has been linked to statin-induced tau hyperphosphorylation (Meske et al., 2003). Furthermore, Rac1, another small GTPase that belongs to Rho family, has been suggested to be involved in N-methyl-D-aspartate (NMDA) receptors mediated synaptic plasticity and memory acquisition (Tejada-Simon et al., 2006). Moreover, Rac1 has been shown to regulate the transcription of the APP gene in neurons (Wang et al., 2009).

Different Rab proteins (Rab-4, 5, 6, 7, 8 and 27) have been shown to be upregulated in AD brains (Elfrink et al., 2012; Ginsberg et al., 2011; Ginsberg et al., 2010b; Scheper et al., 2007). Rab6 upregulation may be involved in the recovery from ER stress insult via modulating unfolded protein response (Elfrink et

al., 2012). Upregulation of other Rab proteins have been linked to endosomal abnormalities observed in AD (Ginsberg et al., 2011; Ginsberg et al., 2010b).

The importance of isoprenoids and protein prenylation for proper neuronal functioning and survival has been demonstrated by the ability of exogenous supply of isoprenoids, mainly GGPP, to prevent statin-induced toxic effects in neurons (Kim et al., 2009; Marz et al., 2007; Schulz et al., 2004; Tanaka et al., 2000). Although one study showed increased level of FPP and GGPP in limited number of AD cases (Eckert et al., 2009) and other studies demonstrated altered expression/protein level of different GTPases in AD brains (Elfrink et al., 2012; Gartner et al., 1999; Gartner et al., 1995; Ginsberg et al., 2010a; Ginsberg et al., 2011; Ginsberg et al., 2010b; Huesa et al., 2010; Scheper et al., 2007; Shimohama et al., 1999), the prenylation status of small GTPases has not been well examined in AD yet.

1.7. Thesis Objectives

In this thesis, we aimed at:

1. Examining the effect of oAβ_{42} on cholesterol homeostasis in primary neurons. We discovered that oAβ_{42} impairs intracellular cholesterol trafficking leading to intracellular cholesterol sequestration at the late endosomes, the Golgi and the plasma membrane. oAβ_{42} increases cholesterol level in lipid rafts. Moreover, oAβ_{42} inhibits anterograde transport of cholesterol from neuronal cell bodies to their distal axons.

2. Analyzing the mechanism underlying oAβ_{42}-induced cholesterol sequestration. We discovered that oAβ_{42} impairs intracellular cholesterol trafficking by inhibiting protein penylation, which plays a role in oAβ_{42}-induced neurotoxicity. The inhibition of protein prenylation results from reduced production of isoprenoids as a) we found that oAβ_{42} inhibits the mevalonate pathway by reducing SREBP-2 cleavage and b) exogenous supply of GGPP could restore the normal level of prenylated Rabs, prevent cholesterol sequestration and reduce neurotoxicity in oAβ_{42} treated neurons.

3. Identifying the mechanism through which oAβ_{42} inhibits SREBP-2 processing. We show for the first time that oAβ_{42} reduces the mature SREBP-2 level, which will lead to impaired protein prenylation (a novel mechanism of oAβ_{42} toxicity). We discovered that oAβ_{42} inhibits SREBP-2 transport from ER to the Golgi via reducing the level of phosphorylated Akt.

1.8. References

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Chapter 2: Materials and methods

2.1. Reagents: $A\beta_{42}$ was purchased from American Peptide Co. (Sunnyvale, CA). The same lot number was used throughout this study. Reverse $A\beta_{42}$ was obtained from Alpha Diagnostic Intl. Inc. Leibovitz L15-CO2 culture medium was from Invitrogen. Mouse nerve growth factor (NGF) (2.5 S) was purchased from Alomone Laboratories Ltd. (Jerusalem, Israel). EZ-Link NHS-S-S-biotin and ImmunoPure immobilized streptavidin were purchased from Pierce. Amplex Red cholesterol assay kit was from Invitrogen-Molecular Probes. Immobilon polyvinylidene difluoride was from Bio-Rad. Enhanced chemiluminescence reagents were from Amersham Biosciences. 3-β-[2-(diethylamino)ethoxy] androst-5-en-17-one (U18666A) was purchased from BIOMOL International. GGPP, filipin, methyl-β-cyclodextrin (MβCD), heptane, diisopropylether, Optiprep, Brefeldin A (cat#B5936) and LY294002 (L9908) were purchased from Sigma-Aldrich. 4-[[N-(Imidazol-4-yl) methyleneamino]-2-(1-naphthyl)benzoyl]leucine (GGTI-2133) and MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal) (cat # 474790) were obtained from Calbiochem. Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) (cat#AB-001) was purchased from Kamiya Biomedical Company. [^{3}H]-acetate was purchased from Moravek Biochemicals and Radiochemicals. [^{3}H]-GGPP was purchased from Perkin Elmer. All other reagents were from Fisher.

2.2. Cell culture: rat basal forebrain and sympathetic neurons were prepared and cultured according to previously published protocols (Saavedra et al., 2007).

2.2.1. Culture of primary rat basal forebrain neurons: Basal forebrain cholinergic neurons were isolated from brains of E17-E18 Sprague-Dawley rats of either sex (Health Science Lab Animal Services, U of Alberta) by enzymatic dissociation with 0.1% w/v of trypsin for 15 minute and plated in Poly-D-lysine

coated plates in Neurobasal medium supplemented with MEM, HEPES, Pen/Strep/ L-Glutamine and B27. All experiments started at day 7–8 in culture.

2.2.2. Culture of primary rat sympathetic neurons: Sympathetic neurons isolated from superior cervical ganglia of newborn Harlan Sprague–Dawley rats of either sex (Health Science Lab Animal Services, U of Alberta) were prepared by enzymatic dissociation with 1% (w/v) of collagenase and 0.1% (w/v) of trypsin followed by mechanical dissociation using a flame polished Pasteur pipette. After filtration of the cell suspension through cell strainer (40 µm Nylon; Falcon), neurons were plated. Standard culture medium was L15 CO2 supplemented with 0.4% methylcellulose. Non-neuronal cells were eliminated with 10–15 µM cytosine arabinoside during the first 5–6 d in culture. Two types of neuronal cultures were used; mass cultures and compartmented cultures. For mass cultures neurons were plated in 24-well dishes at a density of 1–2 ganglia/well in medium supplemented with 2.5% rat serum, 1 mg/ml ascorbic acid and 50 ng/ml NGF. Alternatively, sympathetic neurons were cultured in three-compartment culture dishes as before (Saavedra et al., 2007). Briefly, dissociated neurons were plated in the center compartment at a density of 0.2 ganglia/dish in medium containing 2.5% rat serum, 1 mg/ml ascorbic acid, 10–15 µM cytosine arabinoside and 10 ng/ml NGF. After 5–6 days cytosine arabinoside treatment was discontinued and NGF (50 ng/ml) was confined to the side compartments. All experiments started at day 7–8 in culture.

2.2.3. Culture of primary rat cortical neurons: Rat cortical neurons were prepared from newborn Harlan Sprague–Dawley rats of either sex (Health Science Lab Animal Services, U of Alberta). Brains were dissected and cortices were isolated and enzymatically digested by 1 mg/ml papain for 10 min at 37°C. DNase

was added to the digestion mix in the last 5 min of incubation. After mechanical dissociation using a flame polished Pasteur pipette, the cell suspension was filtered through a cell strainer (40 µm Nylon; Falcon), neurons were plated on poly-D-lysine-coated wells at a density of 1.5×10^5 cells/well in 24 well plate (Brewer and Torricelli, 2007).The culture medium used consisted of Neurobasal A medium (Gibco. Cat# 10888-022) supplemented with B27 (Gibco, # 17504-044), penstrep (Gibco, # 15140) and glutamax (Gibco. #35050-061). Experiments started at day 7 in culture.

2.2.4. Culture of immortalized striatal cells ST14A: Conditionally-immortalized rat embryonic striatal cells ST14A (Cattaneo and Conti, 1998) were maintained in culture at the permissive temperature (33ºC) as previously reported (Rigamonti et al., 2000). Their growth medium was composed of DMEM (Gibco, #12800-017) supplemented with 10% FBS (Gibco, # 12483) and 1% Penstrep (Gibco, # 15140). Cells were plated in 24 well dishes at a density of 0.6 x 10^5 cell/well and cultured for 18hr before starting indicated treatment.

2.3. Oligomers of $A\beta_{42}$ ($oA\beta_{42}$) Preparation: $oA\beta_{42}$ was prepared following a published protocol (Dahlgren et al., 2002). Aβ peptide was initially dissolved to 1mM in hexafluoroisopropanol and separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was dried under a stream of N2, and the peptide film was desiccated at -20 °C. The day previous to the experiment, the peptide was resuspended in DMSO at a concentration of 5 mM. L15-CO2 medium (phenol red-free, antibiotic-free, and serum-free) was added to bring the peptide to a final concentration of 100 µM and the peptide 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) in that they contain monomers and oligomers of Aβ but do not contain fibrils (Figure 2.1).

2.4. Treatment of cells with oAβ_{42}: Sympathetic neurons and ST14A cells were treated with oAβ_{42} in culture medium lacking serum. For cortical and basal forebrain neurons, B27 supplement was replaced by N2 supplement during oAβ_{42} treatment. Cells were harvested as indicated in each experiment.

2.5. Immunofluorescence and Confocal Microscopy: For confocal microscopy studies, neurons were fixed with 4% paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.3% saponin for 15 minutes at room temperature and blocked by incubation with 2% bovine serum albumin (BSA) for 1hour on ice. Primary antibodies were prepared in the same blocking solution. Primary antibodies used include: anti-lamp1 (Santa Cruz, sc-8098) at 1:100, anti-EEA1 (Santa Cruz, sc-33585) at 1:100, anti-COX-IV (Abcam, ab14744) at 1:400, anti-giantin (a generous gift from Dr.Hobman, U of Alberta) at 1:300. Incubation was performed overnight at 4 °C. Secondary antibodies (Alexa 594 or Alexa 488 labelled anti-mouse or anti-rabbit) prepared in blocking buffer (1:1000) were incubated for 2 hours at room temperature. For filipin staining, fixed neurons were incubated with 100µg/ml filipin (Sigma) at room temperature for 2h. Nuclei were stained with Draq5 (Biostatus Limited) at 1:4000 or with Hoechst (Sigma, # 33258) staining at final concentration 1ug/ml. Pictures were taken using a Laser Scanning Confocal Microscope Zeiss LSM 710 equipped with an S-Fluor 40/1.3 oil objective using appropriate filter sets and excitation wavelengths. Within an experiment, all pictures were taken with the same setting for a particular fluorophore. If required for printing purposes brightness and contrast were adjusted using Photoshop software. All images were adjusted with the same parameters. The pictures

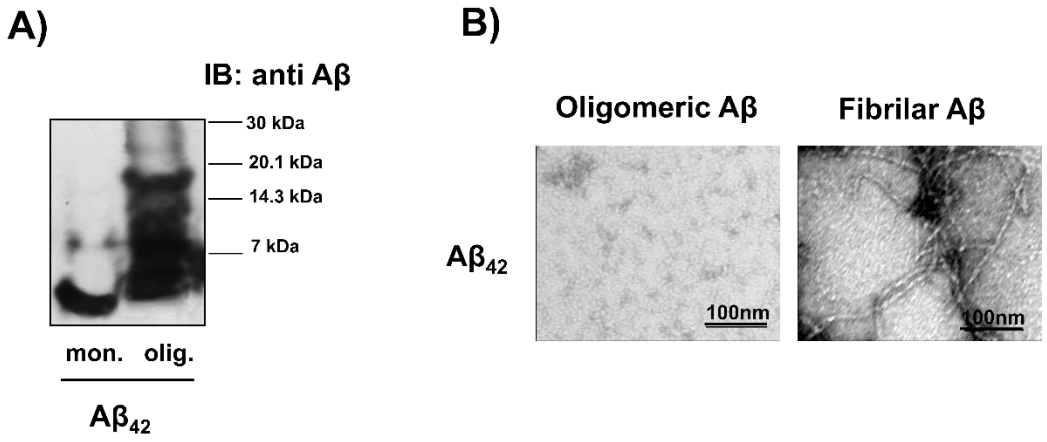

Figure 2.1. Characterization of oAβ_{42} preparation.
Monomers, oligomers and fibrils of Aβ were prepared following the method developed by LaDu and colleagues. Monomers and fibrils were used exclusively for the purposes of characterization of oAβ_{42} preparation. A) Monomeric and oligomeric preparations of Aβ_{42} were analyzed by immunoblot. The lack of oligomers in the monomeric preparations indicates that oligomers are not the result of artifacts during SDS-PAGE separation. B) The absence of fibrils in Aβ oligomeric preparation was confirmed by electron microscopy after negative staining with uranyl acetate. More than 20 fields per sample were examined. Less than 1% of the fields corresponding to oligomeric preparations contained short, isolated fibrils. Conversely every field of the fibrilar preparations at the same peptide concentration contained abundant long fibrils.

selected are representative of at least 3 separate experiments performed under identical conditions.

2.6. Determination of cholesterol mass: Cholesterol mass from cultured neurons was measured using the enzymatic Amplex red cholesterol assay kit and by gas chromatography (Lipid and Lipid Metabolite Analysis Core Facility, U. of Alberta). Results obtained by the two methods were identical.

2.7. Determination of Protein concentration: Protein concentration in the samples were determined using Pierce BCA assay kit (#23227).

2.8. Cholesterol synthesis: Cholesterol synthesis was determined by measuring incorporation of [^{3}H] acetate into [^{3}H] cholesterol using methods described in (de Chaves et al., 1997) and modified as described herein. Neurons received the treatment indicated in each group with the addition of the radioactive cholesterol precursor [^{3}H]acetic acid (100 µCi/ml) for the last 2h. The radioactive medium was removed, neurons were washed twice with cold phosphate buffered saline (PBS), and cellular material was harvested in PBS and sonicated. An aliquot was separated for protein determination. Lipids were isolated from the cell lysate according to the method of Folch (Folch et al., 1959) with chloroform/methanol/water ratio of 2:1:1 (v/v) using 4ml of the solvent mixture per sample. Individual lipid separation was accomplished by thin layer chromatography (TLC). TLC plates were developed in the solvent system heptane: diisopropyl ether: acetic acid (60:40:4, v/v) using unlabeled cholesterol as carrier. The band corresponding to authentic unesterified free cholesterol was scraped from the plate and [^{3}H] cholesterol radioactivity was measured using scintillation counter. Radioactivity was normalized to protein mass and expressed in dpm/ µg protein.

At least 4 cultures per treatment were used. To combine three or more experiments, the results were expressed in comparison to the untreated neurons, which were given a value of 100%.

2.9. Cholesterol trafficking: Neurons cultured in compartmented dishes were treated as indicated and [^{3}H]-acetate (150 µCi/ml) was added to the cell body-containing compartment for the last 24h of treatment. After rinsing, the cellular material of the cell body-containing compartment and the distal axons-containing compartment was harvested separately. Lipid were extracted and separated by TLC as indicated above. Radioactivity of [^{3}H]-cholesterol was expressed as % (total) = dpm in cell bodies or distal axons/ (dpm in cell bodies + dpm in distal axons).

2.10. Estimation of Plasma Membrane Cholesterol: Neurons were incubated with [^{3}H] acetate (150µCi/ml) for 48h prior to the experiment to allow the intracellular pools of cholesterol to achieve equilibrium. After the indicated treatment, neurons were rinsed with PBS and 1.0 mM or 2.5 mM MβCD in L15 medium was added for 5 min to basal forebrain and sympathetic neurons respectively. The MβCD-containing medium was collected. Neurons were rinsed twice and the rinse was pooled with the MβCD medium. Neurons were then harvested separately in PBS and lysed. Lipids from cell lysates and MβCD-containing media were extracted and separated by TLC as above. The results were expressed in percentage of [^{3}H] cholesterol extracted by MβCD = 100 x (dpm in cholesterol from medium)/ (dpm in cholesterol from medium + dpm in cholesterol from cell lysate). The radioactivity of the medium was corrected by subtracting spontaneous cholesterol efflux measured in the absence of MβCD.

2.11. Isolation of lipid rafts/detergent-resistant membranes: The isolation was performed according to (Lingwood and Simons, 2007), with some modifications. All the steps were done on ice in the cold room. The culture medium was removed and the cells were washed two times with cold PBS. Neurons were harvested in TNE buffer (250 mM NaCl, 2 mM EDTA, 50 mM Tris–HCl, pH 7.4) containing protease inhibitor cocktail (= 300 µl/well) . Cells were collected by centrifugation at 1000 xg for 5 min and the buffer was removed. The pellet was lysed in TNE buffer containing protease inhibitor cocktail using 20G needle for 10 strokes and 25G needle for 15 strokes (= 100 µl/well). The lysate was centrifuged at 1000 xg for 10 min and supernatant was transferred to a new tube. Triton X-100 (10%) was added to the supernatant so the final concentration was 1%. The supernatant containing 1% Triton X-100 was incubated on ice for 50 min. The lysate (100 µl) was adjusted to 40% Optiprep with 60% Optiprep (final volume 1.2 ml) and then over layered with 30%-5% discontinuous gradient. Typically the gradient contained 2.4 ml 30% Optiprep and 400ul 5% Optiprep (top). The gradient was centrifuged for 19hr at 100,000 × *g* at 4^0C in an Optima^{TM} MAX-XP Utracentrifuge Beckman Coulter using swing bucket rotor and Beckmann centrifuge tubes # 326819. Thirteen fractions were collected from the top of the gradient separately in labelled tubes. A portion of each fraction was used to measure cholesterol level using Amplex Red kit (invitogen), GM1 level using dot blot and Cholera Toxin-Horseradish Peroxidase conjugate (Molecular probes, Invitrogen). Another portion was used for immunoblot detection of non-raft marker Transferrin receptor (TFR) and raft marker caveolin after trichloroacetic acid (TCA) precipitation.

2.12. Immunoblot Analysis: Proteins were separated by SDS-PAGE using gels at 10% or 12% (only for Ras, Rab7 and Rab9) containing 0.1% SDS. Transfer of

proteins to polyvinylidene difluoride (PVDF) membranes was performed overnight at 4 °C in 25 mM Tris, 192 mM glycine, 16% methanol buffer, pH 8.3. Membranes were blocked for 1 h in Tris-buffered saline, 0.1% Tween 20 (TTBS) containing 5% nonfat milk (blocking buffer) and incubated overnight in the primary antibody solution prepared in TTBS containing 5% nonfat milk except pAkt antibody was diluted in TTBS containing 5% bovine serum albumin. Primary antibodies for beta actin (Cell Signaling, #4967, 1:5000), SREBP-2 (Abcam, #ab-30682,1:500), beta-tubulin (Sigma, # T4026, 1:200), Ras (Upstate, #05-516, 1:500), Flotillin-1 (BD transduction labs, #610821, 1:1000), Rab7 (Sigma, #R8779, 1:1000), Rab9 (Abcam, #ab2810, 1:500), pAkt (cell signaling #4058S, 1:1000), total Akt (New England Biolabs, 1:1000), HA (Sigma #H9658 at 1:5000), Sec24D (Abnova #H00009871-A01, 1:500) and transferrin receptor (Invitrogen-Mouse Anti-Human TFR-cat# 13-6800-1:500) and caveolin (BD transduction- rabbit anti-caveolin-cat#610059-1:3000) were used. Membranes were washed two times with Tris-buffered saline, two times with TTBS, and two times with Tris-buffered saline and then incubated for 1 h with the secondary antibody (1:2000) in blocking buffer at room temperature with gentle agitation. Immunoreactivity was detected by ECL Plus Western Blotting detection system (Amersham Biosciences). UN-SCAN-IT gel 5.3 software was used for semi-quantification of the bands on immunoblots. C_{SREBP} is a control generated by overexpression of (M)SREBP2 in St14A cells using a construct corresponding to the mature form of human SREBP2 kindly provided by Dr. Sipione (Sipione et al, submitted for publication). Cell transfections were performed using Lipofectamine LTX and plus reagent (Invitrogen cat # 15338-100) following the manufacturer's instructions. C_{SREBPP} was used to confirm the band of (M)SREBP-2 in immunoblots.

2.13. Biotinylation and retrograde trafficking of NGF Receptor TrkA: Surface axonal proteins were biotinylated by incubation with 1.5 mg/ml of the membrane impermeable derivative of biotin (sulfo-NHS-S-S-biotin) (Pierce) in Leibovitz L15-CO2 medium (free of NGF, phenol red, antibiotics and serum) for 20 min at 4°C, in the side compartments of neurons cultured in compartmented dishes. Remaining reactive biotin reagent was quenched by incubating cells with 50 mM glycine in modified PBS twice each for 10 min at 4°C. Immediately after, fresh medium containing NGF without or with 20μM oA$\beta_{1\text{-}42}$ was added to the axons and neurons were incubated under regular conditions for 6 and 18 h. NGF induces TrkA activation, endocytosis and retrograde transport. The cellular material from cell bodies-containing compartment and distal axons-containing compartments was collected separately (2 dishes were pooled), and lysed in modified RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholic acid, 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, and 1 mM sodium orthovanadate, pH 7.4, with protease inhibitor mixture) at 4°C for 30 min. The lysate was centrifuged at 16,000 × *g* for 15 min at 4°C, and the resulting supernatants were incubated with 40 μl of 50% StrepAvidin agarose beads (Pierce) overnight at 4°C. The beads were then washed three times with modified RIPA buffer, and the bound proteins were eluted with SDS sample buffer containing dithiothreitol (50 mM final concentration) boiling for 5 min. Biotinylated proteins were separated by SDS-PAGE using 8% gels. Immunoblot analysis of TrkA was performed as before (Song et al., 2006). In all cases the supernatants of incubation with Streptavidin-agarose beads were examined by SDS-PAGE, protein transfer to PDVF membrane and biotinylated protein detection with streptavidin

linked to horseradish peroxidase and ECL to ensure the complete binding of biotinylated proteins to the beads.

2.14. Preparation of Membrane and Cytosol Fractions: Neurons were harvested (3 wells in 24 well plate/treatment) in 10mM HEPES pH7.6 containing 250mM sucrose, 10mM KCl, 1.5 mM MgCl2, 1mM EDTA, 1mM EGTA, 1mM NaF, 2mM NaVO4 and protease inhibitor cocktail. Cellular material was homogenized by passing 20 times through a 25G needle. After resting 20 min on ice the cell homogenate was centrifuged at 1000 × *g* for 10 minutes at 4°C to eliminate unbroken cells and cellular debris. Protein content of the supernatant was determined (BCA, Pierce). Similarly, for experiments in mice brains, frontal cortices from 10 weeks old male wild type and TgCRND8 mice were cut in small pieces and homogenized in the same buffer used for membrane/cytosol separation from cultured neurons by passing through a 22 G needle 15 times. After 20 minutes on ice the homogenate was cleared by centrifugation at 1000 × *g* for 10 minutes at 4°C. Protein content of the supernatant was determined (BCA, Pierce). Equal amounts of protein (= 20-40 µg) from each sample were centrifuged at 105,000 × *g* for 1 hour at 4°C in an Optima^{TM} MAX-XP Utracentrifuge Beckman Coulter using the MLA-130 fixed angle rotor. Supernatants (cytosol fractions) and the pellets (membrane fractions) were separated.

2.15. Protein Prenylation: protein prenylation was examined by extraction of prenylated proteins with Triton-X-114 or recombinant GST-GDI.

2.15.1. Extraction using Triton X-114: Triton X-114 is a non-ionic detergent characterized by forming large micelles in the aqueous solution leading to turbidity, condensation and phase separation at approximately 20ºC (Cloud point) (Bordier,

1981). Therefore, Triton X-114 has been used to solubilize hydrophobic proteins and at its cloud point the solution can be separated into two phases; the detergent-rich phase containing hydrophobic proteins (prenylated Rabs) and the aqueous phase where hydrophilic proteins (unprenylated Rabs) will be (Bordier, 1981; Coxon et al., 2005). For experiments with cultured neurons, neurons (3 wells) were harvested at the end of the corresponding treatments in (100 µl) Tris buffer containing Triton X-114 (20mM Tris HCL pH 7.5 containing 150 mM NaCl, 1% Triton X-114 and protease inhibitor cocktail) and agitated for 15-20 minutes at 4°C. Lysates were cleared by centrifugation at 13,000 × *g* for 15 minutes at 4°C. For experiments in mouse brain, frontal cortices from 10 week-old wild type and APP-Tg (TgCRND8) were lysed separately with (250 µl) Tris buffer containing Triton X-114 as for cultured neurons. Protein content was determined in the clear supernatants. In all cases equal amounts of proteins (= 20µg from neurons and 40µg from cortices) were adjusted to the same volume with Tris buffer containing Triton X-114, were loaded on top of a (30 µl) cushion solution (20mM Tris HCl pH 7.4 containing 150 mM NaCl, 6% sucrose, and 0.06% Triton X-114, protease inhibitor cocktail) and were incubated at 37 °C for 10 minutes. After centrifugation at 16,000 × *g* for 5 minutes the top aqueous clear layer was separated from the detergent-rich, oil-like droplet. Detergent phase was washed with Triton X-114 free buffer and aqueous phase was washed with Triton X-114 (0.5%) containing buffer. The detergent level in both phases was approximately balanced by adding Triton X-114 free Tris buffer and Triton X-114 containing Tris buffer to the detergent and aquaeous phases respectively. Both layers were boiled with SDS sample buffer and proteins were resolved by 12% SDS-PAGE. Immunoblot analysis was performed with anti-Rab9 and/or anti-Rab7. Notice that prenylated Rabs always

run faster than unprenylated Rabs under our experimental SDS-PAGE conditions. The lower band is present in the detergent phase.

2.15.2. Rab-GDI capture: GDI capture was performed according to (Narita et al., 2005). Neuronal lysates were prepared by homogenization of cultured neurons (3 wells) in buffer containing 250mM sucrose, 3mM imidazole pH 7.4, with 1mM DTT and protease inhibitors by passing through a 22 G needle 10 times on ice followed by centrifugation at 300 × *g* for 5 minutes to eliminate unbroken cells and cellular debris. Alternatively cytosol and membranes fractions from neuronal cultures or mouse brain prepared as indicated above were used. Protein content was measured and equal amounts of proteins (= 30 µg) from all samples were diluted fivefold with extraction buffer (30mM HEPES, 75 mM potassium acetate, 5mM MgCl2, 100µM ATP, 500µM GDP) containing 10µM GDI fused to glutathione S-transferase (GDI-GST) and incubated for 20min at 30°C. Rabs bound to GDI were recovered by incubating with glutathione-Sepharose 4B beads (Amersham) overnight at 4ºC. The beads were washed three times with 1 ml buffer containing 75mM potassium acetate, 30mM HEPES pH 7.4 and 5 mM MgCl2. Prenylated Rab bound to GDI-GST were eluted by boiling with SDS-sample buffer for 10 min, and analyzed by SDS-PAGE and immunoblotting with anti Rab-7 or anti Rab-9 antibodies. As indicated for Triton X-114 extraction, prenylated Rabs ran faster than unprenylated Rabs. The lower band is the only band present in membrane preparations and is the band that binds to GDI.

2.16. Incorporation of exogenous GGPP in prenylated proteins. Neurons were treated with 20 µM oAβ_{42} or 50µM pravastatin. [^{3}H]GGPP (22.4 Ci/mmol) was added at a concentration of 200 µCi/ml (9.1 µM). After 24h neurons were rinsed several times, harvested and lysed. Radioactivity was counted in an aliquot of the

neuronal lysate and was used to calculate GGPP uptake. Proteins in neuronal lysates were separated and identified by SDS PAGE and immunoblotting for Rab7 and actin. PDVF membranes were dyed with Ponceau Red and bands corresponding to the molecular weight of Rabs were cut for radioactivity counting. Radioactivity of the rest of the lane was counted separated. The results were referred to actin content and expressed in dpm in Rabs/actin pixels or total protein dpm/actin pixels.

2.17. Evaluation of Metabolic Activity: metabolic activity was evaluated using the cell MTT reduction assay (Promega). This assay is based on the conversion of MTT (3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide) to a blue/purple formazan crystal by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge and Tan, 1993). After neuron treatment MTT was added to the medium and incubation continued for 1 h in CO2 incubator at 37°C. The blue formazan product of MTT reduction was visualized under the microscope. Direct observation of decreased color formation in bright field images is indicative of decreased metabolic activity.

2.18. Detection of nuclear apoptosis and caspase 3 activation. Cortical neurons were treated with oAβ_{42} in the absence or presence of the general caspase inhibitor Z-VAD-FMK for 24h. 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 Hoechst 33258 for 10–20 min. The percentage of apoptotic neurons was estimated by counting condensed and/or fragmented nuclei versus evenly stained nuclei. Nuclei were visualized using a Nikon TE300 inverted fluorescence microscope equipped with a Nikon digital camera DXM-1200 (Nikon Canada, Toronto, Ont.). Images were

analyzed using Northern Elite V6.0 image capture and analysis software (Empix Imaging, Missisauga, Ont.). Five hundred to one thousand neurons per treatment were counted by an observer 'blinded' to the neuronal treatment. Data were analyzed using the Kruskal–Wallis test. In parallel some neurons were harvested and processed for immunoblot analysis of cleaved caspase 3.

2.19. Detection of nuclear translocation of SREBP-2: ST14A cells were transfected with EGFP-hn-SREBP-2 construct corresponding to the mature form of human SREBP2 (kindly provided by Dr. Sipione (Sipione et al. submitted for publication)) using Lipofectamine LTX and plus reagent (Invitrogen cat # 15338-100) following the manufacture's instructions. After 12 hs to allow protein expression the cells were treated with oAβ_{42} (20 μM) for 24 hs. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min and stained with 500 ng/ml Hoechst 33258 for 10–20 min. The nuclear translocation of nSREBP-2 was identified by co-localization of EGFP-hn-SREBP-2 with Hoechst nuclear staining. Pictures were taken using a Laser Scanning Confocal Microscope Zeiss LSM 710 equipped with an S-Fluor 40/1.3 oil objective using appropriate filter sets and excitation wavelengths. Within an experiment, all pictures were taken with the same setting for a particular fluorophore. If required for printing purposes brightness and contrast were adjusted using Photoshop software. All images were adjusted with the same parameters. The pictures selected are representative of at least 3 separate experiments performed under identical conditions.

2.20. Expression of myr-Akt. Primary cortical neurons were infected with recombinant adenoviruses expressing hemagglutinin-tagged myristoylated Akt obtained from CVRC Molecular Biology Core, University of Alberta, or empty virus. The viral particles (at MOI 100-500) were incubated with poly-L-lysine (Sigma, #

p4707) for 30 min at RT to enhance viral infection (Le and Nabi, 2003). Neurons were exposed to the viral particles for 24 hr. The next day, the medium was changed to fresh medium and the experiments started after 48 hr of protein expression. The level of Akt expression was followed by immunoblot analysis using anti HA antibody.

2.21. Statistical analysis: Prism 4 GraphPad computer software was used. Data were from 3 to 5 experiments with 3 to 5 determinations each. Values were expressed as means ± SE. Statistical significance was analyzed using one-way ANOVA-Kruskal-Wallis statistic with Dunn's Multiple Comparison Test and is indicated by * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). In experiments with only two groups, T test was used.

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Chapter 3: oAβ_{42} causes intracellular sequestration of endogenous cholesterol in primary neurons

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3.1. Introduction:

AD, the most common form of age-related dementia, is characterized by accumulation of Aβ and neurofibrillary tangles in the brain (Selkoe, 2004). Mounting evidence indicates the involvement of cholesterol in AD pathology (discussed in details in Chapter 1). The inheritance of ε4 allele of apoE gene is the main risk factor for the development of sporadic AD (Corder et al., 1993; Poirier et al., 1993). Some epidemiological studies suggests that hypercholesterolemia increases the risk of developing AD (reviewed in (Shepardson et al., 2011)). Moreover, large number of studies demonstrated the ability of cholesterol to regulate Aβ production, internalization, degradation and toxicity (Posse de Chaves, 2012). Interestingly, increased cholesterol level has been shown in AD brain (Bandaru et al., 2009; Cutler et al., 2004; Lazar et al., 2013; Sparks, 1997; Xiong et al., 2008). The cholesterol sequestration in AD brain adds to the similarities between AD and NPC, which include endosomal abnormalities, brain accumulation of Aβ, neurofibrillary tangles and influence of apoE genotype (Jin et al., 2004; Koh and Cheung, 2006; Nixon, 2004; Yamazaki et al., 2001). Based on these similarities, 2-hydroxy propyl-β-cyclodextrin (2HP-β-CD), which improved cholesterol trafficking and prevented neurodegerenation in NPC mouse model (Aqul et al., 2011; Liu et al., 2010a; Ramirez et al., 2010), was examined in AD mouse model (Yao et al., 2012). Interestingly, 2HP-β-CD significantly reduced Aβ deposition, tau pathology, lysosomal abnormalities and improved learning and memory in Tg19959, AD mouse model (Yao et al., 2012), suggesting that cholesterol sequestration plays a major role in AD pathology.

Intraneuronal accumulation of Aβ precedes neurofibrillary tangles and Aβ plaques deposition (D'Andrea et al., 2001; Wirths et al., 2002); and has been

causally linked to cell death (Casas et al., 2004; Christensen et al., 2010; Christensen et al., 2008; Chui et al., 2001; Tomiyama et al., 2010; Zhang et al., 2002), synaptic dysfunction (Oddo et al., 2003; Takahashi et al., 2002) and cognitive impairment (Billings et al., 2005). Studies in human AD brains have shown cholesterol accumulation specifically in Aβ-immunopositive neurons (Gomez-Ramos and Asuncion Moran, 2007; Ohm et al., 2003; Xiong et al., 2008). Moreover, brain cholesterol accumulation was preceded by Aβ accumulation in the AD mouse model APP/PS1, suggesting that Aβ could regulate cholesterol homeostasis and/or trafficking (Fernandez et al., 2009). Therefore, in this chapter we examined the effects of $oA\beta_{42}$ treatment on neuronal cholesterol homeostasis. We demonstrated for the first time that $oA\beta_{42}$ induces intracellular cholesterol sequestration and impairs intracellular cholesterol trafficking. These findings suggest that cholesterol sequestration observed in AD could be caused by Aβ.

3.2. Results:

3.2.1. $oA\beta_{42}$ causes intracellular accumulation of endogenous cholesterol.

Due to poor degradation, $A\beta_{42}$ accumulates inside neurons with dramatic consequences. We observed that in basal forebrain as well as sympathetic neurons exposed to toxic concentrations of $oA\beta_{42}$ there was extensive accumulation of unesterified cholesterol as detected by filipin staining (Figure 3.1). This effect was specific to $oA\beta_{42}$ since reverse $A\beta_{42}$ ($revA\beta_{42}$) did not induce cholesterol accumulation (Figure 3.1). The pattern of cholesterol staining with filipin in $oA\beta_{42}$-treated neurons resembled, although was not identical to that displayed by neurons treated with the amphiphilic amine U18666A, conventionally used to mimic the accumulation of cholesterol characteristic of the NPC phenotype (Karten et al., 2002).

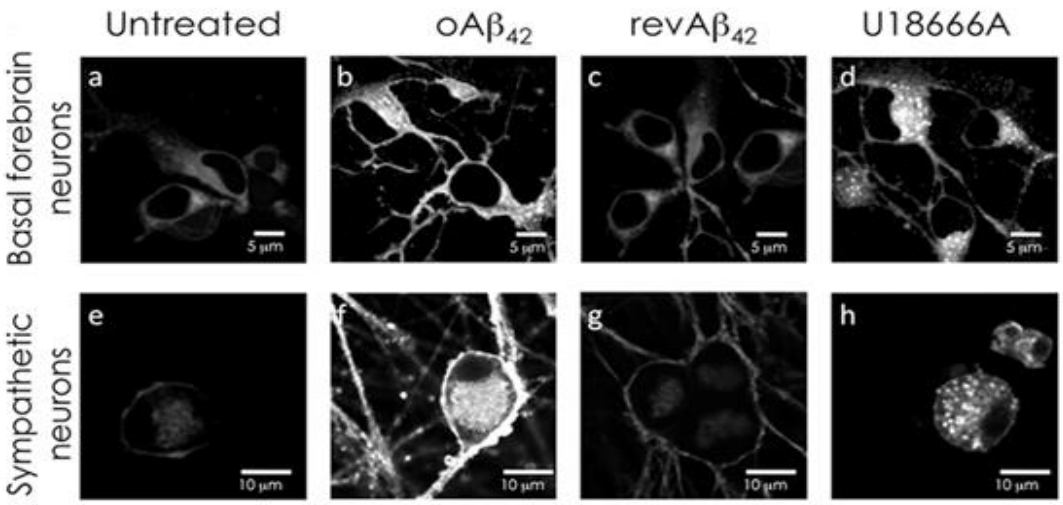

Figure 3.1 oAβ$_{42}$ causes intracellular accumulation of endogenous cholesterol. Basal forebrain and sympathetic neurons were treated with 20μM oAβ$_{42}$, 20μM revAβ$_{42}$ or 3μM U18666A for 24h in serum-free media. Neuronal cholesterol was detected by filipin staining and confocal microscopy.

3.2.2. Statin could not protect against oAβ_{42}-induced intracellular cholesterol sequestration.

Under our experimental conditions cholesterol sequestered in neurons challenged with oAβ_{42} derives from endogenous synthesis exclusively, since neurons were cultured in absence of serum during the treatments with oAβ_{42}. Thus, we attempted to prevent cholesterol accumulation by inhibiting cholesterol synthesis with pravastatin (de Chaves et al., 1997; Saavedra et al., 2007). Contrary to our expectations, cholesterol still accumulated in neurons that received pravastatin with oAβ_{42} (Figure 3.2).

3.2.3. Subcellular localization of sequestered cholesterol in oAβ_{42}-treated neurons.

In NPC, cholesterol accumulates in late endosomes/MVBs (Maxfield and Tabas, 2005; Vanier and Millat, 2003). Since intracellular filipin staining was more diffused in oAβ_{42}-treated than in U18666A-treated neurons, we investigated the subcellular localization of cholesterol in oAβ_{42} challenged neurons. Cholesterol significantly co-localized with lysosomal associated membrane protein-1 (LAMP1), a marker for MVBs/lysosomes, and partially co-localized with giantin a marker for the Golgi (Figure 3.3). We observed no evidence of co-localization of cholesterol with early endosome associated protein-1 (EEA1), a marker for early endosomes, or cytochrome C oxidase subunit IV (COX-IV), a marker for mitochondria.

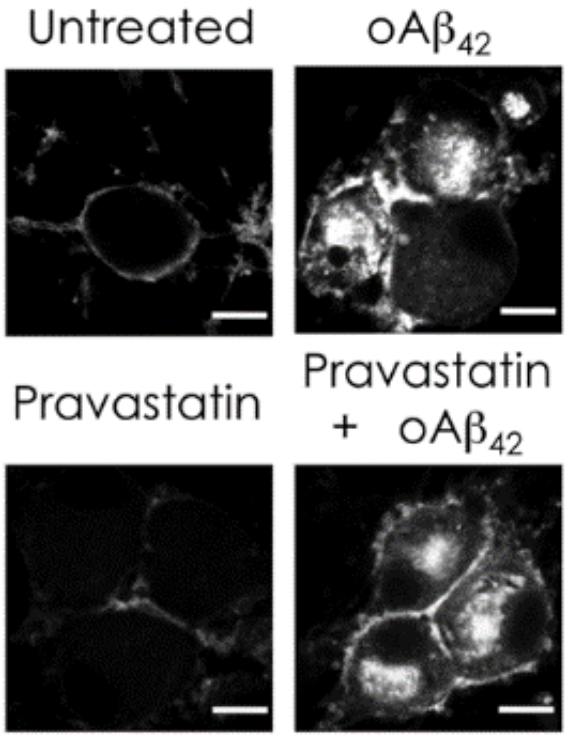

Figure 3.2 Inhibition of cholesterol synthesis does not prevent cholesterol sequestration in oAβ$_{42}$ treated neurons. Sympathetic neurons were treated with 20μM oAβ$_{42}$, 50μM pravastatin or 20μM oAβ$_{42}$ and 50μM pravastatin for 24h in serum-free media. Cholesterol was detected with filipin and examined by confocal microscopy (Scale bars 10μm).

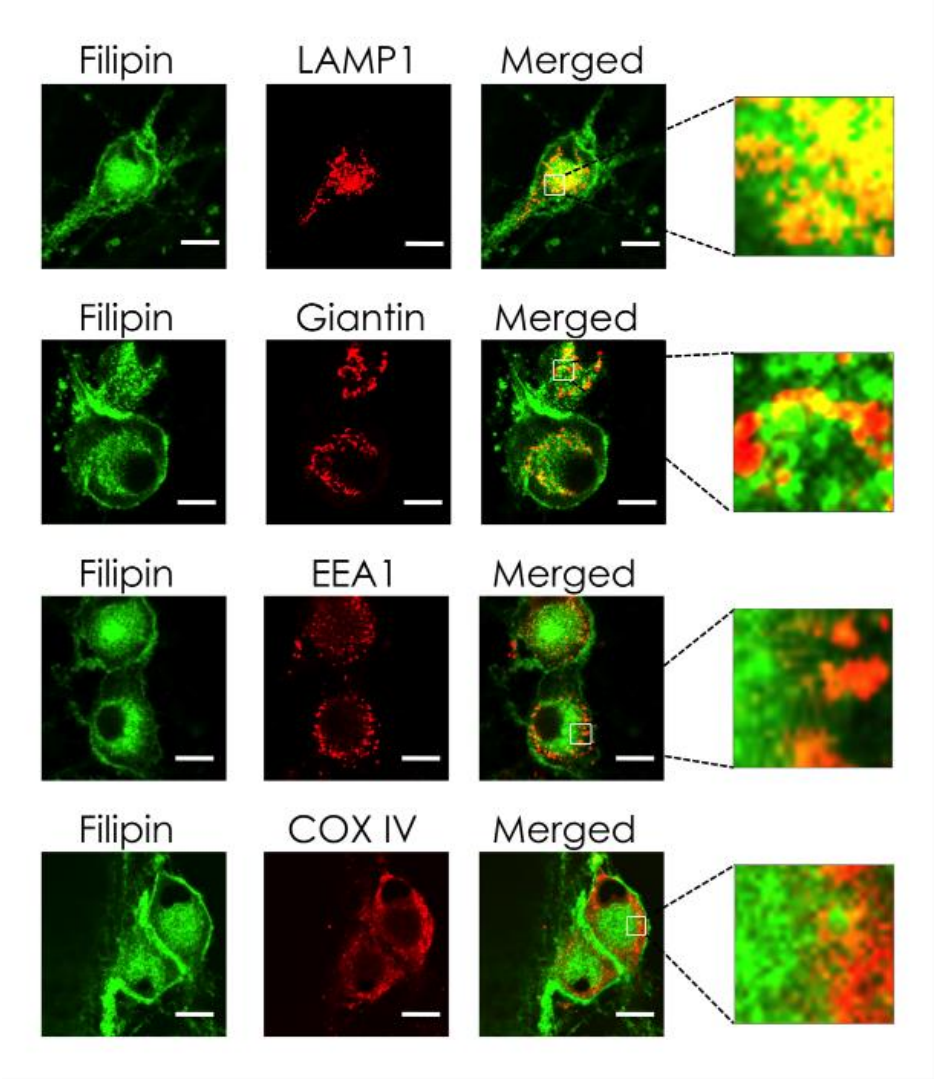

Figure 3.3 Subcellular localization of sequestered cholesterol in oAβ$_{42}$-treated neurons. Sympathetic neurons were treated with 20μM oAβ$_{42}$ for 24h. Intracellular localization of cholesterol accumulation was examined by immunofluorescence confocal microscopy using LAMP1 as a marker of late endosome/MVBs, Giantin as a Golgi marker, EEA1 as a marker of early endosomes and COX IV as mitochondria marker. Filipin was used to detect cholesterol (green pseudo color). (Scale bars 10μm)

A significant difference of the cholesterol sequestration pattern in oAβ_{42} and U18666A treated neurons is that oAβ_{42} but not U18666A caused cholesterol accumulation at the cell surface (Figure 3.1-f &h). To examine the changes in cholesterol level at the plasma membrane, we standardized the use of MβCD to extract cholesterol selectively from plasma membrane. MβCD is a very efficient cholesterol acceptor that binds specifically to cholesterol especially below 10mM of MβCD (Leventis and Silvius, 2001; Zidovetzki and Levitan, 2007). The increase in membrane cholesterol content has been previously shown to result in more cholesterol transferred to cyclodextrins (Lange et al., 2004). As shown in (Figure 3.4-A), there is spontaneous efflux of cholesterol in the absence of MβCD, which does not change with time. Incubation of neurons with MβCD for 5 minutes already allows detection of increased extraction of cholesterol in a concentration dependent manner. Moreover, the MβCD concentrations used are sufficiently low so that increasing the incubation time results in an equivalent increase in cholesterol extraction. Based on this experiment, we estimated the content of plasma membrane cholesterol in sympathetic and forebrain neurons using 2.5 or 1 mM MβCD for five minutes. We found that the intense filipin labeling at the cell surface of oAβ_{42}-treated neurons was associated with a larger pool of surface cholesterol extracted with MβCD (Figure 3.4-B&C). Surface cholesterol accumulation became significant after 24h of exposure to oAβ_{42}, and it did not result from decrease in spontaneous cholesterol efflux (Figure 3.4-D).

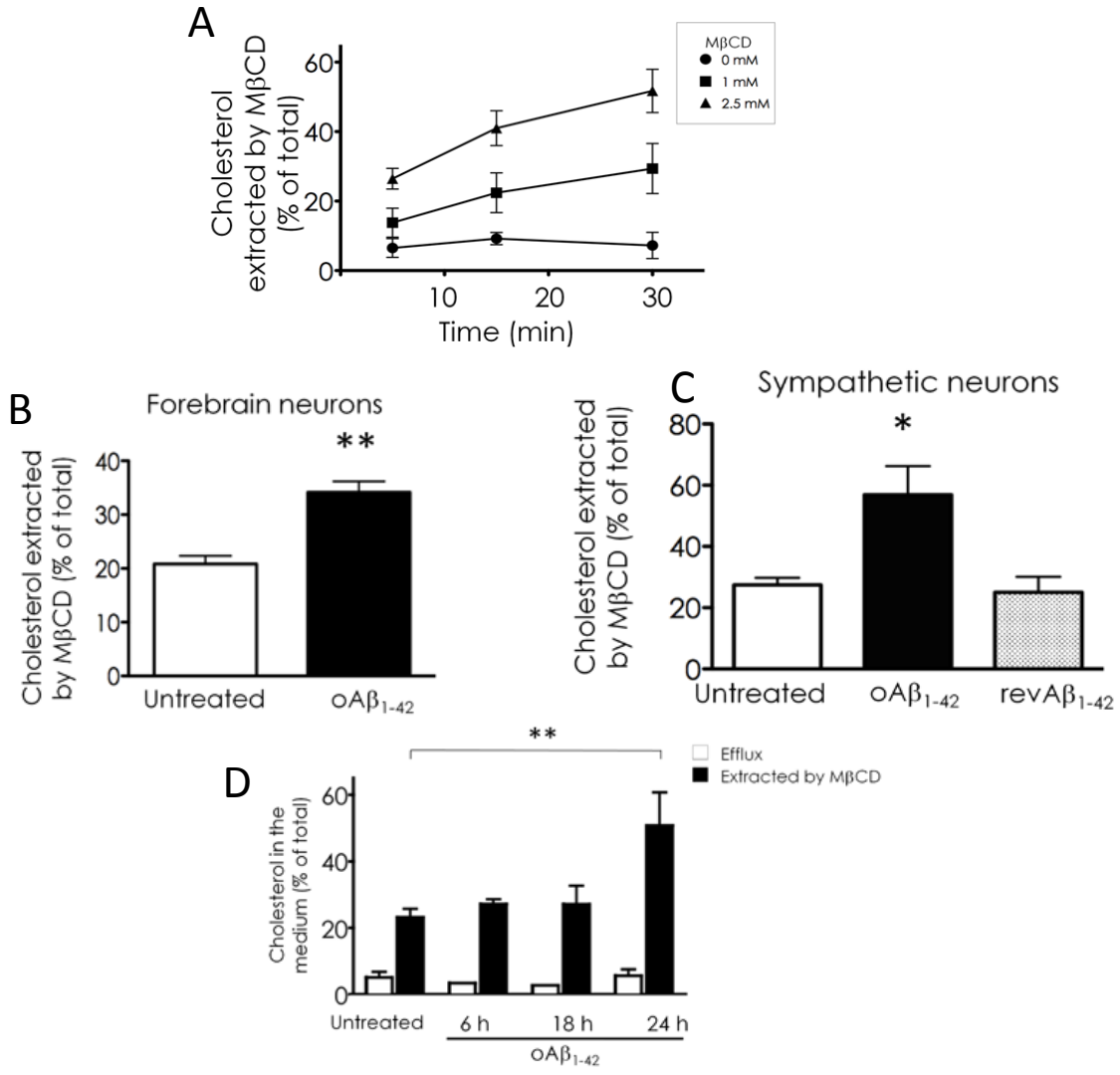

Figure 3.4 oAβ$_{42}$ increases cholesterol level at the neuronal plasma membrane. A. Sympathetic neurons were incubated with [^{3}H]-acetate (150µCi/ml of medium) for 72h to label cellular pools of cholesterol. After carefully rinsing the neurons, neurons were exposed to different concentrations of (0-2.5 mM) of MβCD for various durations (5, 15, 30 min). MβCD-containing medium and cells were collected separately. Lipids were extracted from the cell lysate and the MβCD-containing medium. [^{3}H]-cholesterol separated by TLC was quantified using scintillation counter. The results are expressed as percentage of cholesterol extracted by MβCD = 100 x (dpm in cholesterol from medium)/(dpm in cholesterol from medium + dpm in cholesterol from cell lysate). B. and C. The content of the plasma membrane cholesterol was estimated in basal forebrain and sympathetic neurons incubated with [^{3}H]-acetate (150µCi/ml of medium) for 72h and with 20 µM oAβ$_{42}$ or revAβ$_{42\text{-}1}$ for the last 24h. After carefully rinsing the neurons, 1mM or 2.5 mM MβCD was added for 5 min to basal forebrain (B) and sympathetic neurons (C) respectively and the MβCD-containing medium and cells were collected separately. Lipids were extracted from the cell lysate and the MβCD-

containing medium. The results are expressed as percentage of cholesterol extracted by MβCD. D. Sympathetic neurons were incubated with [^{3}H]-acetate (150µCi/ml of medium) for 72h. After carefully rinsing the neurons, a group of neurons was exposed to 2.5 mM MβCD for 5 min. Other group of neurons was kept untreated. Media and cells were collected separately. Lipids were extracted from the cell lysate and the media. Radioactive cholesterol was separated by TLC and quantified as indicated. The results are expressed as percentage of cholesterol in the medium = 100 x (dpm in cholesterol from medium)/(dpm in cholesterol from medium + dpm in cholesterol from cell lysate).

At the plasma membrane cholesterol localizes primarily in lipid rafts where it interacts with sphingolipids (Simons and Ikonen, 1997). Therefore, we examined the content of cholesterol in lipid rafts isolated from neurons treated with Aβ. We found that the distribution of cholesterol between raft and non-raft fractions of the membrane is the same in untreated and Aβ treated neurons. However, the actual mass of cholesterol that exists in lipid rafts (fraction1-6) is 20-30% higher in Aβ-treated neurons compared to untreated neurons (Figure 3.5-A). The distribution of caveolin-1, a raft marker, transferrin receptor, a non-raft marker, and ganglioside GM1 were similar in oAβ_{42} treated neurons compared to untreated neurons (Figure 3.5-B, C and D). All together these experiments indicate that Aβ leads to an enrichment of cholesterol in lipid rafts without obvious change in lipid raft structure.

3.2.4. Impaired anterograde trafficking of endogenous cholesterol in oAβ_{42}-treated neurons.

To determine if the increased filipin fluorescence displayed by oAβ_{42}-treated neurons represented a *bona fide* increase in cellular cholesterol content, we measured cholesterol mass but we did not detect any significant change induced by oAβ_{42} (Figure 3.6-A). In NPC neurons, the simultaneous occurrence of increased cellular filipin staining with normal cellular cholesterol levels has been explained by mis-localization of cellular cholesterol due to intracellular trafficking dysfunction. Notably, previous work from our laboratory using neurons cultured in compartmented dishes suggested that oAβ_{42}-induced apoptosis was due in part to decrease in trafficking of survival signals to cell bodies (Song et al., 2006).

Therefore, we directly tested the effect of oAβ_{42} on cholesterol trafficking in neurons in compartmented cultures (Figure 3.6-B). By continuous radioactive labeling of endogenous cholesterol, we found that intracellular trafficking of

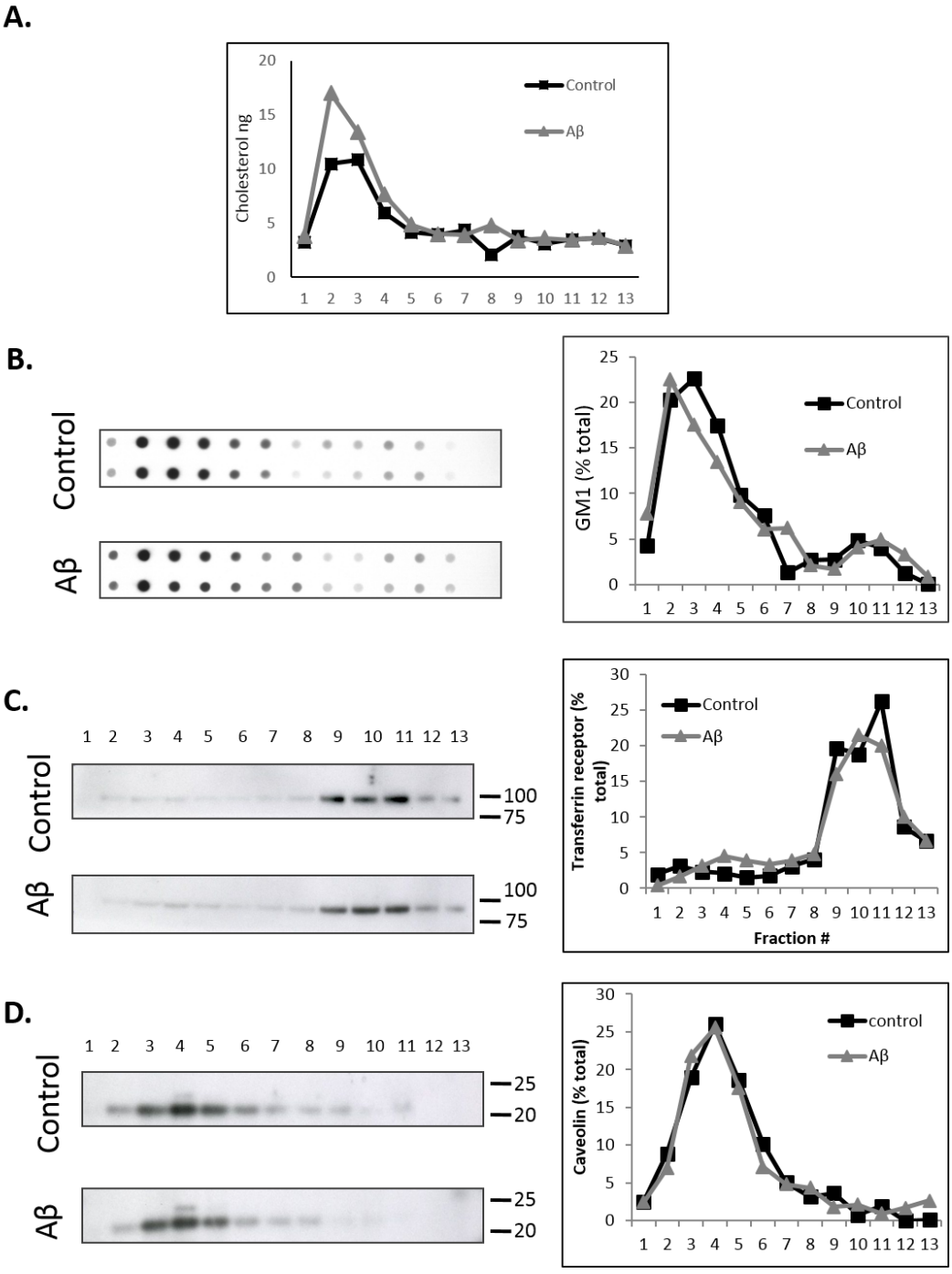

Figure 3.5 oAβ$_{42}$ increases cholesterol level in lipid rafts. Untreated or Aβ$_{42}$-treated sympathetic neurons were incubated with 1% Triton X-100 for 50 minutes on ice. Homogenate was subjected to Optiprep gradient ultracentrifugation and fractionation from the top fraction 1 to 13. A. Cholesterol level in each fraction was measured using Amplex red kit. B. GM1 was detected by loading equal volume of each fraction to a dot blot followed by incubation with cholera toxin subunit B-HRP conjugate. C. and D. transferrin receptor (non-raft marker) and caveolin (lipid raft

marker) were detected by immunoblot analysis. The level of each marker is presented in each fraction as a % of the total level of the marker in all the fractions.

cholesterol from cell bodies to axons was significantly reduced in neurons challenged with oAβ_{42} as it was in U18666A-treated neurons (Figure 3.6-C). The inhibitory effect of U18666A on axonal transport of endogenous cholesterol had been previously reported (Karten et al., 2003). We also investigated the trafficking of proteins by assessing the retrograde transport of the NGF receptor tropomyosin-receptor kinase A (TrkA), which reaches the cell bodies by vesicular transport. We observed significant decrease in TrkA retrograde trafficking in neurons challenged with oAβ_{42} (Figure 3.6-D). Together our findings indicate that oAβ_{42} inhibits intracellular trafficking and causes sequestration of cholesterol in late endosomes /MVBs, partially at the Golgi and at the plasma membrane.

3.3. Discussion

The phenomenon of cholesterol sequestration has received attention only recently in AD. There was some initial evidence that Aβ causes free cholesterol accumulation in hippocampal neurons (Liu et al., 1998). In addition, studies in AD brains reported cholesterol accumulation specifically in Aβ-immunopositive neurons (Gomez-Ramos and Asuncion Moran, 2007; Ohm et al., 2003; Xiong et al., 2008) and studies in APP/PS1 mouse brains showed that Aβ accumulation preceded cholesterol accumulation (Fernandez et al., 2009). In the past couple of years studies using time-of-flight secondary ion mass spectrometry (TOF-SIMS) imaging confirmed cholesterol sequestration in brains of Tg2576 (AD mouse model) (Sole-Domenech et al., 2013) and AD patients (Lazar et al., 2013). This is important because a causal relationship between cholesterol sequestration and cell death was identified in NPC (Aqul et al., 2011). Perhaps more importantly a recent study showed that *in vivo* treatment of mice models of AD with 2HP-β-CD,

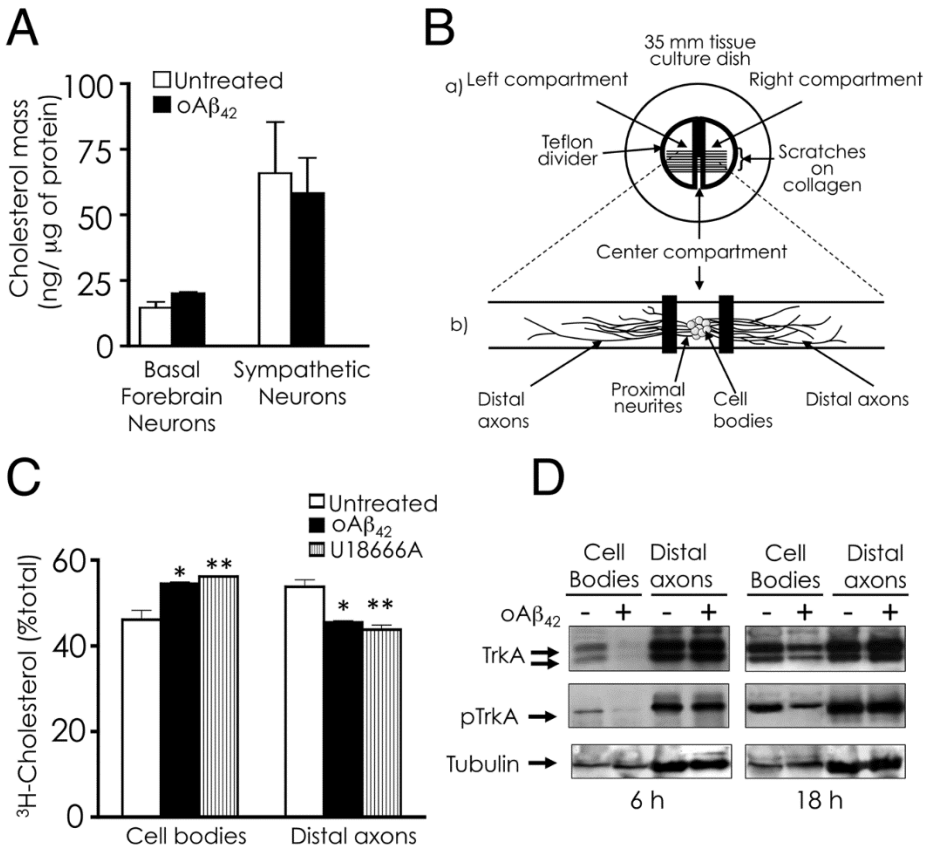

Figure 3.6 oAβ$_{42}$ does not increase cholesterol mass but impairs intracellular trafficking. ***A***, Basal forebrain neurons and sympathetic neurons were treated with 20 µM oAβ$_{42}$ for 24 h in the absence of serum. ***B***, The three-compartment model for culture neurons (***a***) illustrates an entire culture and (***b***) is an enlargement of a single track of a culture in which neurons are plated in the center compartment. Neurites extend under silicone grease barriers and into complete separate fluid environments of left and right compartments. Cellular material from the center (cell bodies/proximal axons) and side (distal axons) compartments can be harvested and processed separately. ***C***, Cholesterol trafficking. Sympathetic neurons cultured in compartmented dishes were treated with 20 µM oAβ$_{42}$ in all compartments or 1.5µM U18666A in cell bodies/proximal axons for 48 h. During the final 24 h of incubation, 150 µCi/ml [^{3}H]-acetate was included in the medium of the center compartment. At the end of the incubation, cell bodies/proximal neurites and distal axons were harvested separately. Three dishes were combined for each sample. Cellular lipids were extracted and separated by TLC, and radioactivity was measured in unesterified cholesterol. Data are expressed as radioactivity in unesterified cholesterol in distal axons as percentages of total radioactivity present in unesterified cholesterol in cell bodies/proximal neurites + distal axons. Data represent means ± SE of three independent experiments performed in quintuplicate. *p < 0.05, **p<0.01, one-way ANOVA. . ***D***, Retrograde transport of NGF receptor TrkA. Sympathetic neurons were cultured in 3-compartment dishes. Surface axonal proteins were biotinylated in the

absence of NGF at 4°C for 30min just before the addition of 20µM oAβ_{42} in the axons. Treatment with oAβ_{42} was performed in the presence of 50 ng/ml NGF. After 6 and 18h the material from the cell body-containing compartment and distal axon-containing compartment was independently harvested (2 dishes were pooled). Biotinylated proteins were pooled down from the cell lysate with streptavidin-agarose and the proteins in the pellet were resolved by SDS-PAGE. Total TrkA and activated TrkA (pTrkA) were detected by immunoblot analysis. Non-biotinylated proteins in the supernatant were analyzed by SDS-PAGE and immunoblot analysis for tubulin as loading control. The experiment was repeated twice.

an agent used to counteract cholesterol sequestration, is neuroprotective and significantly improves spatial learning and memory deficits (Yao et al., 2012). Collectively, this suggests that a pathological mechanism involving abnormal cholesterol trafficking could take place in AD.

In this chapter, we demonstrated that oAβ_{42} induces intracellular cholesterol sequestration in primary neurons. Filipin-staining pattern of oAβ_{42}-treated neurons is similar but not identical to that exhibited by neurons treated with U18666A or NPC fibroblasts (Karten et al., 2002; Narita et al., 2005). Similarly, filipin staining of AD brains showed a diffuse pattern of cholesterol accumulation intracellularly compared to the granular pattern seen in NPC brains (Ohm et al., 2003).

Pravastatin could not prevent Aβ-induced cholesterol sequestration. This is because cholesterol sequestration in oAβ_{42}-treated neurons seems to be induced by impaired cholesterol trafficking and not an increase in cholesterol mass (more details about pravastatin in our model are given in chapter 4).

In oAβ_{42}-treated neurons, cholesterol accumulates in lysosomes/MVBs and partially at the Golgi. These are the organelles that also accumulate intracellular Aβ. We (Mohamed and Posse de Chaves, 2011) and others (Langui et al., 2004; Liu et al., 2010b; Takahashi et al., 2002) have detected accumulation of Aβ_{42} in lysosomes/MVBs. More specifically Aβ_{42} seems to insert in lysosomal membranes (Liu et al., 2010b), accumulating in the outer membrane of MVBs first and moving later to the intravesicular membranes of abnormal endosomes (Takahashi et al., 2004). Internal vesicles from MVBs and recycling endosomes contain most of the cholesterol found in the endocytic pathway (Mobius et al., 2003). There is evidence

that Aβ directly interacts with and partitions into negatively charged membranes that contain cholesterol (Avdulov et al., 1997; Chochina et al., 2001; Igbavboa et al., 2003) and the ganglioside GM1 (Kimura and Yanagisawa, 2007). This interaction may cause changes in lipid packing and in the physicochemical properties of the membrane constituents (Ashley et al., 2006; Chochina et al., 2001). Aβ could accumulate in intravesicular membranes and "anchor" cholesterol at these sites. With respect to the Golgi, the presence of filipin staining in the Golgi of Aβ-treated neurons coincides with the intracellular localization of Aβ in brains of APP/PS1 model of AD (Langui et al., 2004) and in astrocytes treated with $A\beta_{42}$ (Igbavboa et al., 2003; Igbavboa et al., 2009). Accumulation of Aβ in the Golgi of astrocytes disrupts cholesterol homeostasis (Igbavboa et al., 2003). In our study, we did not find increased filipin staining in mitochondria; neither we have detected Aβ in these organelles before (Saavedra and Posse de Chaves, personal communication). This is different from what has been shown in other work (Hansson Petersen et al., 2008). The pathway of endocytosis involved in Aβ uptake could determine the different fate of Aβ within the cells.

Although, cholesterol accumulation in late endosomes has been linked to cell death in NPC (Aqul et al., 2011), others demonstrated that cholesterol accumulation in the endocytic pathway could exhibit a protective effect against lysosomal membrane permeabilization-induced cell death (Appelqvist et al., 2011; Reiners et al., 2011). Yet, we foresee that the simultaneous accumulation of Aβ and cholesterol would have more severe consequences to lysosomes than accumulation of cholesterol by itself based on reports that suggest that lysosomal accumulation of $A\beta_{42}$ causes lysosome disruption and/or increased permeability (Ditaranto et al., 2001; Liu et al., 2010b; Song et al., 2011). Moreover, cholesterol

accumulation in the late endosomes in oAβ_{42}-treated neurons could promote its own production as observed in NPC leading to excessive Aβ accumulation (Burns et al., 2003; Runz et al., 2002) and consequently potentiate Aβ toxicity.

A striking difference between oAβ_{42}-treated neurons and to U18666A-treated neurons is the intense filipin staining at the plasma membrane, which correlates with a significant increase in the size of the cell surface cholesterol pool assessed by MβCD. This finding agrees with previous evidence showing that Aβ_{40} causes redistribution of cholesterol to the plasma membrane in hippocampal neurons (Liu et al., 1998). In addition, membrane cholesterol increases in AD brain with the increase in the severity of the disease (Cutler et al., 2004). Cyclodextrin can extract sequestered cholesterol from the lysosomes and direct it to the ER to be esterified (Abi-Mosleh et al., 2009; Rosenbaum et al., 2010). However, the possibility that the increased cholesterol level extracted by cyclodextrin from Aβ-treated neurons in our experiments has a lysosomal origin is very low, since the duration of exposure to MβCD is only 5 minutes compared to hours or even days in the other studies (Abi-Mosleh et al., 2009; Rosenbaum et al., 2010).

Accumulation of cholesterol at the plasma membrane has important implications in the life of cells and has been linked to Aβ-induced toxicity, although the nature of this relationship is unclear. Some studies showed that an increase in membrane cholesterol confers resistance to death induced by Aβ and other insults (Arispe and Doh, 2002; Cecchi et al., 2005; Cecchi et al., 2009; Pensalfini et al., 2011; Yip et al., 2001), while in other cases higher membrane cholesterol levels has led to increased cellular death susceptibility (Abramov et al., 2011; Nicholson and Ferreira, 2009). Increased plasma membrane cholesterol could facilitate Aβ incorporation into membranes (Ashley et al., 2006; Micelli et al., 2004) and cause

an increase of cytosolic calcium leading to cell death (Abramov et al., 2011), or facilitate Aβ-dependent calpain mediated tau cleavage and cell death (Nicholson and Ferreira, 2009). Furthermore, it has been shown that cholesterol level at the plasma membrane regulates Aβ production via modulating the membrane fluidity (Fassbender et al., 2001; Kojro et al., 2001). Aβ itself was shown to stimulate its own production by reducing the plasma membrane fluidity through Aβ binding to GM1 (Peters et al., 2009). It is therefore conceivable that Aβ-induced high cholesterol level at the plasma membrane will reduce membrane fluidity and would be an additional mechanism through which Aβ could stimulate its own production leading to a vicious cycle.

With the observed increase in plasma membrane cholesterol in oAβ_{42}-treated basal forebrain and sympathetic neurons, we found an increase in cholesterol level in lipid rafts. Similarly, higher cholesterol level was observed in lipid rafts isolated from NPC-null CHO cells compared to wild type cells (Kosicek et al., 2010). The increase of cholesterol in lipid rafts could lead to recruitment of proteins that are normally excluded from the rafts changing the function of these proteins. For example, increased cholesterol level in lipid rafts isolated from oAβ_{42}-treated neurons may recruit APP into the lipid raft and so enhances APP interaction with β-secretase there leading to more Aβ production, similar to what has been observed in NPC cell model (Kosicek et al., 2010).

We could not detect any change in total mass of cholesterol in oAβ_{42}-treated neurons. Perhaps the effect is too small to be detected. However, this finding was not surprising since cholesterol mass is not elevated in NPC neurons or in U18666A-treated neurons despite they sequester cholesterol intracellularly (Karten et al., 2002; Tashiro et al., 2004). In addition, it has been shown that Aβ-

induced cholesterol redistribution to the Golgi in astrocytes was not accompanied with any change in total astrocytic mass of cholesterol (Igbavboa et al., 2003).

Continuous labelling of endogenous cholesterol allows us to detect impaired anterograde transport of labelled cholesterol from cell bodies to distal axons in oAβ_{42} treated neurons. Similarly, U18666A reduces anterograde transport of labelled endogenous cholesterol in sympathetic neurons (Karten et al., 2003). On the other hand, impaired anterograde transport of endogenously synthesized cholesterol in NPC null sympathetic neurons was accompanied with reduced mass of cholesterol in distal axons and increased mass in cell bodies (Karten et al., 2003). Axonal growth depends on cholesterol trafficking from cell bodies to distal axons, since cholesterol is not synthesized in the distal axons (Vance et al., 1994). Therefore, impaired anterograde transport of cholesterol could lead to reduced axonal growth, impaired axonal trafficking and consequently axonal degeneration observed in AD and NPC (Karten et al., 2009; Stokin and Goldstein, 2006). Moreover, impaired axonal trafficking has been shown to enhance APP amyloidogenic processing and Aβ production (Stokin et al., 2005).

In this chapter we demonstrated for the first time that oAβ_{42} induces intracellular cholesterol sequestration and impairs intracellular cholesterol trafficking. This work suggests that cholesterol accumulation and impaired cholesterol trafficking observed in AD (Bandaru et al., 2009; Cutler et al., 2004; Kagedal et al., 2010; Lazar et al., 2013; Sparks, 1997; Xiong et al., 2008) might be induced by oAβ, the main player in AD pathology. Furthermore, our novel discovery that oAβ_{42}-induced cholesterol sequestration and impaired cholesterol trafficking

might be a new mechanism underlying Aβ-induced increased APP processing and Aβ production leading to morbid vicious cycle.

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Chapter 4: oAβ_{42} inhibits proteins prenylation: an underlying mechanism of Aβ-induced cholesterol sequestration and Aβ neurotoxicity.

Portions of this chapter have been published in: Mohamed, A., Saavedra, L., Di Pardo, A., Sipione, S., and Posse de Chaves, E. 2012. Beta-amyloid inhibits protein prenylation and induces cholesterol sequestration by impairing SREBP-2 cleavage. J. Neurosci. 32(19): 6490-500.

4.1. Introduction:

We demonstrated that oAβ_{42} causes intracellular cholesterol sequestration and impairs cellular cholesterol trafficking. Supporting these findings, impaired cholesterol trafficking in AD was suggested by the discovery of increased mRNA and protein levels of NPC1 observed in AD brains (Kagedal et al., 2010). Rab proteins regulate intracellular vesicular trafficking (Figure 4.1) (Hutagalung and Novick, 2011; Pereira-Leal and Seabra, 2001; Seabra et al., 2002; Stenmark, 2009). Rab7 and Rab9 overexpression in NPC models prevents cholesterol accumulation (Choudhury et al., 2002; Kaptzan et al., 2009; Narita et al., 2005) indicating the involvement of Rab7 and Rab9 in intracellular cholesterol trafficking and cholesterol egress out of the lysosomes. Rab proteins are small GTPases that need to be post-translationally prenylated for their membrane binding and their proper functioning (McTaggart, 2006).

FPP and GGPP are the substrates for pernyltransferases, the enzymes catalyzing post-translational prenylation of small GTPases (Casey and Seabra, 1996; Leung et al., 2006). FPP and GGPP are produced through the mevalonate pathway (Holstein and Hohl, 2004). Inhibition of the mevalonate pathway by statins in neurons leads to deleterious effects, which cannot be corrected by exogenous cholesterol supply. However, statin-induced toxic effects in neurons were reduced by exogenous supply of GGPP or low level of mevalonate, which has been shown to be sufficient to maintain normal level of isoprenoids but not cholesterol level (Kim et al., 2009; Marz et al., 2007; Meske et al., 2003; Schulz et al., 2004; Tanaka et al., 2000). These studies indicate the essential role of the isoprenoids and protein prenylation in maintaining proper neuronal functions.

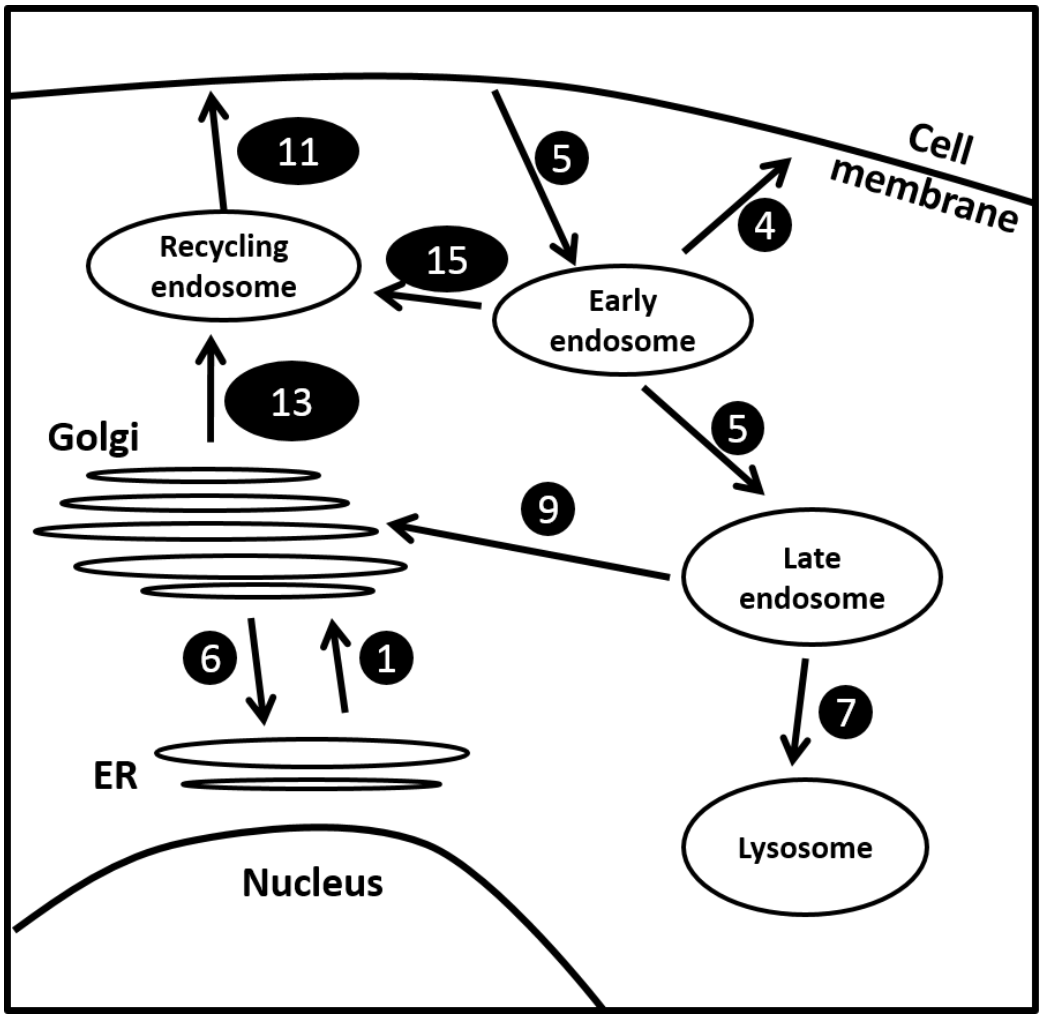

Figure 4.1 Rab proteins regulate intracellular vesicular trafficking. Numbers indicate Rab protein involved.

One of the major transcriptional regulations of the mevalonate pathway is carried out by SREBP-2. (P)SREBP-2 exists in the ER and is transported to the Golgi, where it is proteolytically cleaved to produce (M)SREBP-2. (M)SREBP-2 enters the nucleus to regulate gene transcription (Brown and Goldstein, 1997; Horton, 2002; McPherson and Gauthier, 2004; Sakai et al., 1998; Ye et al., 2000). (M)SREBP-2 increases the expression of many enzymes involved in the mevalonate pathway (Horton et al., 2003).

In this chapter, we investigated the mechanism underlying oAβ_{42}-induced cholesterol sequestration. We found that oAβ_{42} inhibits the mevalonate pathway/cholesterol synthesis by reducing SREBP-2 cleavage. oAβ_{42} inhibits protein prenylation and exogenous GGPP restores normal protein prenylation in oAβ_{42}-treated neurons. Interestingly, exogenous GGPP prevents cholesterol sequestration and reduces neuronal cell loss in oAβ_{42}-treated neurons. Therefore, we demonstrate that inhibition of protein prenylation is a novel mechanism of oAβ_{42}-induced cholesterol sequestration and oAβ_{42}-induced neurotoxicity.

4.2. Results:

4.2.1. oAβ_{42} inhibits cholesterol synthesis.

Cellular cholesterol levels are sensed by the pool of cholesterol in ER (Lange et al., 1999). We rationalized that since oAβ_{42} impairs cholesterol trafficking, oAβ_{42} could prevent cholesterol transport to the ER thus increasing cholesterol synthesis. However we found that oAβ_{42} significantly reduced cholesterol synthesis in basal forebrain, cortical and sympathetic neurons (Figure 4.2-A). oAβ_{42} inhibited cholesterol synthesis only at the concentration (20μM) at which it caused neuronal apoptosis (Figure 4.2-B, C), suggesting a link between

these two phenomena. Yet, the lack of significant apoptosis in neurons that received pravastatin (Figure 4.2-D) indicate that cholesterol synthesis inhibition is not sufficient to induce apoptosis under our experimental conditions.

4.2.2. oAβ_{42} reduces SREBP-2 processing.

Regulation of cholesterol synthesis at the molecular level is mediated by the transcription factor SREBP-2 (Goldstein et al., 2006). Therefore, we examined if SREBP-2 is involved in Aβ-induced inhibition of cholesterol. For activation, SREBP-2 requires to be transported from the ER to the Golgi, where it undergoes proteolytic cleavage, and (M)SREBP-2 translocates into the nucleus (Goldstein et al., 2006). Finding that oAβ_{42} inhibited cholesterol synthesis and impaired intracellular trafficking prompted us to examine whether oAβ_{42} regulates proteolytic activation of SREBP-2. As in other cells, in neurons SREBP-2 cleavage was increased in response to sterol depletion (serum deprivation), however in the presence of oAβ_{42}, proteolytic activation of SREBP-2 was significantly hindered (Figure 4.3). This clearly demonstrates that oAβ_{42} inhibited SREBP-2 proteolytic cleavage.

4.2.3. oAβ_{42} inhibits protein prenylation.

SREBP-2 controls transcription of all genes of the mevalonate pathway, a biosynthetic pathway for cholesterol and non-steroid isoprenoids; FPP and GGPP among others (Horton et al., 2002; Horton et al., 2003). Isoprenoids are essential for post-translational modification of more than 100 proteins (Hooff et al., 2010).

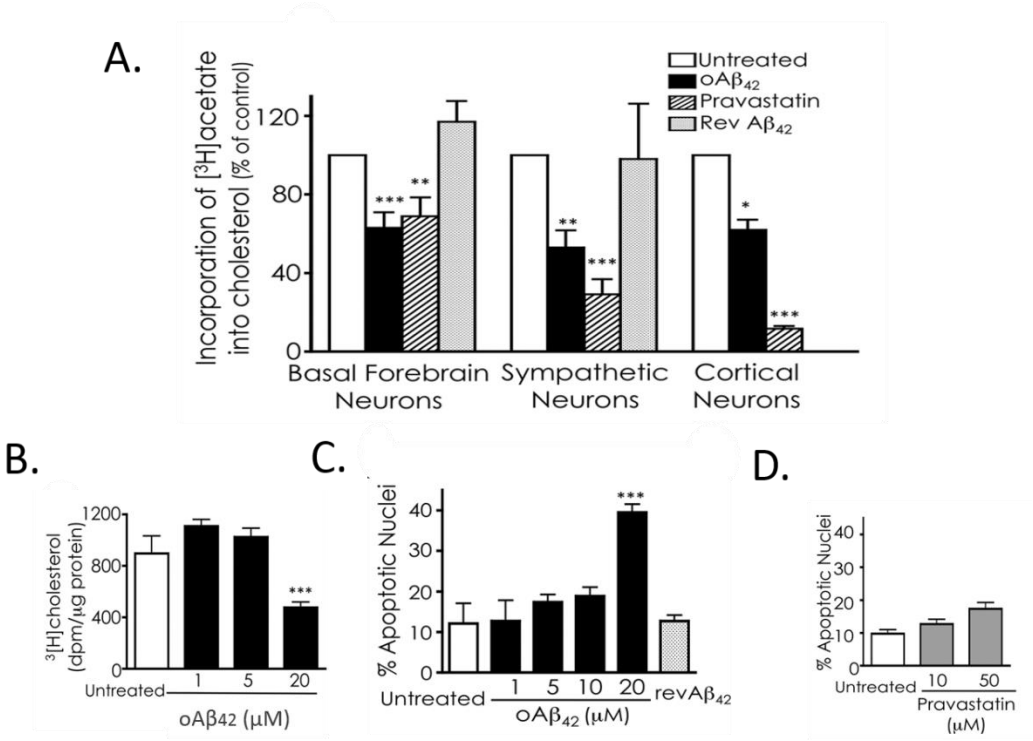

Figure 4.2 oAβ$_{42}$ inhibits cholesterol synthesis. A) oAβ$_{42}$ inhibits cholesterol synthesis. Basal forebrain, sympathetic and cortical neurons were treated with oAβ$_{42}$ (20 µM), pravastatin (50 µM) or revAβ$_{42}$ (20 µM) for 24h (sympathetic and basal forebrain neurons) or 48h (cortical neurons). [^{3}H]-acetate (100 µCi/ml) was added for the last 2h of treatment. Lipids were extracted and separated by TLC. Incorporation of [^{3}H]-acetate into unesterified cholesterol was calculated as dpm/µg protein and expressed as percent of values obtained with untreated neurons. The results are the means ± S.E. of three experiments performed in 3-5 replicates. **B)** Dose response of oAβ$_{42}$ effect on cholesterol synthesis in sympathetic neurons. The same protocol than in **A** was used. The results are the means ± S.E. of three experiments performed in 3-5 replicates. **C)** Dose response of oAβ$_{42}$–induced apoptosis. Sympathetic neurons were incubated with different concentrations of oAβ$_{42}$ or with 20 µM of revAβ$_{42}$. After 36 h the percentage of apoptotic nuclei was evaluated by Hoechst 33258 staining. Data are expressed as means± S.E. of three experiments. Each experiment was performed in quintuplicate and 500–1000 neurons per treatment were counted. **D)** Effect of pravastatin on neuronal survival. Neurons were incubated with different concentrations of pravastatin. After 36 h the percentage of apoptotic nuclei was evaluated by Hoechst 33258 staining. Data are expressed as means± S.E. of three experiments. Each experiment was performed in quintuplicate and 500–1000 neurons per treatment were counted. Data are means ±S.E. For all experiments *($p<0.05$), **($p<0.01$) and ***($p<0.001$) one-way ANOVA.

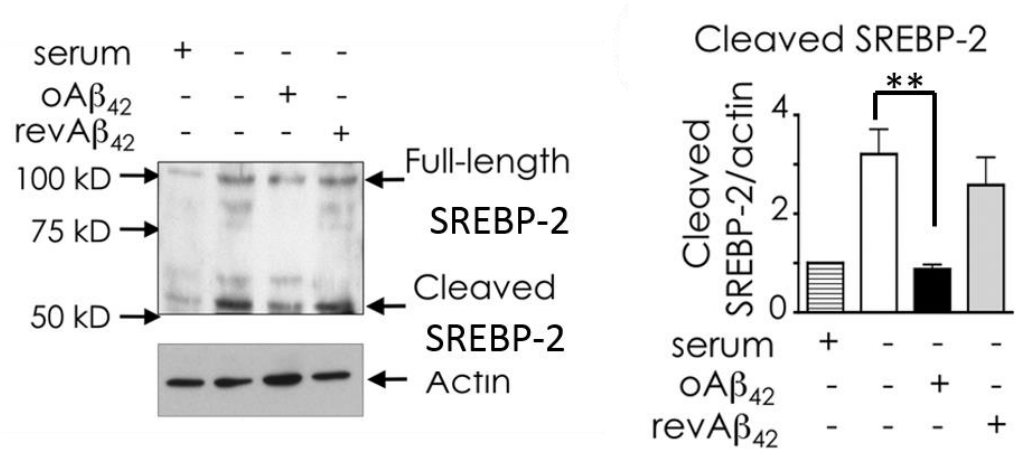

Figure 4.3 oAβ$_{42}$ reduces SREBP-2 processing. Neurons were incubated in medium containing serum, no serum, oAβ$_{42}$ (20μM), or reverse Aβ (20μM) as indicated for 24h, and then were harvested and lysed. Proteins were separated by SDS PAGE and SREBP-2 was detected by immunoblot analysis of total neuronal lysates. Densitometric analysis of SREBP-2 cleavage combines five experiments. Data are means ±S.E. **($p<0.01$) one-way ANOVA.

Hence, we hypothesized that, by inhibiting the mevalonate pathway, oAβ_{42} would affect protein prenylation. We examined prenylation of Rab proteins mainly Rab7 and Rab9 which regulate vesicular trafficking to late endosome/lysosomes and trans Golgi (Feng et al., 1995; Lombardi et al., 1993; Stenmark, 2009). Rabs require prenylation for membrane targeting and for binding to Rab GDP dissociating inhibitor (Rab-GDI). Rab-GDI keeps prenylated Rabs in a soluble state in the cytosol and delivers them to proper membrane locations (Figure 4.4-A) (Pfeffer et al., 1995). To assess the membrane and cytosolic pools of prenylated Rabs we used two alternative approaches. First, we performed extraction of prenylated proteins with Triton-X114 and found reduced prenylation of Rab 9 in oAβ_{42}-treated neurons to an extent similar to prenylation reduction by treatment with geranylgeranyl transferase inhibitor (GGTI-2133) (Figure 4.4-B). Next, we performed extraction of prenylated proteins with recombinant Rab-GDI. GDI capture of prenylated Rabs from cellular membranes may be physically hindered by cholesterol accumulation within membranes (Choudhury et al., 2004; Ganley and Pfeffer, 2006; Lebrand et al., 2002). Thus, to avoid confounding physical effects due to the accumulation of cholesterol in neurons treated with oAβ_{42} we performed the GDI Rab capturing experiments in cytosolic and membrane fractions separately and confirmed that protein prenylation is reduced in oAβ_{42}-treated neurons (Figure 4.4-C,D).

We next investigated if the decrease in protein prenylation induced by oAβ_{42} in cultured neurons was recapitulated in the brain of the TgCRND8 mouse overexpressing APP (Chishti et al., 2001). We found that prenylated pools of Rab7 and Rab9 were reduced in cortex of TgCRND8 mice compared to aged-matched wild type mice (Figure 4.5- A, B).

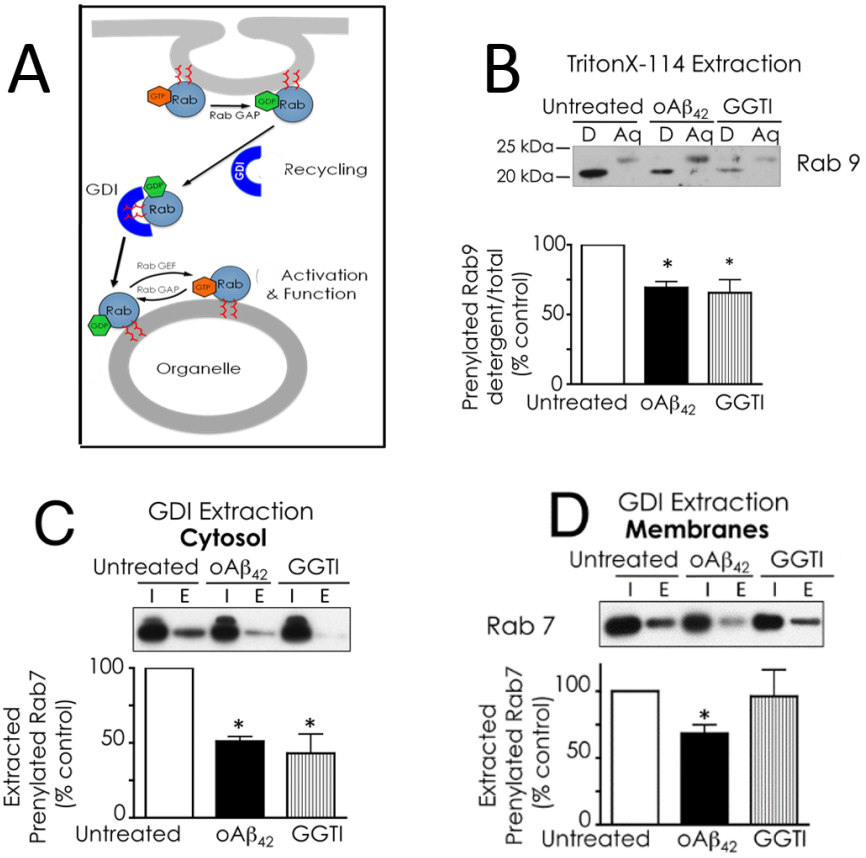

Figure 4.4 oAβ$_{1-42}$ inhibits protein prenylation. **A) Schematic presentation showing Rab-GDI mediating prenylated Rab recycling between different membranes.** In B), C) and D) Neurons were treated with 20μM oAβ$_{42}$ or 5μM GGTI for 24h. **B) Analysis of Rab prenylation by extraction with Triton X-114:** Neuronal lysates (equal amount of protein) were extracted with Triton X-114. Proteins in detergent (D, prenylated proteins) and aqueous (Aq, unprenylated proteins) phases were analyzed by SDS-PAGE and immunoblotting. Ratios between total pixels of Rab9 in D/total pixels of Rab 9 in D plus Aq. were calculated. The data are presented as % control (untreated neurons). Data are means ±S.E. of 3 experiments. **C) & D) GDI capture from cytosol and membranes**. Neuronal lysates were fractionated in membranes and cytosol prior to GST-GDI extraction. Half of each fraction was used to assess total protein input (I). The other half was used to extract prenylated proteins with recombinant GST-GDI (E). Proteins were analyzed by immunoblot analysis. Ratios between total pixels of Rab7 in GDI-bound (E)/total pixels of Rab 7 in lysate before extraction (I) were calculated. Densitometric analysis of I included both bands. Data are presented as % control (untreated neurons). In all experiments data are means ±S.E. of values. *($p<0.05$) one-way ANOVA.

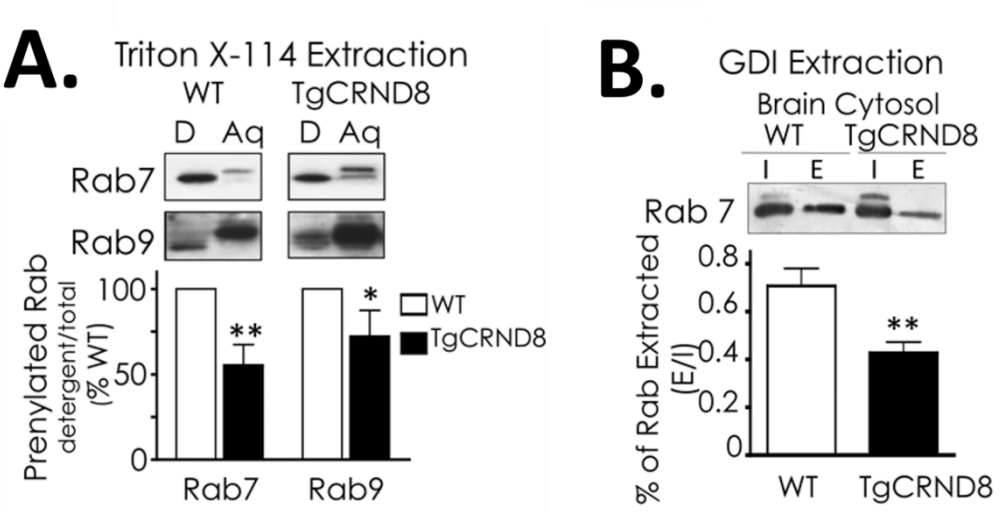

Figure 4.5 Brain cortices of TgCRND8 mice have reduced level of prenylated Rab proteins. A) Triton X-114 extraction of brain cortices of TgCRND8 mice: prenylated and unprenylated Rab proteins were separated by Triton X-114 extraction from mouse brain cortex homogenates. Densitometric analysis of prenylated (detergent)/prenylated +unprenylated (aqueous) combined results from 4 brains in each group. **B) Rab-GDI capture from brains of TgCRND8 mice:** brain cortex membrane and cytosol fractions were prepared and prenylated rabs were extracted from cytosol fraction with GST-GDI as for cultured neurons. (I) represents total Rab protein input and (E) represents the extracted prenylated Rab with recombinant GST-GDI. Ratios between total pixels of Rab7 in GDI-bound (E)/total pixels of Rab 7 in lysate before extraction (I) were calculated. Results obtained from 6 individual brains for each group have been combined in the quantification plot. In all experiments data are means ±S.E. of values. *($p<0.05$) and **($p<0.01$) T-test.

To exclude that the inhibition of prenylation by oAβ_{42} was specific to Rab proteins we examined the level of membrane-associated Ras, which is an indication of Ras prenylation. oAβ_{42} caused significant decrease of Ras in neuronal membranes (Figure 4.6-A). Although Ras proteins are preferentially modified by farnesylation, geranylgeranylation of KRAS and to a lesser extent NRAS becomes important when farnesylation is blocked (Downward, 2003). All together these results indicate that oAβ_{42} effectively inhibits protein prenylation.

4.2.4. Isoprenoid supply prevents oAβ_{42}-induced inhibition of protein prenylation.

Since oAβ_{42} inhibits the mevalonate pathway and causes reduction of prenylation, we hypothesize that decreased prenylation is due to shortage of isoprenoids and therefore could be prevented by addition of GGPP. The finding that extracted prenylated Rab7 and membrane Ras levels were unchanged in neurons received GGPP with oAβ_{42} confirmed our hypothesis (Figure 4.6- A, B). The use of GGPP can be limited by its relative membrane impermeability, but there is documented evidence of GGPP ability to enter cultured cells (Kukar et al., 2005), and to counteract some effects of inhibitors of the mevalonate pathway (Kim et al., 2009; Marz et al., 2007; Meske et al., 2003; Schulz et al., 2004; Tanaka et al., 2000). We confirmed GGPP uptake by measuring the incorporation of [^{3}H]GGPP in cellular proteins. Neurons were able to take up 0.2 ± 0.015 % of the total [^{3}H]GGPP added to the medium in 24h and [^{3}H]GGPP uptake was not affected by Aβ. However, neurons treated with oAβ_{42} incorporated 2-3 fold more GGPP in proteins than untreated neurons or neurons treated with pravastatin (156.94 dpm/actin pixel in oAβ_{42}-treated neurons vs 48.51 dpm/actin pixel in untreated neurons and 56.35 dpm/actin pixel in pravastatin-treated neurons). This

experiment has two main implications: first, oAβ_{42} significantly reduces the endogenous pool of GGPP; and second, GGPP does not neutralize oAβ_{42} effects but truly serves as a substrate for protein prenylation. In addition these data also show that under our experimental conditions pravastatin does not significantly affect isoprenoids levels, which agrees with the lack of effect of pravastatin on protein prenylation (Figure 4.6-C) and cell survival (Figure 4.2- D).

4.2.5. Isoprenoid supply prevents oAβ_{42}-induced cholesterol sequestration and protects against oAβ_{42}-induced neurotoxicity.

Based on the evidence presented above we hypothesized that cholesterol sequestration in oAβ_{42}-treated neurons results from protein prenylation inhibition, and could be prevented by GGPP. Supporting this hypothesis the prenylation inhibitor GGTI caused intracellular cholesterol accumulation similar to treatment with oAβ_{42} (Figure 4.7-A). Administration of GGPP prevented oAβ_{42}-induced, but not GGTI-induced cholesterol sequestration (Figure 4.7- A). Furthermore, GGPP blocked the detrimental effect of oAβ_{42} on neuronal metabolic activity (Figure 4.7-B) and significantly reduced oAβ_{42}-induced but not GGTI-induced apoptosis (Figure 4.7- C).

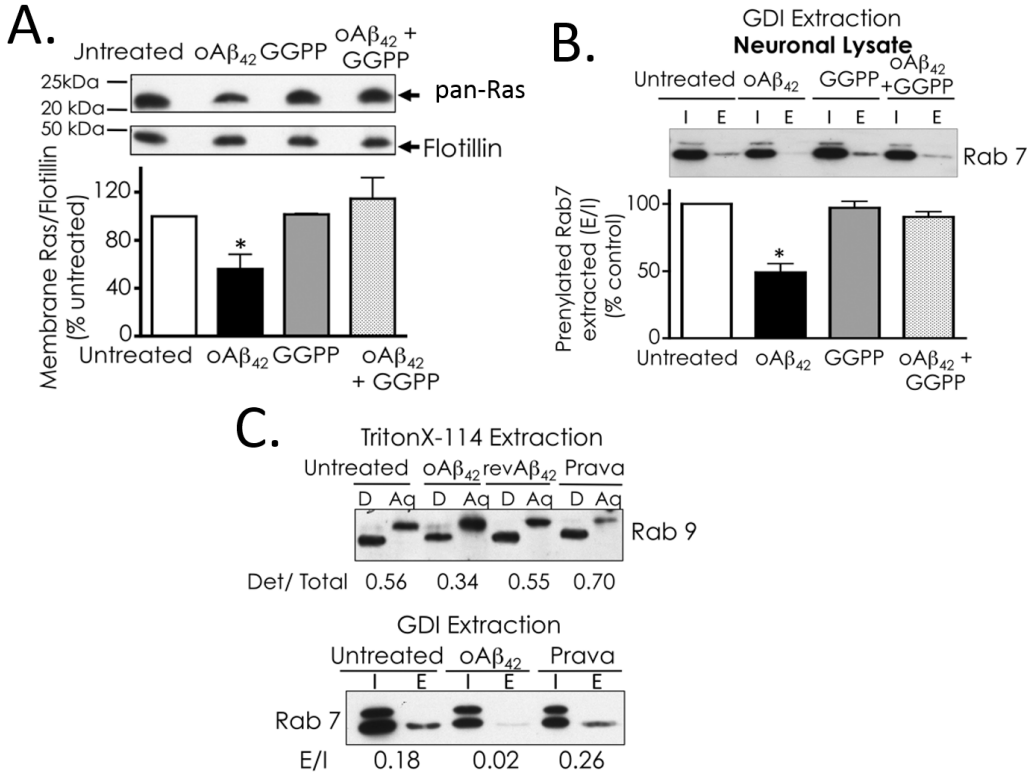

Figure 4.6 oAβ$_{42}$ but not pravastatin inhibits Ras and Rab prenylation. GGPP cotreatment reduces oAβ$_{42}$-induced inhibition of protein prenylation. A) Ras delivery to membranes. Sympathetic neurons were treated with 20μM oAβ$_{42}$ with or without the isoprenoid GGPP (10 μM) for 24h. Membrane fractions were prepared from treated neurons. Equal amounts of membrane proteins were separated by SDS-PAGE and analyzed by immunoblotting. Densitometric analysis of Ras/flotillin combined 4 experiments. Values expressed as percentage of untreated neurons. **B) Analysis of Rab prenylation by Rab-GDI capture.** Neuronal lysates (equal amount of protein) were prepared. Half sample was used to assess total protein input (I). The other half was used to extract prenylated proteins with recombinant GST-GDI (E). Proteins were analyzed by immunoblot analysis. Ratios between total pixels of Rab7 in GDI-bound (E)/total pixels of Rab 7 in lysate before extraction (I) were calculated. Densitometric analysis of I included both bands. Data are presented as % control (untreated neurons). Data are means ±S.E. of 3 experiments. **C) Pravastatin does not impair protein prenylation.** Neurons were treated with oAβ$_{42}$ (20μM), revAβ$_{42\text{-}1}$ (20μM) or pravastatin 50 μM for 24h. Protein prenylation was examined by extraction with Triton X-114 or GST-GDI. In all experiments data are means S.E. of values. *($p<0.05$) one way ANOVA.

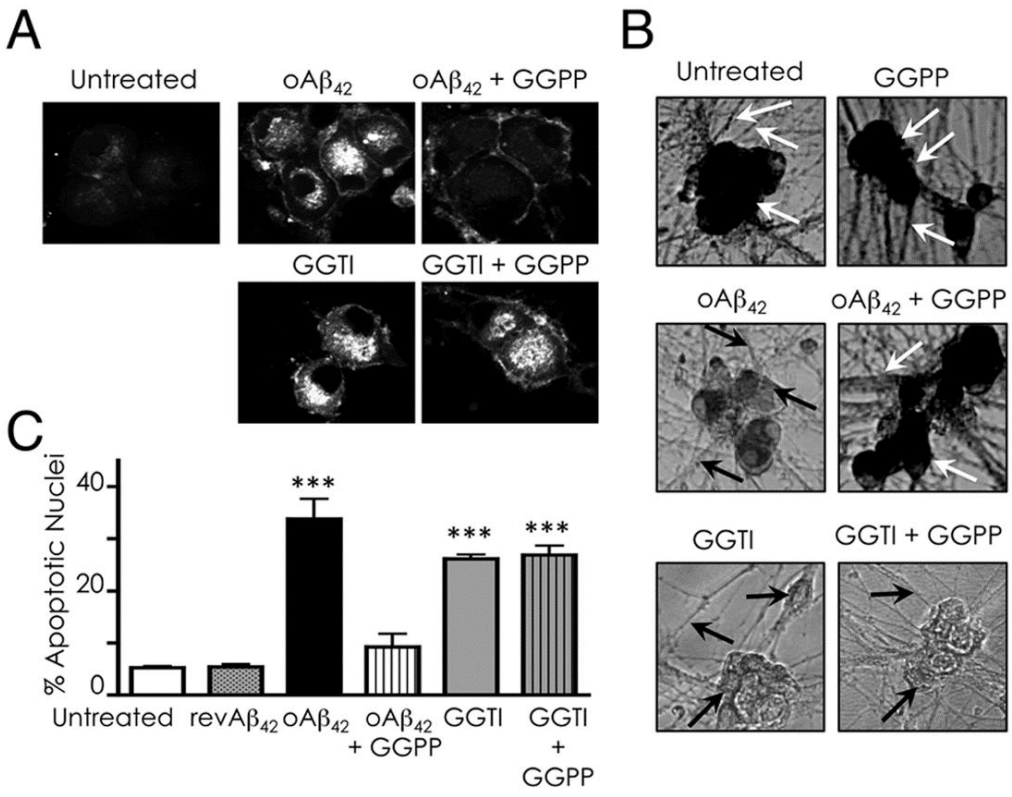

Figure 4.7 GGPP reduces oAβ$_{42}$–induced cholesterol sequestration and oAβ$_{42}$–induced neurotoxicity. Neurons were treated with 20 µM oAβ$_{42}$ or 5 µM GGTI with or without 10 µM GGPP for 36h. **A)** Cholesterol sequestration was detected by filipin staining. **B)** Metabolic activity was examined by MTT assay "in situ". White arrows point to healthy cell bodies and neurites containing the formazan compound. Black arrows indicate cell bodies or neurites devoid of the MTT reduction product due to reduced metabolic activity. **C)** Apoptosis was quantified by counting nuclei stained with Hoechst 33258. Data are expressed as means± S.E. of three experiments. Each experiment was performed in quintuplicate and 500–1000 neurons per treatment were counted. ***($p<0.001$) one-way ANOVA.

4.3. Discussion:

We found that oAβ_{42} inhibits cholesterol synthesis, which at first seemed paradoxical to the intensive filipin staining. Nevertheless U18666A, a potent inhibitor of cholesterol synthesis (Jin et al., 2004; Runz et al., 2002) also causes endosomal cholesterol accumulation (Cenedella, 2009; Karten et al., 2002). Inhibition of cholesterol synthesis by Aβ_{40} but not Aβ_{42} has been previously reported (Gong et al., 2002; Grimm et al., 2005). The consequences of cholesterol synthesis inhibition in neurons are unclear. Neurons have lower rate of cholesterol synthesis than glia (Nieweg et al., 2009), and adulthood neurons outsource cholesterol from astrocytes (Aqul et al., 2011; Funfschilling et al., 2007). However, some studies showed that neurons need to maintain some level of cholesterol synthesis for normal neuronal functions (Korade et al., 2009; Mailman et al., 2011; Valdez et al., 2010). Our data suggest that inhibition of cholesterol synthesis “per se” is not a direct cause of neuronal death since cholesterol synthesis inhibition by pravastatin did not cause neuronal death (see also (de Chaves et al., 1997; Saavedra et al., 2007).

Our results indicate that one mechanism of oAβ_{42} inhibition of the mevalonate pathway is a decrease in SREBP-2 processing. This is different from what has been shown for Aβ_{40}. Gong and collaborators found that Aβ_{40} did not affect SREBP-2 proteolysis (Gong et al., 2002), and Hartmann’s group found no inhibition of cholesterol synthesis when mevalonate was used as a precursor, indicating that HMGCR was the target of Aβ_{40} (Grimm et al., 2005) but also suggesting that Aβ_{40} does not affect SREBP-2. Importantly, in Gong’s and Hartmann’s studies Aβ was used at non-toxic concentrations and our data indicate

that only toxic concentrations of oAβ_{42} inhibit cholesterol synthesis. We examined the mechanism of inhibition of SREBP-2 cleavage by oAβ_{42} in chapter 5.

In addition to cholesterol, the mevalonate pathway produces other intermediates such as isoprenoids, which are required for protein prenylation. We show that Rab and Ras prenylation were significantly reduced in oAβ_{42}-treated neurons; and protein prenylation in brain cortex from the TgCRND8 mouse model of AD was significantly lower than in cortex of age-matched wild type animals. Decreased prenylation in neurons challenged with oAβ_{42} was due to shortage of isoprenoids since supply of GGPP prevented prenylation inhibition and cholesterol sequestration suggesting that normal intracellular trafficking was restored. Inhibition of protein prenylation appears as a pivotal mechanism of neuronal death. GGTI inhibited protein prenylation, caused cholesterol accumulation and induced neuronal death. GGPP was readily incorporated into prenylated proteins and significantly reduced oAβ_{42}-induced neuronal death but, as expected, it could not rescue neurons treated with GGTI.

In addition to the direct effect of Aβ on prenylation, Aβ could also affect indirectly the function of remaining prenylated Rabs by means of cholesterol accumulation within membranes. The level of cholesterol in endocytic compartments plays a role in Rabs fate and function (Choudhury et al., 2004; Ganley and Pfeffer, 2006; Lebrand et al., 2002). GDI-capture of prenylated Rab proteins from membranes of oAβ_{42}-treated neurons is reduced, suggesting that in Aβ-treated neurons there are membrane structural changes similar to those present in NPC cells (Choudhury et al., 2004; Ganley and Pfeffer, 2006). These changes could result from a combination of cholesterol accumulation and the direct interaction of Aβ with cholesterol and other lipids such as GM1 (Kimura and

Yanagisawa, 2007). Therefore, reducing membrane cholesterol accumulation and improving intracellular cholesterol trafficking by HP-β-CD would restore the activity of these membrane Rab proteins by allowing their GDI-capturing. This could play a role in the neuroprotective effect of HP-β-CD observed in AD mouse model (Yao et al., 2012).

Isoprenoids and protein prenylation play important roles in the nervous system, especially in AD (Hooff et al., 2010). Reduced isoprenoid production explained the decrease of long-term potentiation in hippocampal slices (Kotti et al., 2008; Kotti et al., 2006; Matthies et al., 1997), the increase in tau hyperphosphorylation (Meske et al., 2003), and isoprenoids prevented statin-induced neurotoxicity (Meske et al., 2003; Schulz et al., 2004; Tanaka et al., 2000). Protein prenylation regulates APP trafficking and cleavage, therefore, reduction of protein prenylation results in increased or decreased Aβ production, depending on the cell type (Cole et al., 2005; Ostrowski et al., 2007). Moreover, reduction of Ras prenylation represents a mechanism of neurodegeneration (Zipp et al., 2007). In our studies the decrease in protein prenylation is accompanied by cholesterol accumulation within intracellular membranes, which might favour APP cleavage and Aβ production.

Recent studies showed that GGPP, FPP and the mRNA of their respective synthases are elevated in AD brains (Eckert et al., 2009). The significance of this elevation is still unknown since protein prenylation was not examined in this study and elevation of isoprenoids does not warrant an increase in protein prenylation. Our findings suggest that a decrease in isoprenoid synthesis will take place specifically in cells that accumulate Aβ, most likely neurons. Depending on the size of the cell population that contains intracellular Aβ, this might, or might not impact

the overall content of isoprenoids in the brain. An interesting model has been proposed in which SREBPs in astrocytes are involved in lipid synthesis for supply to neurons for neurite outgrowth, synaptogenesis and synaptic plasticity (Camargo et al., 2009). According to this model glia SREBPs may work as control points of neuronal function providing neurons with appropriate lipids when neurons cannot make their own. Whether astrocytes can supply isoprenoids has not been investigated to our knowledge, but the increase of isoprenoids in AD brains could represent an astrocytic attempt to compensate a decrease in SREBP-dependent metabolic pathways in neurons.

In this chapter we illustrated that oAβ_{42} inhibits cholesterol synthesis/the mevalonate pathway via reducing SREBP-2 cleavage. Moreover, oAβ_{42} diminishes protein prenylation, which could be corrected by exogenous supply of GGPP. This indicates that oAβ_{42}-induced reduction of protein prenylation results from depletion of cellular isoprenoids caused by oAβ_{42}-induced inhibition of cholesterol synthesis. Furthermore, we demonstrated oAβ_{42}-induced inhibition of protein prenylation as a novel underlying mechanism of oAβ_{42}-induced cholesterol sequestration and oAβ_{42}-induced neurotoxicity.

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Chapter 5: oAβ_{42} inhibits SREBP-2 cleavage by reducing cellular levels of activated Akt

Aversion of this chapter has been submitted for publication.

5.1. Introduction

In the past several years it has become clear that cholesterol plays significant roles in AD and that cholesterol homeostasis is perturbed in AD (Di Paolo and Kim, 2011; Posse de Chaves, 2012). In addition, there is increasing evidence of the importance of protein prenylation in aging and in the pathophysiology of AD (Hooff et al., 2010; Li et al., 2012; Ostrowski et al., 2007). Cholesterol and short isoprenoids used for protein prenylation are products of the mevalonate pathway. We have demonstrated that oAβ_{42} inhibits cholesterol synthesis and protein prenylation. The effects of oAβ_{42} are due to reduction of the maturation of the transcription factor SREBP-2. SREBP-2 belongs to a family of transcription factors known to regulate cholesterol and fatty acid homeostasis. SREBP-2 regulates preferentially cholesterol and isoprenoid synthesis by increasing the expression of the majority of the enzymes of the mevalonate pathway (Horton et al., 2003).

SREBPs regulation is complex. The main regulators of SREBP-2 maturation are cholesterol and oxysterols (Adams et al., 2004; Nohturfft et al., 2000; Radhakrishnan et al., 2007; Radhakrishnan et al., 2004; Sun et al., 2007). SREBP2 is synthesized as an inactive (P)SREBP-2 that localizes to the ER by binding to the ER protein SCAP. SCAP is both a sterol sensing protein and an escort protein (Goldstein et al., 2006). If cellular cholesterol levels are high, the complex SCAP/SREBP-2 is retained at the ER (Yabe et al., 2002; Yang et al., 2002). Conversely, under conditions of low cellular cholesterol, SCAP undergoes conformational changes that allow it to bind to COPII. COPII clusters the SCAP/SREBP-2 complex into coated vesicles that bud from the ER and travel to the Golgi complex (Sun et al., 2007). At the Golgi, (P)SREBP-2 is cleaved

releasing the N-terminal, (M)SREBP-2, which enters the nucleus and activates several genes involved in cholesterol synthesis and uptake (Sakai et al., 1998; Ye et al., 2000). The exit of SCAP/SREBP-2 from the ER represents a critical point in feedback regulation of cholesterol metabolism (Brown et al., 2002; Yang et al., 2002). Recently, Brown and collaborators identified the activation of Akt as a new mechanism that regulates the trafficking of SCAP/SREBP-2 from ER to Golgi, and therefore contributes to the control of the proteolytic cleavage of SREBP-2 (Du et al., 2006; Luu et al., 2012).

The main aim of this study is to identify the mechanism through which oAβ_{42} reduces the levels of (M)SREBP-2. Our work is important in light of the crucial roles of lipids in AD and the more recent understanding that SREBPs' roles extend well beyond lipid metabolism to crucial physiological processes some of which are altered in AD.

5.2. Results:

5.2.1. oAβ_{42} reduces (M)SREBP-2 levels in primary neurons.

We have demonstrated that oAβ_{42} (20μM) significantly decreases SREBP-2 cleavage in sympathetic neurons. Here we extended our studies to cortical neurons, which are significantly affected in AD. We show that the inhibitory effect of oAβ_{42} on SREBP-2 maturation is not exclusive to sympathetic neurons; oAβ_{42} effectively reduces SREBP-2 maturation in a dose-dependent manner also in cortical neurons (Figure 5.1-A, B, D, E). Interestingly, we observed that (P)SREBP-2 is also reduced in oAβ_{42} -treated neurons. It has been shown that SREBP-2 gene has SRE sequence allowing auto-regulation of SREBP-2 gene expression (Sato

et al., 1996). Therefore, reduced level of (M)SREBP-2 in oAβ_{42} –treated neurons would reduce SREBP-2 gene expression and so reduced (P)SREBP-2 level.

Moreover, frontal cortices of TgCRND8 mouse, a mouse model for AD (Chishti et al., 2001), have lower levels of (M)SREBP-2 compared to frontal cortices of age-matched mice (Figure 5.1-C, F). The effect of oAβ_{42} on SREBP-2 maturation is specific since the reverse peptide rAβ_{42-1} does not alter SREBP-2 cleavage (Figure 5.1-G).

5.2.2. oAβ_{42}-induced reduction of (M)SREBP-2 levels is independent of induction of apoptosis and of SREBP-2 degradation.

To identify the mechanism by which oAβ_{42} inhibits SREBP-2 cleavage we first excluded the possibility that the decrease of (M)SREBP-2 was due to Aβ-induced apoptosis. For that purpose we use the caspase inhibitor Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone). Z-VAD effectively decreased oAβ_{42}-induced caspase-3 cleavage (Figure 5.2-A) and reduced oAβ_{42}-induced nuclear fragmentation (Figure 5.2-B,C). Nevertheless, oAβ_{42} still decreased (M)SREBP-2 in the presence of Z-VAD (Figure 5.2-D,E). Next, we investigated if the decrease in (M)SREBP-2 induced by oAβ_{42} was the result of enhancement of SREBP-2 degradation. (M)SREBP-2 is degraded by the proteasome (Hirano et al., 2001) and inhibition of proteosomal degradation by MG132 increases the level of (M)SREBP-2 (Sundqvist et al., 2005; Yamauchi et al., 2011). Thus, we compared the effect of oAβ_{42} on (M)SREBP-2 in the absence and presence of MG132. As expected, the level of (M)SREBP-2 in untreated neurons was elevated in the presence of MG132, but MG132 did not prevent the decrease of (M)SREBP-2 induced by oAβ_{42} (Figure 5.2-F,G), indicating that oAβ_{42}

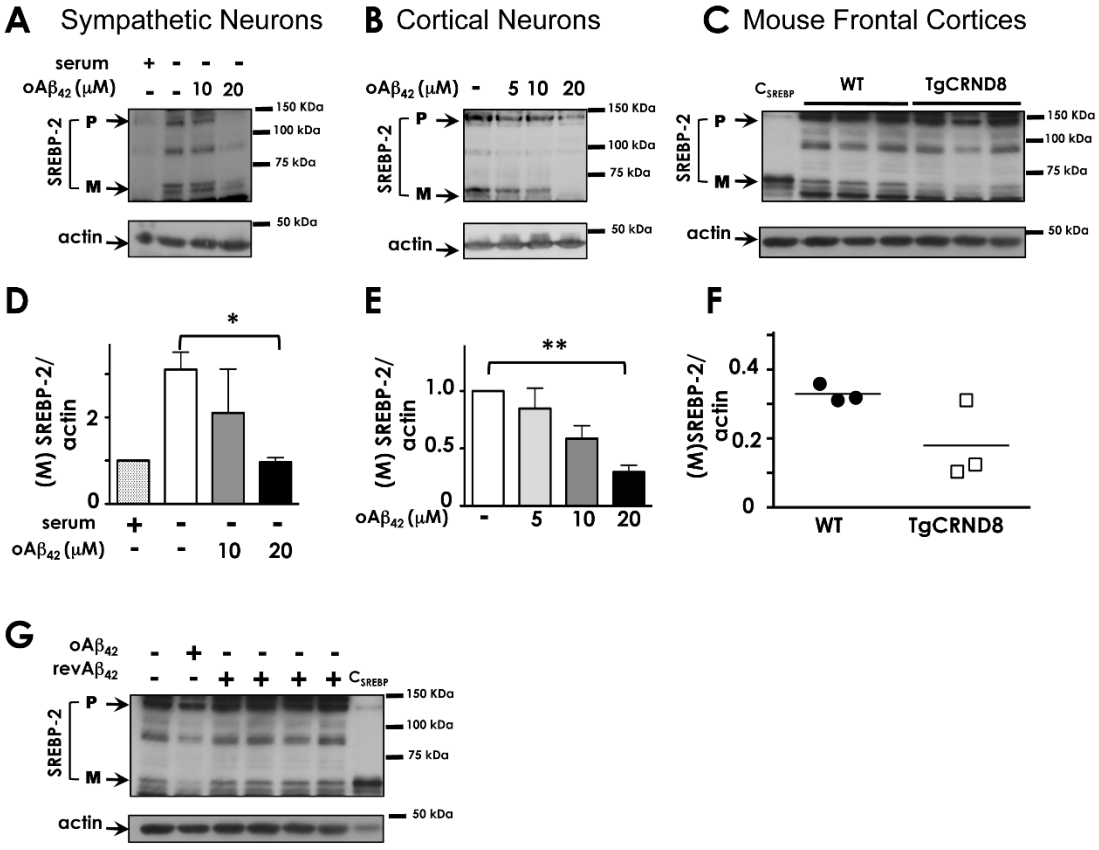


Figure 5.1: oAβ$_{42}$ reduces the level of (M)SREBP-2 in neurons. A-C and G) Analysis of SREBP-2 processing. Neurons were incubated as indicated at the top of the blots for 24h at which time they were harvested and lysed. Frontal cortices of wild type mice and TgCRND8 mice were collected, homogenized and equal amount of protein of each homogenate were loaded on SDS-PAGE. Proteins were separated by SDS-PAGE and SREBP-2 was detected by immunoblot analysis. C_{SREBP} is a control generated by overexpression of (M)SREBP2 in St14A cells and used to confirm the band of (M)SREBP-2 in inmmunoblots. D-F**)** Densitometric analysis of SREBP-2 cleavage combining five experiments (for neurons) and 3 animals (for brains). Data are means ±S.E. For all experiments *($p<0.05$), **($p<0.01$) and ***($p<0.001$) one-way ANOVA.

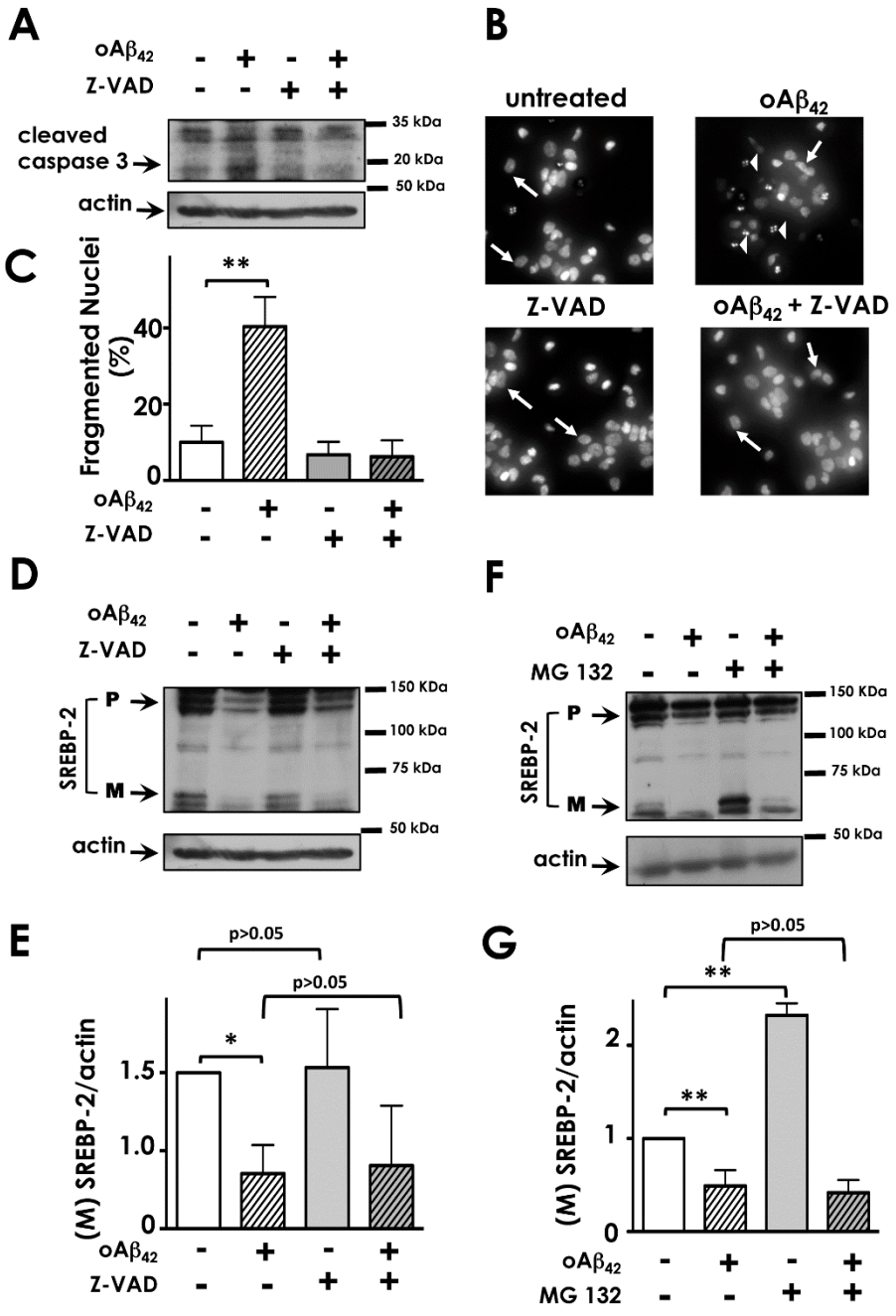


Figure 5.2: The decrease of (M)SREBP-2 by oAβ$_{42}$ is independent of apoptosis and SREBP-2 degradation. A-C) Caspase-3 activation and apoptosis induced by oAβ$_{42}$. Cortical neurons were incubated in medium containing oAβ (20μM) or ZVAD (50μM) or a combination of both for 24hr. Neurons were harvested and lysed. Proteins were analyzed by SDS-PAGE and cleaved caspase 3 was detected by immunoblot analysis. Nuclear fragmentation was evaluated by Hoechst 33258 staining. Data in B) are expressed as means ± S.E.M. of three experiments. Each experiment was performed in quintuplicate and 500–1000 neurons per treatment were counted. Statistically significant differences from cultures given no Aβ ($p < 0.01$)

are indicated by the symbol ** and were evaluated by the Kruskal–Wallis test with Dunn's multiple post hoc comparison test. C) Fluorescence microscopy of neurons treated as in A). White arrows point to normal nuclei and white arrowheads indicate fragmented nuclei. D, E) SREBP-2 cleavage was examined in neurons receiving oAβ_{42} with and without the caspase inhibitor ZVAD (50 μM). F,G) SREBP-2 cleavage was examined in neurons treated with oAβ_{42} for 24 in the absence or presence of the proteasome inhibitor MG132 (10 μM) for the last 2hr. For experiments in E and G densitometric analysis combines 3 experiments. Data are means ±S.E.*($p<0.05$), **($p<0.01$) one-way ANOVA.

does not affect SREBP-2 degradation but more likely oAβ_{42} acts directly on SREBP-2 processing.

5.2.3. oAβ_{42} does not affect the enzymatic proteolysis of SREBP-2.

The physiological mechanism of SREBPs maturation warrants that, unless required by the cell, SREBPs present in the ER remain separated from the enzymes that cleave them, which reside in the Golgi. In order to determine if oAβ_{42} affects the enzymatic proteolysis of SREBP-2 “per se”, we used Brefeldin A (BFA), a fungal metabolite that causes the redistribution of Golgi components to the ER (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989) and allows the cleavage of (P)SREPB-2 without the requirement of transport of the complex SCAP/SREBP-2 to the Golgi (DeBose-Boyd et al., 1999). We first examined the cellular distribution of the Golgi complex in neurons treated with BFA by detecting the Golgi resident protein giantin by indirect immunofluorescence microscopy (Figure 5.3-A). Treatment with BFA abolished the perinuclear dense Golgi staining observed in untreated cells indicating the reorganization of the Golgi and ER. We next tested BFA at different concentrations in cortical neurons and confirmed the increased in SREBP-2 cleavage caused by the collapse of ER and Golgi (Figure 5.3-B and 5.3-C, -Ly group). In the same experiment we used the phosphoinositide-3-kinase (PI3K) inhibitor LY294002, which inhibits SREBP-2 cleavage in CHO cells by preventing transport of SCAP/SREBP-2 to the Golgi (Du et al., 2006; Luu et al., 2012; Yamauchi et al., 2011). We show here that in cortical neurons LY294002 also inhibits SREBP-2 cleavage in the absence of BFA but not in the presence of BFA, as expected from its mechanism of action (Figure 5.3-B and 5.3-C, Ly-treated group). Similarly, oAβ_{42} reduced SREBP-2 cleavage in the absence of BFA but was unable to inhibit SREBP-2 maturation in the presence of BFA (Figure 5.3-D

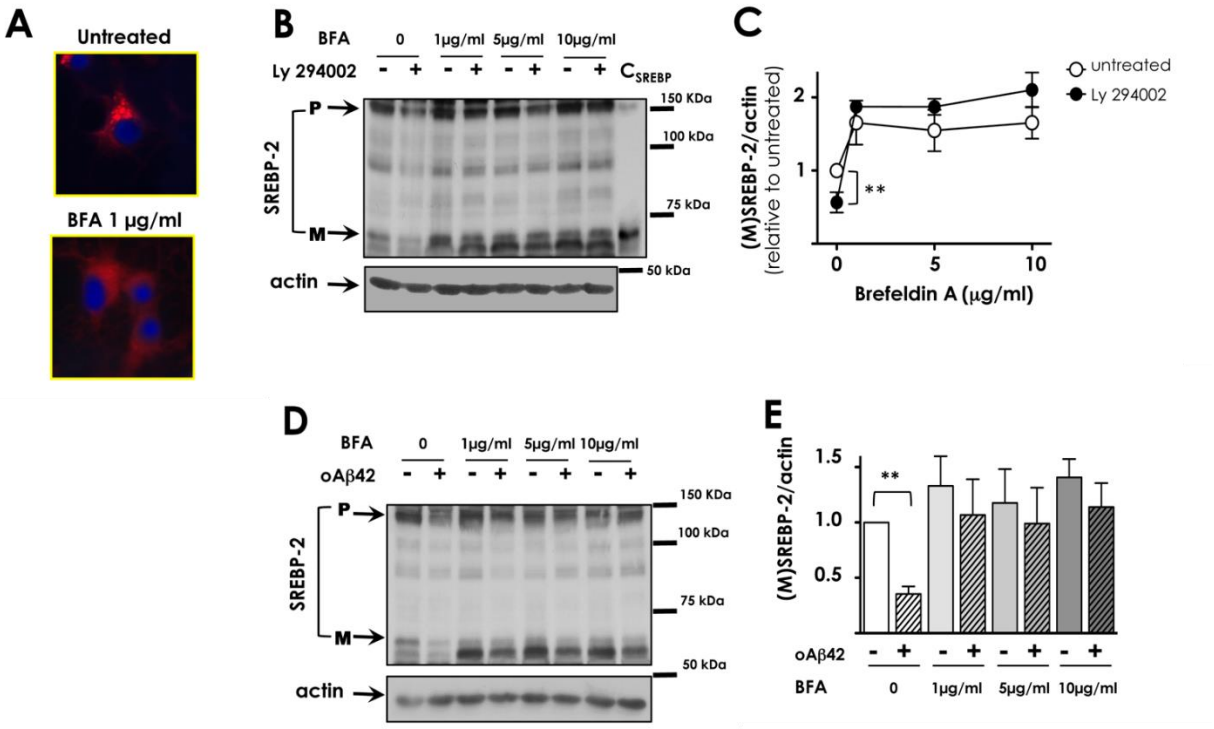


Figure 5.3: oAβ$_{42}$ does not affect SREBP-2 proteolytic cleavage at the Golgi A) Brefeldin A causes disassembly of the Golgi complex in cortical neurons. Notice the dense perinuclear Golgi staining in untreated neurons compared with the diffused red fluorescence in neurons that have received 1µg/ml BFA for 5 hs. B-E) BFA precludes the inhibition of SREBP-2 cleavage by the PI3K inhibitor LY294002 and by oAβ$_{42}$. Cortical neurons were incubated with LY294002 (B,C) or oAβ$_{42}$ (D,E) for 24hr. In the last 5hr BFA was added to the medium at different final concentrations (1, 5, 10 µg/ml). Neurons were harvested and lysed. Proteins were separated by SDS-PAGE and SREBP-2 was detected by immunoblot analysis (B,D). C and E represent the densitometeric analysis of SREBP-2 cleavage combining 3 and 4 experiments respectively. Data are means ±S.E. **($p<0.01$) one-way ANOVA.

and 5.3-E). This indicates that oAβ_{42} does not affect the enzymatic cleavage of SREBP-2 and suggests that oAβ_{42} alters a step in SREBP-2 maturation that lays upstream SREBP-2 cleavage, possibly the transport of (P)SREBP-2 from ER to Golgi.

5.2.4. oAβ_{42} does not affect the nuclear transport of (M)SREBP-2.

In order to confirm that the SREBP-2 pathway downstream of the Golgi is normal in the presence of oAβ_{42}, we expressed a nuclear form of SREBP-2 (EGFP-human nSREBP-2) in the cell line ST14A and examined the subcellular localization by confocal microscopy. First, we confirmed that oAβ_{42} reduces the level of (M)SREBP-2 in ST14A cells in a dose-dependent manner similar to cortical and sympathetic neurons (Figure 5.4-A and B). Second, all EGFP-human nSREBP-2-transfected cells treated with oAβ_{42} at the concentration that reduces SREBP-2 cleavage, displayed EGFP-nSREBP-2 present in the nucleus (Figure 5.4-C) indicating that oAβ_{42} does not affect nuclear translocation of (M)SREBP-2.

5.2.5. oAβ_{42} reduces cellular level of phosphorylated Akt and Sec24D.

Cholesterol and oxysterols have been recognized as the main regulators of transport of SCAP/SREBP-2 from ER to the Golgi (Adams et al., 2004; Nohturfft et al., 2000; Radhakrishnan et al., 2007; Radhakrishnan et al., 2004). In addition, Brown's team has recently discovered the involvement of the PI3K/Akt pathway in this process (Du et al., 2006; Luu et al., 2012). Inhibition of the PI3K/Akt pathway reduces the level of (M)SREBP-2 by disrupting the transport of the SREBP-SCAP complex from ER to Golgi (Du et al., 2006). Based on previous evidence indicating that Aβ inhibits the PI3K/Akt pathway (Jimenez et al., 2011; Lee et al., 2008; Takashima et al., 1996) we tested the hypothesis that inhibition of SREBP-2 maturation by oAβ_{42} resulted from inhibition of the PI3K/Akt pathway. Using

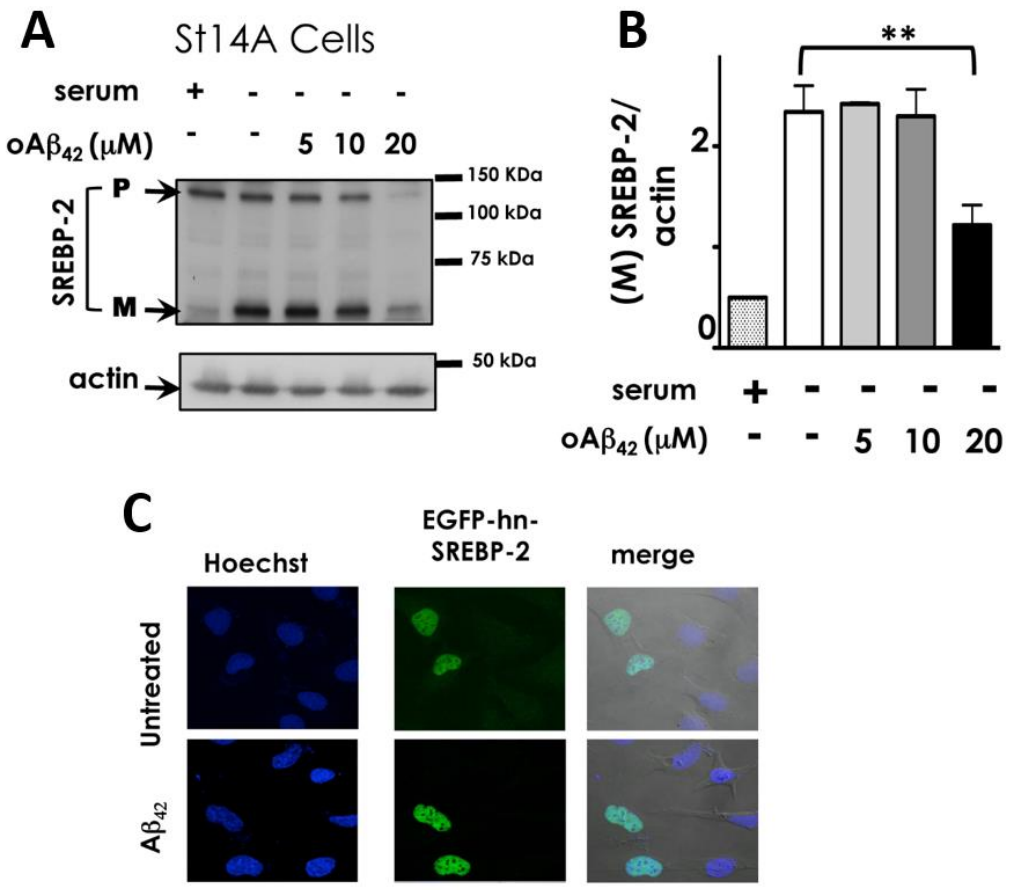
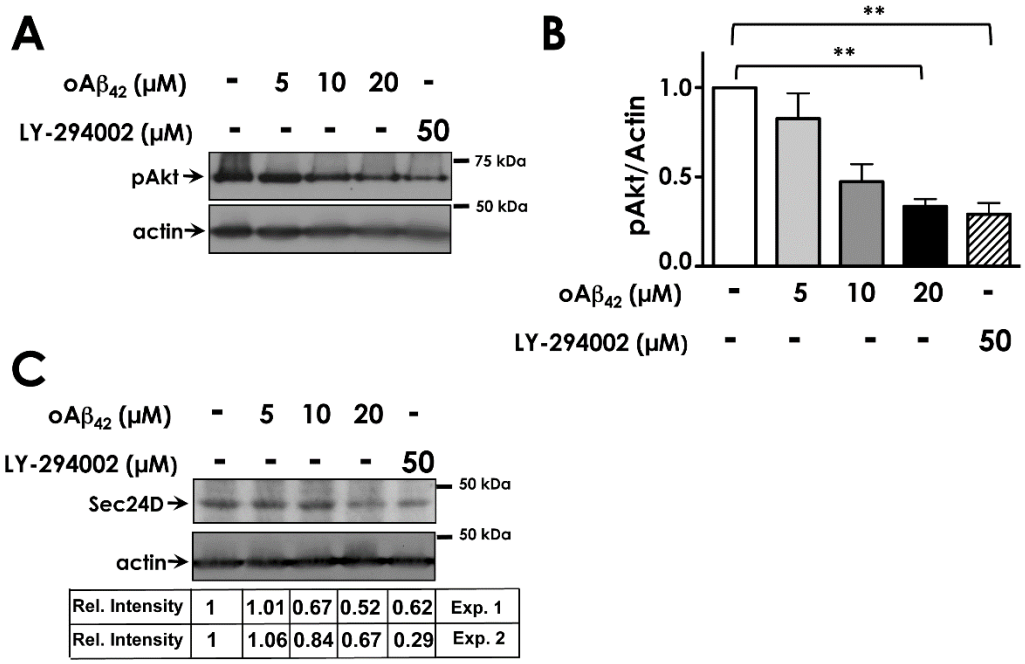


Figure 5.4: oAβ_{42} does not affect (M)SREBP-2 nuclear transport. A) Neuronal ST14A cells were incubated as indicated at the top of the blot for 24h at which time cells were harvested, lysed and examined for SREBP-2 cleavage by immunoblot analysis. B) Densitometric analysis of SREBP-2 cleavage combining five experiments. Data are means ±S.E. For all experiments *($p<0.05$), **($p<0.01$) and ***($p<0.001$) one-way ANOVA. C) Neuronal ST14A cells expressing a human form of (M)SREBP-2 (EGFP-hn-SREBP-2) were exposed to oAβ_{42}(20μM) for 24 hr, then cells were fixed and stained with Hoechst. Representative fields are shown.

immunoblot analysis with an antibody specific for the active form of Akt (p^{Ser473}Akt), we examined the effect of oAβ_{42} on Akt activation. As a control we used the PI3K inhibitor LY-294002, which causes severe reduction of pAkt in primary neurons (Song and Posse de Chaves, 2003). Neurons exposed to oAβ_{42} at the concentration that affects SREBP-2 (20μM) have reduced levels of cellular pAkt (Figure 5.5-A and B). Inhibition of Akt phosphorylation by PI3K and Akt inhibitors reduces transport of the complex SCAP-SREBP-2 from ER to Golgi by reducing interaction of SCAP with some components of COP-II vesicles, mainly sec24D (Sharpe et al., 2011). Inhibition of Akt by LY-294002 causes reduction in sec24D protein level (Sharpe et al., 2011). Similarly, we found that sec24D is reduced in neurons exposed to oAβ_{42} (Fig. 5.5-C). This supports the hypothesis that oAβ_{42}-induced reduction in pAkt level is the mechanism underlying the oAβ_{42}–induced inhibition of SREBP-2 cleavage.

5.2.6. Expression of constitutively active Akt prevents inhibition of SREBP-2 cleavage by oAβ_{42}.

In order to prove that the reduction in pAkt levels by oAβ_{42} is the cause of the decrease in SREBP-2 cleavage, we examined if oAβ_{42} is able to inhibit SREBP-2 cleavage in primary neurons expressing a constitutively active form of Akt (myristoylated Akt) (Kohn et al., 1996). oAβ_{42} significantly reduced (M)SREBP-2 in non-infected cortical neurons and in cortical neurons infected with adenoviral particles carrying an empty vector but neuronal infection with adenovirus expressing myrAkt prevented oAβ_{42}-induced inhibition of SREBP-2 cleavage (Figure 5.6-A, B and C). This finding indicates that oAβ_{42} reduces nuclear SREBP-2 cleavage via inhibition of Akt activation.



Rel. Intensity	1	1.01	0.67	0.52	0.62	Exp. 1
Rel. Intensity	1	1.06	0.84	0.67	0.29	Exp. 2

Figure 5.5: oAβ$_{42}$ reduces cellular level of phosphorylated Akt and Sec24D. Cortical neurons were treated with oAβ$_{42}$ or LY294002 as indicated on the top of the blots for 24 hr after which neurons were harvested and analyzed for p^{Ser473}Akt (A,B) and Sec24D (C) by western blot analysis. Densitometric analysis of pAkt (B) combines 4 experiments. Data are means ±S.E.*($p<0.05$), **($p<0.01$) one-way ANOVA.

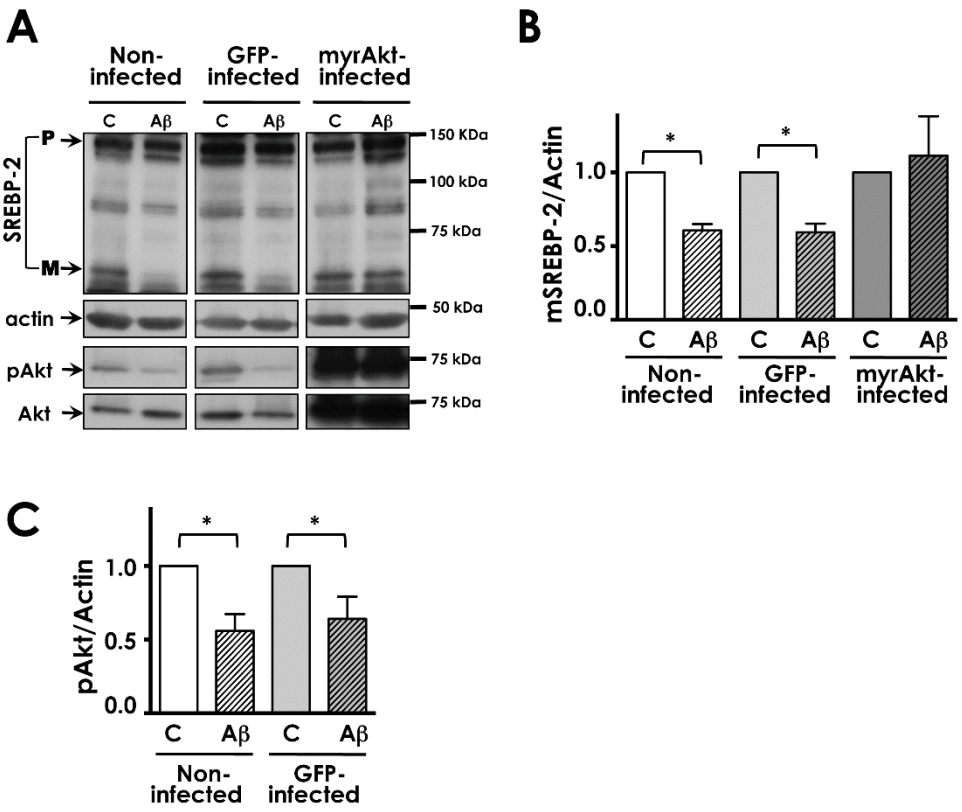


Figure 5.6: Constitutively active Akt prevents oAβ_{42}-induced reduction of (M)SREBP-2 level. Uninfected, GFP infected and myr-Akt infected cortical neurons were exposed to oAβ_{42} (20μM) for 24 hr. The cells were harvested and lysed. Proteins were analyzed by SDS-PAGE. A) SREBP-2, pAkt, Akt and actin were detected by immunoblot anlaysis. B, C) Densitometric analysis combining 3-4 experiments. Data are means ±S.E.*($p<0.05$) one-way ANOVA.

5.3. Discussion:

Several discoveries have indicated that cholesterol plays important roles in AD (reviewed in (Bu, 2009; Maulik et al., 2013; Posse de Chaves, 2012; Posse de Chaves and Narayanaswami, 2008; Shepardson et al., 2011; Verghese et al., 2011). The pools of cholesterol in the brain and in the periphery are strictly separated by the BBB, and brain cholesterol homeostasis is regulated independently of peripheral organs (Dietschy and Turley, 2004). Cholesterol synthesis *in situ* in the brain is sufficient to meet the demands of the CNS during development and in adult life. This local synthesis may decrease with age (Dietschy, 2009; Dietschy and Turley, 2004; Thelen et al., 2006; Yanagisawa, 2002). Cholesterol biosynthesis involves successive enzymatic reactions collectively known as the mevalonate pathway. In addition to providing cholesterol, the mevalonate pathway is also the source of short-chain isoprenoids FPP and GGPP used for post-translational modification of proteins (protein prenylation). There is limited information on the status and regulation of the mevalonate pathway in AD. One report on HMGR in AD, found no change in the transcript levels of HMGR (Yasojima et al., 2001). However the Dhcr24 gene product seladin-1/DHCR24 is downregulated in AD brains (Greeve et al., 2000; Iivonen et al., 2002), suggesting that overall cholesterol synthesis could be inhibited. At the subcellular level there is a reciprocal regulation between cholesterol and Aβ production. We, and others have shown that Aβ peptides inhibit cholesterol synthesis (Gong et al., 2002; Grimm et al., 2005). We demonstrated that oAβ_{42} inhibit SREBP-2 maturation in cultured neurons. Here we show that the levels of (M)SREBP-2 are reduced in the frontal cortex of the TgCRND8 mouse suggesting that the negative regulation of SREBP-2 may also occur *in vivo* in AD.

Inhibition of cholesterol synthesis in neurons that accumulate Aβ might not have dramatic consequences since neurons, especially in adulthood, might not synthesize cholesterol but outsource it from astrocytes (Aqul et al., 2011; Funfschilling et al., 2007). Conversely, inhibition of protein prenylation has significant consequences such as neuronal death (Marz et al., 2007; Tanaka et al., 2000) (and data presented in chapter-4), accumulation of full length-APP, APP fragments, and intracellular Aβ with parallel decrease of secreted Aβ (Cole et al., 2005; Ostrowski et al., 2007) among others. The role of protein prenylation in AD has emerged from studies using statins, which inhibit HMGR. Since Aβ acts on the mevalonate pathway through SREBP-2 and SREBP-2 regulates several enzymes both upstream and downstream HMGR, including FPP and GGPP synthases it is expected that the effect of Aβ on protein prenylation would be more severe than the effect of HMGR inhibitors. Recently, it was reported that APP also controls neuronal cholesterol synthesis through the SREBP pathway (Pierrot et al., 2013). This study showed that APP levels inversely correlates with precursor SREBP-1 level in mice and human brains and demonstrated that inhibition of the mevalonate pathway by APP impairs neuronal activity. The interaction of APP and SREBP-1 in the Golgi prevents the release of mature SREBP-1 and the translocation of SREBP-1 to the nucleus. Our data, on the other hand, indicate that $A\beta_{42}$ does not affect SREBP-2 enzymatic cleavage. Under conditions in which the ER and the Golgi are forced to fuse, the cleavage of SREBP-2 is normal in the presence of $oA\beta_{42}$, suggesting that it is the transport of (P)SREBP-2 from the ER to the Golgi which is affected. In addition, we also show that $oA\beta_{42}$ does not alter the translocation of (M)SREBP-2 to the nucleus, neither increases (M)SREBP-2 degradation by the proteasome. The mechanism of action of $oA\beta_{42}$ on SREBP-2 regulation that we uncovered resembled closely to the mechanism of the PI3K/Akt

inhibitor LY294002 (Du et al., 2006). Several studies had linked Akt to the SREBPs such that pharmacological inhibition of PI3K/Akt decreased (M)SREBP-1 in response to insulin, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGF) (Demoulin et al., 2004; Hegarty et al., 2005; Zhou et al., 2004). Later it was discovered that inhibition of the PI3K/Akt pathway also regulates SREBP-2 by targeting the ER-to-Golgi transport (Du et al., 2006; Luu et al., 2012). Akt phosphorylates Sec24, an essential coat protein II (COPII) component involved in mediating cargo selection for ER-to-Golgi trafficking (Sharpe et al., 2011), which could explain the enhancement of SCAP transport from ER to the Golgi induced by Akt activation as well as the inhibition by inhibitors of the PI3K/Akt pathway (Du et al., 2006). The evidence described above prompted us to investigate the status of Akt activation in neurons challenged with $oA\beta_{42}$. We found that levels of activated p^{Ser473}Akt are significantly reduced in $oA\beta_{42}$-treated neurons. Moreover, a decrease in Akt activation explains the inhibition of SREBP-2 cleavage by $oA\beta_{42}$ because Aβ was unable to inhibit SREBP-2 cleavage in neurons expressing constitutively active Akt.

Our findings are in agreement with several previous studies that show a reduction of Akt activation due to Aβ (Jimenez et al., 2011; Lee et al., 2009; Lee et al., 2008; Magrane et al., 2005; Townsend et al., 2007), although other reports show that Aβ peptides could activate Akt (Heras-Sandoval et al., 2012). PI3K and Akt activity are significantly reduced in AD brain (Jolles et al., 1992; Lee et al., 2009; Liu et al., 2011). The mechanisms that explain the inhibition of Akt by Aβ have been investigated. Lee and collaborators suggested that extra and intracellular Aβ have different targets in the PI3K/Akt pathway (Lee et al., 2009). Their work shows that intraneuronal Aβ might block the interaction between

phosphoinositide dependent protein kinase-1 (PDK1) and Akt, thereby preventing Akt activation. There is also evidence that Aβ binds to the insulin receptor (IR) or to a receptor complex that includes IRs and interferes with insulin-induced IR autophosphorylation and consequently interferes with the PI3K/Akt cascade activated downstream the IRs (Townsend et al., 2007; Xie et al., 2002; Zhao et al., 2008). Others have demonstrated that Aβ causes down-regulation of IRs at the plasma membrane and redistribution of IRs to the cell bodies of neurons (De Felice et al., 2009; Zhao et al., 2008). Thus, the decrease of Akt activation may be indirect, through the inhibition of IRs or other growth factors receptors.

Akt phosphorylates a large number of targets (Hemmings and Restuccia, 2012). One Akt target that has received attention in the context of AD, in particular for the crosstalk between AD and diabetes is GSK-3β. GSK-3β is involved in tau phosphorylation (Hooper et al., 2008; Takashima, 2006) and is inhibited by Akt. Therefore, down-regulation of insulin signalling could ultimately lead to both decreased glucose metabolism and increased tau phosphorylation through GSK-3 activation (Liu et al., 2011; Takeda et al., 2011). Our work reports a novel consequence of Aβ-induced Akt inhibition which is the inactivation of SREBP-2, which in turn causes inhibition of cholesterol synthesis and inhibition of protein prenylation. Novel functional roles for SREBPs have been revealed by genome-wide analysis (Reed et al., 2008; Seo et al., 2009; Seo et al., 2011). These global studies provided evidence that SREBPs play a more comprehensive role in physiology and metabolism through activation of additional key target genes that participate in several cellular processes such as autophagy, phagocytosis, hypoxia, …etc (Jeon and Osborne, 2012). In this context our findings here indicate that SREBP-2 may also plays a role in disease development.

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Chapter 6: General Discussion & Conclusions

The main discoveries presented here are; 1) oAβ_{42} causes intracellular sequestration of cholesterol, 2) oAβ_{42} reduces maturation of SREBP-2, inhibits cholesterol synthesis and 3) oAβ_{42} inhibits protein prenylation.

oAβ_{42} and cholesterol sequestration.

Several studies demonstrated cholesterol sequestration in AD (Bandaru et al., 2009; Cutler et al., 2004; Lazar et al., 2013; Sparks, 1997; Xiong et al., 2008). It has also been suggested that cholesterol trafficking might be impaired in AD based on a compensatory increase in NPC1 expression observed in AD brain (Kagedal et al., 2010). Moreover, 2HP-β-CD, a cyclodextrin that reduced cholesterol sequestration and neurodegereneration in NPC, improved memory and learning in AD transgenic mouse model (Yao et al., 2012). All these studies indicate that cholesterol sequestration and impaired cholesterol trafficking are involved in AD pathology, however, nothing is known about the origin/cause of these changes in cholesterol homeostasis in AD. In this thesis, we presented a novel evidence that oAβ_{42} induces cholesterol sequestration and impairs intracellular cholesterol trafficking in neurons.

Amyloidogenic processing of APP to Aβ is increased by cholesterol sequestration in NPC models (Burns et al., 2003; Jin et al., 2004; Kosicek et al., 2010; Mattsson et al., 2011; Runz et al., 2002). It is also stimulated by Aβ itself leading to a morbid vicious cycle. Several mechanisms have explained this vicious cycle (Bahr et al., 1998; Tampellini et al., 2009; Yang et al., 1995). One study correlated the reduction of membrane fluidity that takes place upon Aβ interaction with ganglioside GM1 with the enhancement of Aβ production (Peters et al., 2009). In addition, Aβ-induced oxidative stress, inflammation and perturbed calcium homeostasis enhance β-secretase1 (BACE1) activity and consequently increase

APP processing and Aβ production (Chami and Checler, 2012). Our novel finding that oAβ_{42}-induced cholesterol sequestration, impaired cholesterol trafficking and cholesterol enrichment in lipid rafts could represent a new mechanism underlying Aβ-induced increase of its own production.

oAβ_{42} and SREBP-2.

We discovered that oAβ_{42} inhibits cholesterol synthesis by reducing SREBP-2 cleavage. SREBP-2 is a transcription factor that preferentially regulates transcription of most of the enzymes involved in the mevalonate pathway (Horton et al., 2003). SREBP-2 is a positive regulator of the transcription factor LXR, through generation of oxysterol ligands for LXR (Wong et al., 2006). Very importantly, LXR agonists are being regarded as possible therapeutic agents in AD based on studies in which these agents showed beneficial effects with respect to reduced APP processing into Aβ, reduced levels of Aβ peptides, and improved memory function (Hirsch-Reinshagen et al., 2009; Sodhi and Singh, 2013). LXR activation positively regulates transcription of the cholesterol efflux gene, ABCA1, among other genes. Our work suggests that Aβ via reducing (M)SREBP-2 level and consequently the production of LXR agonists would reduce LXR activation and ABCA1 transcription. In line with this idea Aβ_{42} reduces expression of ABCA1 in an astrocytic cell line and ABCA1 protein expression is reduced in brain of APP/PS1 mice (Canepa et al., 2011). Interestingly, ABCA1 level has been negatively correlated to Aβ production (Cui et al., 2011; Sun et al., 2003) and positively correlated to Aβ degradation (Fitz et al., 2010; Lee et al., 2012). Therefore, our work suggests that Aβ would reduce LXR activation and ABCA1 expression.

One important LXR target is *Dhcr24,* which encodes Seladin-1/DHCR24 (Wang et al., 2008). *Dhcr24* is downregulated in AD brains (Greeve et al., 2000; Iivonen et al., 2002). Seladin-1 is considered neuroprotective due to its antiapoptotic activity and its involvement in hormone-mediated neuroprotection in AD (Peri et al., 2011). The available data indicate that reduction of LXR and Seladin-1 are important in AD but the causes for their downregulation have not been elucidated yet. Our work opens the possibility that Aβ could regulate Seladin-1 levels and LXR activation by inhibiting SREBP-2 cleavage and reducing formation of oxysterols.

oAβ_{42} and protein prenylation.

Seladin-1 downregulation in AD brain may indicate an overall reduction of the cholesterol synthesis in AD (Greeve et al., 2000; Iivonen et al., 2002). Through the mevalonate pathway, cholesterol and isoprenoids are synthesized. FPP and GGPP are required for protein prenylation (Holstein and Hohl, 2004). Exogenous supply of isoprenoids or small concentration of mevalonate restored normal neuronal functions and prevented statins-induced toxic effects in neurons (Kim et al., 2009; Marz et al., 2007; Meske et al., 2003; Schulz et al., 2004; Tanaka et al., 2000). These studies indicate the essential role of protein prenylation for proper neuronal functions and neuronal viability. Although one study showed increased level of FPP and GGPP in the brains of thirteen males with AD (Eckert et al., 2009), protein prenylation has not been studied in AD. We demonstrated in this thesis that oAβ_{42} inhibits protein prenylation, which could be corrected by exogenous supply of GGPP. Therefore, our work suggests that oAβ_{42} reduces SREBP-2 cleavage leading to inhibition of the mevalonate pathway, reduction of cellular levels of isoprenoids and consequent inhibition of protein prenylation. We established

oAβ_{42}-induced inhibition of protein prenylation as a novel mechanism underlying oAβ_{42}-induced cholesterol sequestration, impaired cholesterol trafficking and neurotoxicity.

In our studies we looked at prenylation of small GTPase. Small GTPases are important in the nervous system and in AD pathology (reviewed in (Hooff et al., 2010)), thus our work opens a new area of research which aims at studying the role of oAβ_{42}-induced inhibition of protein prenylation of different small GTPases in the development of pathological changes observed in AD as discussed below.

Endosomal abnormalities and axonal impairments in AD:

Endosomal abnormalities are one of the earliest pathological changes in AD (Cataldo et al., 1997; Nixon, 2005). Endosomal abnormalities have been linked to increased Aβ production in AD and DS (Cataldo et al., 2004). Neurons are very sensitive to any impairment in the endosomal system as endosomes are involved in intra-cellular communication between neuronal cell bodies and axons (anterograde and retrogarade transport) (Nixon, 2005). Bidirectional axonal transport is highly essential for neuronal survival and function (Hirokawa and Takemura, 2005). Impaired axonal transport has been shown to predispose to neurodegeneration (Chevalier-Larsen and Holzbaur, 2006).

Retrograde transport of neurotrophins/neurotrophic receptors is essential for neuronal viability. Impaired retrograde transport of nerve growth factor (NGF) induces neurodegeneration of cholinergic basal forebrain neurons (Mufson et al., 1995; Salehi et al., 2006; Salehi et al., 2004). Reduced brain derived neurotrophic factor (BDNF) retrograde transport has been observed in Tg2576, a transgenic animal model of AD (Poon et al., 2011). Interestingly, endosome associated Rab7

has been shown to regulate retrograde transport of neurotrophins and neurotrophic receptors (Deinhardt et al., 2006). Therefore, oAβ_{42}-induced inhibition of protein prenylation of Rab7 may be responsible for the reduced retrograde transport of TrkA that we demonstrated and reduced retrograde transport of neurotrophins/neurotrophin receptors in AD.

Anterograde transport is required for axonal growth and maintenance. oAβ_{42} may reduce axonal growth via inhibition of protein prenylation of Rab33a, which participates in anterograde transport of synaptophysin-positive vesicles and their fusion with the growth cones required for axonal growth (Nakazawa et al., 2012). Moreover, oAβ_{42} may reduce dendrite formation via inhibition of protein prenylation of Rab17 that mediates membrane trafficking required for dendritogenesis in hippocampal neurons (Mori et al., 2012). In addition, we demonstrated that oAβ_{42}-induced inhibition of protein prenylation causes intracellular cholesterol sequestration and impaired anterograde transport of cholesterol, which could lead to axonal loss observed in AD (Dickson and Vickers, 2001; Song et al., 2006; Spires and Hyman, 2004).

Tau protein is axonally transported and is involved in regulation of axonal transport. Tau hyperhosphorylation has been linked to impaired axonal transport in AD (Johnson and Stoothoff, 2004; Utton et al., 2005). Rho GTPases (RhoA, Rac, Cdc42) regulate the dynamics and stability of the cytoskeleton (Linseman and Loucks, 2008). One study showed that statin-induced neuronal degeneration was accompanied by cytoskeletal abnormalities, reduced level of membrane associated RhoA and hyperphosphorylation of tau (Meske et al., 2003). These effects were completely reversed by co-treatment with GGPP and mevalonate (Meske et al., 2003). This study suggested that inhibited RhoA prenylation by

statins was involved in statins-induced hyperphosphorylation of tau although prenylation was not directly examined. In AD brain, total RhoA was significantly reduced and highly colocalized with hyperphosphorylated tau (Huesa et al., 2010). Therefore, oAβ_{42}-induced inhibition of protein prenylation of RhoA could be a novel mechanism that underlies the previously observed Aβ-induced tau hyperphosphorylation (De Felice et al., 2008; Zheng et al., 2002) and consequent Aβ-induced axonal abnormalities.

Impaired autophagy in AD:

Impaired autophagy is one of the major pathological changes observed in AD brains (Nixon et al., 2005) and have been linked to increased Aβ production (Nixon, 2007; Yu et al., 2005), increased neuronal loss (Yang et al., 2008) and consequently development of learning and memory deficits (Yang et al., 2011). Autophagy is the cellular degradation of cytoplasmic cargos via their delivery to the lysosomes (Levine and Kroemer, 2008). Macroautophagy, usually referred to as autophagy, starts with the formation of isolation membrane called phagophore (nucleation step). This phagophore sequesters a region of the cytoplasm forming a double membrane vesicle called autophagosome (expansion/elongation step). Then, these autophagosomes fuse with the lysosomes forming autophagolysosomes (fusion step), in which lysosomal enzymes degrade autophagolysosomal inner membrane and its contents (degradation step) (Levine and Kroemer, 2008; Nixon, 2006; Nixon, 2007). Cortical biopsies from AD patients showed accumulation of autophagic vacuoles mainly in the dystrophic neurites. These autophagic vacuoles includes autophagosomes that did not fuse with lysosome (pre-lysosomal) and autophagolysosomes (late autophagic vacuoles) (Nixon et al., 2005). The accumulation of these autophagic vacuoles in AD brains

indicates that fusion with the lysosomes and/or further degradation are inhibited (Nixon et al., 2005; Nixon et al., 2008). Different Rab proteins are involved in autophagy (Hirota, 2013). Excitingly, Rab7 is involved in the autophagosome-lysosome fusion (Gutierrez et al., 2004; Hyttinen et al., 2013; Jager et al., 2004). We propose that reduced Rab7 prenylation we observed in oAβ_{42}-treated neurons could play a role in the development of impaired autophagy in AD and exogenous supply of GGPP could restore the normal autophagic flow in these neurons. Preliminary experiments in our laboratory support this hypothesis.

Memory deficits and excitotoxicity in AD:

Aberrant glutamatergic transmission plays a major role in Aβ-induced excitotoxicity, suppression of LTP and development of memory deficits observed in AD (Danysz and Parsons, 2012). Aβ directly or indirectly activates NMDA receptors resulting in excessive neuronal calcium influx and excitotoxic cell loss (Danysz and Parsons, 2012; Paula-Lima et al., 2013) . Rab5b participates in NMDA receptor endocytosis leading to reduced neuronal vulnerability to excitotoxicity (Arnett et al., 2004; Baskys et al., 2007; Blaabjerg et al., 2003). Thus oAβ_{42}-induced inhibition of protein prenylation of Rab5b would lead to increased neuronal vulnerability to excitotoxicity.

Furthermore, Aβ accumulation in the glutamateric presynaptic terminals observed in AD brains (Sokolow et al., 2012) would reduce neurotransmitter release (Chiang et al., 2009), which could lead to inhibition of LTP. Interestingly, Rab3 and Rab11 are involved in calcium-triggered synaptic exocytosis and neurotransmitter release (Castillo et al., 1997; Khvotchev et al., 2003). Accordingly, oAβ_{42}-induced inhibition of protein prenylation of Rab3 and Rab11

may be one of the underlying mechanisms of presynaptic Aβ-induced reduced neurotransmitter release.

Several studies illustrated small GTPases as potential mediators of synaptic transmission. Ras plays a role in AMPA and NMDA receptors signaling required for synaptic plasticity and LTP (Imamura et al., 2003; Yun et al., 1999; Yun et al., 1998; Zhu et al., 2002). Overexpression of constitutively active Ras has been associated with enhanced LTP, which is mediated via NMDA receptor activation (Arendt et al., 2004). Furthermore, RhoA, RhoB and Rac1 are involved in NMDA receptor dependent synaptic plasticity and memory formation (Hooff et al., 2010; Norenberg et al., 1999; O'Kane et al., 2003; Tejada-Simon et al., 2006). Moreover, Rab 8, 10 and 11 regulates AMPA receptor endosomal membrane trafficking into the synaptic membrane which is essential for synaptic plasticity and LTP (Brown et al., 2007; Gerges et al., 2004; Glodowski et al., 2007; Seebohm et al., 2012). Therefore, o$A\beta_{42}$-induced inhibition of protein prenylation of Ras, Rho and Rab proteins could play a role in Aβ-induced modulation of glutamatergic transmission.

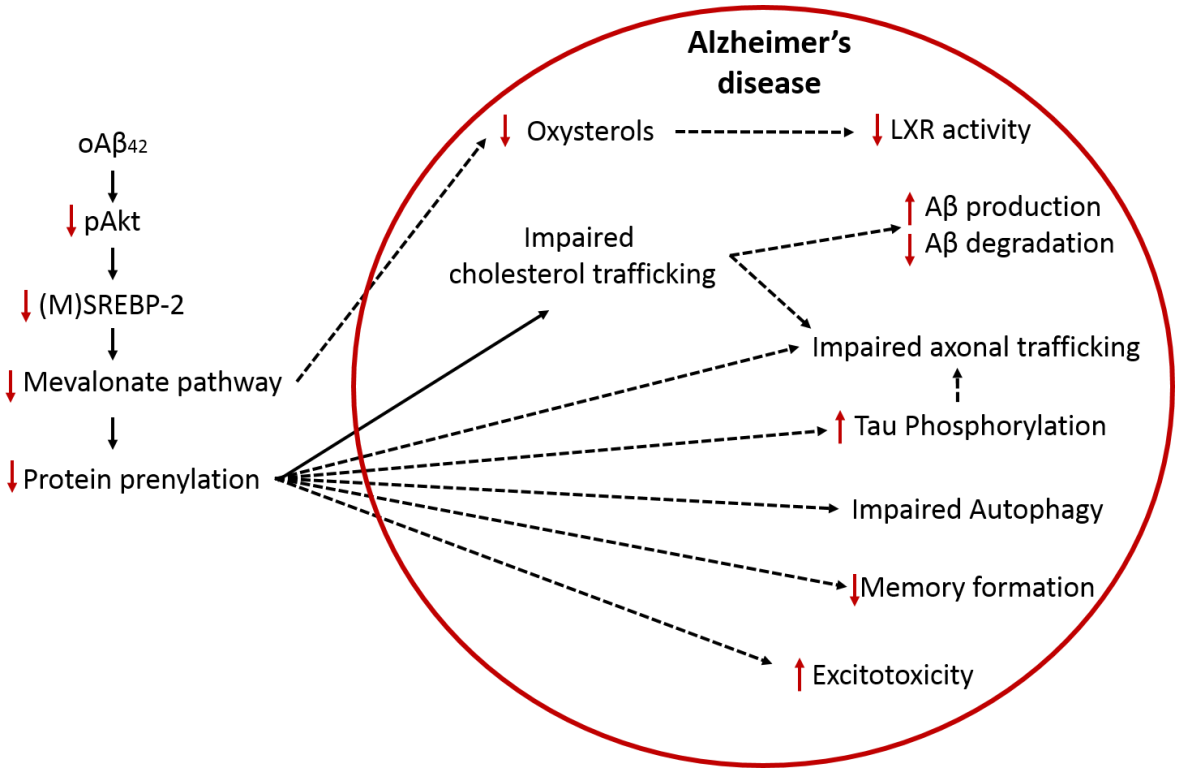

Figure 6.1: Final model summarizing the major findings and the future implications.

Conclusion:

Mounting evidence demonstrated the regulation of Aβ production, degradation, internalization and toxicity by cholesterol, however, very little is known about the regulation of cholesterol by Aβ. Our study provided novel evidence that $oA\beta_{42}$ impairs cholesterol trafficking leading to cholesterol sequestration in neurons. Therefore, this study suggests that cholesterol sequestration and impaired cholesterol trafficking observed in AD could be induced by $oA\beta_{42}$. Moreover, we demonstrated that $oA\beta_{42}$-induced reduction of cellular pAkt level decreases SREBP-2 cleavage and consequently inhibits cholesterol synthesis. $oA\beta_{42}$-induced inhibition of cholesterol synthesis depletes cellular isoprenoids leading to inhibition of protein prenylation of small GTPases. We discovered that $oA\beta_{42}$-induced inhibition of protein prenylation is a novel mechanism underlying $oA\beta_{42}$-induced cholesterol sequestration and neurotoxicity. Our work opens a new broad area of research investigating the role of $oA\beta_{42}$-induced reduction of protein prenylation in the development of different pathological changes observed in AD.

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