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MITOCHONDRIA IN ALZHEIMER DISEASE: REGULATORY MECHANISMS AND CELL DEATH

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Cover picture: GFP transfected hippocampal neuron visualized with confocal microscopy.

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Dedicated to my family

"*It is always wise to look ahead, but difficult to look further than you can see*"
Winston Churchill

ABSTRACT

Synaptic loss is the major correlate for cognitive decline in Alzheimer disease (AD). Processes taking place in the synapses are highly energy demanding and needs strict regulation, which makes the mitochondria and ER crucial at these sites so as to supply energy and spatially regulate intracellular calcium signaling. The ER and mitochondria interact with each other at a highly specialized region of ER called the mitochondria-associated ER membrane (MAM). At the MAM several processes are regulated, including calcium handling, metabolism of glucose, phospholipids and cholesterol as well as apoptosis, all of which are deranged in AD. The aim of this thesis was to obtain deeper understanding of processes that could be behind mitochondrial dysfunction and caspase activation, and thereby cause synapse loss. Caspases are activated both during normal plasticity and in apoptosis, and their activation is associated with elevated Aβ production. In **Paper I,** we studied this relationship and showed that during caspase activation intracellular Aβ42/Aβ40 ratio increases due to caspase cleavage of presenilin 1 (PS1) residing in active γ-secretase complexes. Intracellular Aβ is cytotoxic and interferes with various processes for example intra-mitochondrial accumulation cause damage to mitochondrial functions. Aβ is imported into the mitochondria via the TOM40 pore. A specific polymorphic poly-T variant (rs10524523), in the *TOMM40* gene had been postulated to cause earlier disease onset of late-onset AD (LOAD) in APOE ε3/ε4 carriers. Knowing the importance of TOM40 protein we set out, in **Paper II,** to investigate the functional implication of this polymorphism. However, we could not identify any deficits in mitochondrial function or morphology. Nevertheless, the mitochondria are evidently affected by AD, as indication include altered calcium homeostasis and metabolism. These alterations can be linked to the MAM region, which is a region scarcely investigated in the brain. Therefore, in **Paper III,** studying MAM, we showed that it exists in synapses and is essential for both neuronal and astrocytic survival. Furthermore, we showed that MAM is altered in human AD brain as well as in $APP_{Swe/Lon}$ mice, and is so before the appearance of plaques. Moreover, MAM can be functionally modulated by the amyloid-β peptide (Aβ). Based on evident alterations in mitochondrial function in AD, treatments enhancing mitochondrial resistance could be a promising strategy. The final study, **Paper IV**, concerned a potential novel drug, Dimebon (Latrepirdine), intended for treatment of AD. We found it to enhance mitochondrial function both in absence and presence of stress, and, in turn, partially protect cells to maintain cell viability. Since mitochondrial function is essential for synaptic integrity drugs targeting the mitochondria could have disease-modifying effect.

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. **Louise Hedskog**, Camilla A. Hansson Petersen, Annelie I Svensson, Hedvig Welander, Lars O Tjernberg, Helena Karlström, Maria Ankarcrona.
γ-Secretase complexes containing caspase-cleaved presenilin-1 increase intracellular Aβ42/Aβ40 Ratio.
*J Cell Mol Med. Oct **15**, 2150-2163 (2011)*

II. **Louise Hedskog**, Jesper Brohede, Birgitta Wiehager, Catarina Moreira Pinho, Priya Revathikumar, Lena Lilius, Elzbieta Glaser, Caroline Graff, Helena Karlström, Maria Ankarcrona.
Biochemical studies of poly-T variants in the Alzheimer disease associated *TOMM40* gene.
J. Alzheimer Dis. May 16 (2012) [Epub ahead of print]

III. **Louise Hedskog**, Catarina Moreira Pinho, Riccardo Filadi, Laura Hertwig, Birgitta Wiehager, Annica Rönnbäck, Sandra Gellhaar, Anna Sandebring, Marie Westerlund, Caroline Graff, Bengt Winblad, Dagmar Galter, Homira Behbahani, Paola Pizzo, Elzbieta Glaser, Maria Ankarcrona.
MAM is essential for neuronal survival and modulated by Alzheimer disease pathology.
Submitted for publication

IV. Shouting Zhang*, **Louise Hedskog***, Camilla A Hansson Petersen, Bengt Winblad, Maria Ankarcrona. * *contributed equally*
Dimebon (Latrepirdine) enhances mitochondrial function and protects neuronal cells from death.
*J. Alzheimers Dis. **21**, 389-402 (2010)*

CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
ABAD	Aβ-binding alcohol dehydrogenase
AD	Alzheimer disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
Aph1	Anterior pharynx defective-1
APLP	APP-like protein
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE	B-site APP cleaving enzyme
BBB	Blood brain barrier
caspCTF	Caspase-cleaved C-terminal fragment
COX	Cytochrome c oxidase
CTF	C-terminal fragment
ΔΨm	Mitochondrial membrane potential
Drp1	Dynamine-related protein 1
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Familial AD
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
ICC	immunocytochemistry
IMM	Inner mitochondrial membrane
IP3R3	Inositol 1, 4, 5-triphosphate receptor type 3
Nct	Nicastrin
NICD	Notch intracellular domain
NTF	N-terminal fragment
LTD	Long term depression
LTP	Long term potentiation
mtDNA	Mitochondrial DNA
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PreP	Presequence protease
MCI	Mild cognitive impairment
NFT	Neurofibrillary tangles
MAM	Mitochondria-associated ER membrane
Mfn2	Mitofusin-2
NMDA	N-methyl-D-aspastate receptor
PACS2	Phosphofurin acidic cluster sorting protein 2
PS	Presenilin
PSS1	Phosphatidylserine synthase
PTP	Permeability transition pore
RIP	Regulated intramembrane proteolysis
RFP	Red fluorescent protein
Sigma1R	Sigma-1 receptor
ROS	Reactive oxygen species
VDAC1	Voltage-dependent anion channel
TCA	Tricarboxylic acid cycle
TOM	Translocase of the outer membrane
TLC	Thin liquid chromatography

1 INTRODUCTION

1.1 ALZHEIMER DISEASE

Alzheimer disease (AD) is the most common form of dementia and affects millions of people worldwide. It is a complex, age-related, multifactorial disease affecting mainly the basal forebrain, cortex and hippocampus causing wide spread neurodegeneration, characteristic atrophy and enlargement of the ventricles (Wenk, 2003). Clinically, AD is characterized by gradual decline in memory and cognitive functioning, specifically planning, language, orientation, reasoning and performance of everyday activities, ultimately leading to death. At present there is no cure for AD and the drugs available on the market can only ameliorate symptoms. New technical advances have enabled researchers to study the disease in living patients and to investigate early events that occur before clinical symptoms are apparent. Presumably, the key to cure this devastating disease would be to diagnose it earlier and thereby enable treatment to be initiated much earlier than is the case today. Now treatment begins after symptoms are observed, though, by then, the patient already has wide spread pathology and neuronal loss. Still, the etiology of AD is largely unknown and there is a huge need for exploration of cellular pathways underlying the pathogenesis in the early phases in order to find promising, novel drug targets.

Mitochondria play essential and diverse roles in the physiology of eukaryotic cells. Besides ATP production, mitochondria participate in numerous intermediate metabolic reactions and play a central role in calcium homeostasis, apoptosis, cell signaling, proliferation, and differentiation. Impairment of mitochondrial function has been implicated in a wide variety of human diseases, including neurodegenerative disorders (Seppet et al., 2009). This thesis will explore some cellular processes disturbed in AD with its focus on the aberrant caspase activation, increased intracellular production of amyloid β-peptide (Aβ) and the mitochondria-associated endoplasmic reticulum (ER) membrane (MAM). The latter, a region, involved in regulating several pathways, including glucose, lipid metabolism, calcium homeostasis and apoptosis, all of which are altered in AD. Expanding knowledge about the mitochondria is important since mitochondrial abnormalities can play important roles in the etiology and progression of AD (Reddy and Beal, 2005).

1.1.1 Neuropathology

Molecular lesions arising from an accumulation of misfolded proteins in the aging brain are thought to cause oxidative and inflammatory damage, which in turn leads to energy failure and synaptic dysfunction. The neuropathological hallmarks of AD are characterized by extracellular neuritic plaques and intracellular neurofibrillary tangles (NFTs), which were first described in 1907 by Alois Alzheimer (Alzheimer et al., 1995). Not until 1980s were the main constituents of these lesions identified as Aβ and hyperphosphorylated tau, in plaques and NFTs, respectively (Masters et al., 1985; Grundke-Iqbal et al., 1986). At present, a definitive diagnosis of AD is made postmortem and includes neuropathological findings of plaques and NFTs. The pathological lesions are usually distributed in a characteristic way by which tau pathology is observed in the hippocampus and entorhinal cortex while prefrontal, parietal and temporal cortices exhibit most of the amyloid pathology (Braak and Braak, 1991). Another prominent feature is inflammation as both microglial activation and astrocytosis are important mediators of the disease progression (Tuppo and Arias, 2005;

Town, 2010). The pathology affects mainly the cholinergic and the glutamatergic neurotransmitter systems. The cholinergic system, thought to be particularly vulnerable to aging, resides in the basal forebrain and is heavily involved in higher cognitive functions, such as, learning and memory. Loss of cells in both the nucleus basalis of Maynert and the medial septal nucleus projecting to neocortex and hippocampus, respectively, are prominent features of AD. Glutamate is a major transmitter in the brain and the transmitter of cortical and hippocampal pyramidal neurons. The formation of new memories, through the mechanism of long-term potentiation (LTP), involves glutamate and its receptors. In AD, histological studies indicate loss of pyramidal neurons and their synapses (Francis, 2003). The pathological change correlating most strongly with cognitive decline is synapse loss, thought to start in hippocampus and entorhinal cortex. Neurons depend heavily on mitochondria for energy supply, and also as modulators of signaling pathways that involve intracellular calcium along their profoundly polarized shape at distances far away from soma. Mitochondrial dysfunction is an early feature in AD that might be implicated in synaptic dysfunction. This will be discussed separately in the section below entitled "Mitochondria".

1.1.2 Pathophysiologic hypothesis

It is generally believed that Aβ is important in the development of the neuropathic hallmarks of the disease, including: synapse loss, neurodegeneration, amyloid plaques, and neurofibrillary tangles (Hardy and Selkoe, 2002). This hypothesis is called the "**amyloid hypothesis**" and suggests that alteration in cleavage, processing and clearance of the amyloid precursor protein (APP) causes the pathogenesis of AD. This hypothesis has dominated the Alzheimer field as a pathophysiological explanation for about 20 years. This hypothesis has mainly survived as the top candidate due to the discovery of mutations causing familial AD (FAD) in the genes coding for amyloid precursor protein (APP) and presenilin 1 (PS1) and presenilin 2 (PS2), each of which influence production of Aβ. Nevertheless, this hypothesis has received criticism due weak correspondence between the amount of amyloid plaque and the observed progression of clinical symptoms. Therefore, this hypothesis was recently updated to "**toxic Aβ oligomer hypothesis**" as an attempt to explain the lack of correlation between plaque load and neurodegeneration and cognitive impairment (Benilova et al., 2012). Many other pathophysiological mechanisms have also been suggested. The oldest one is the "**cholinergic hypothesis**", which is based on the relative selective loss of cholinergic neurons in nucleus basalis of Maynert (Bartus et al., 1982). The most commonly used drugs for treatment of AD, the acetylcholinesterase inhibitors (e.g. Donepezil, Tacrine and Galantamine), are targeted to compensate for the falling acetylcholine levels by reversibly blocking acetylcholine esterase. These drugs can only ameliorate symptoms and give modest improvements in memory and do not retard neurodegeneration.

Another major theory today that correlates well with clinical symptoms is the "**neuronal cytoskeletal degeneration hypothesis**" which suggests that cytoskeletal changes that give rise to the formation of NFTs are the cause of the pathogenesis (Braak and Braak, 1991). Hyperphosphorylated tau, which comprises NFTs, is a microtubule-associated protein. Tau is primarily expressed in axons of the neurons and is important to stabilize microtubules during their assembly. Hyperphosphorylation results in decreased affinity for microtubules, which deteriorates axonal trafficking (Alonso et al., 1997; Iqbal et al., 2005). There are mutations in tau but none known to cause AD. Instead, such mutations, are linked to other neurodegenerative diseases like frontotemporal dementia (Foster et al., 1997). It has been suggested that hyperphosphorylation of tau might be triggered by Aβ accumulation. However, by what

mechanism Aβ affects tau is not fully clarified. One suggestion is that Aβ induces caspase cleavage of tau, since this truncation has been shown to proceed and be required for tangle formation (Hardy and Selkoe, 2002; de Calignon et al., 2010).

Additional new hypotheses have emerged e.g. the "**excitotoxicity hypothesis**", which suggests that glutamate toxicity could be one of the underlying pathogenic mechanisms of AD (Molinuevo et al., 2005). The drug, memantine, is a NMDA receptor antagonist and the latest of drugs approved by the U.S Food and Drug Administration (FDA) for treatment of AD. This drug is usually used in combination with acetylcholinesterase inhibitors in the later stages of the disease.

Another is the "**mitochondrial hypothesis**" that postulates that age-related changes in mitochondria influence the susceptibility and vulnerability for environmental factors triggering AD pathology (Swerdlow et al., 2010). Aging mitochondria show increased production of reactive oxygen species (ROS) (Navarro and Boveris, 2007). Oxidative stress, in turn, leads to cellular lipid, protein and nucleic acid damage, eventually triggering apoptosis (Markesbery and Carney, 1999; Mattson, 2000). Accumulation of Aβ in the mitochondria has been detected in our laboratory as well as in other labs. Inside the mitochondria, Aβ disturbs essential mitochondrial functions leading to disturbances in energy production and induction of apoptosis. Recently, additional data supporting this hypothesis has emerged. Leuner et al. showed that oxidative stress, *per se*, triggers elevation of Aβ production to toxic levels, which in turn impairs the mitochondria, thereby initiating a vicious cycle that incorporates ROS and Aβ (Leuner et al., 2012). These events may indeed, be important in AD pathogenesis and may account for disturbed mitochondrial metabolism and synaptic dysfunction detected at early stages in the disease.

The triggering events behind AD are still somewhat of a mystery and the heterogeneity of AD implies that there are probably several triggers. It is possible that all the above-mentioned hypotheses intervene with each other in a complex, hard-to-dissect manner. Nevertheless, Aβ probably has a central role in AD pathogenesis. However, with the many failures in drug development, which have mainly aimed to reduce Aβ levels, research has now tended to shift from mainly Aβ oriented to a wider, multi-candidate perspective. Other pathophysiological candidate areas for investigation that are being described today include hypometabolism, mitochondrial dysfunction, inflammation, dysfunctional autophagy, oxidative stress (NO), dysregulated calcium homeostasis, altered cholesterol and phospholipid metabolism. To understand what the triggers are, focus must be brought to pathophysiological events that precede Aβ aggregation and tau hyperphosphorylation.

1.1.3 Genetics and risk factors

Alzheimer disease is a multifactorial and heterogeneous disease where both genotype and environmental factors influence one's susceptibility. Most of the AD cases are sporadic (without known genetic background) and only roughly 1% of the cases are autosomal-dominantly inherited familial AD (FAD). Familial AD has been linked to mutations in three genes, including *APP*, located on chromosome 21; *PSEN1* and *PSEN2*, located on chromosomes 14 and 1, respectively (Goate et al., 1991; Levy-Lahad et al., 1995; Sherrington et al., 1995). Most of the aggressive, early-onset forms of FAD are caused by mutations in the *PSEN1* gene. The mutations in *APP*, *PSEN1* and *PSEN2* affect Aβ production in several ways, including increasing total Aβ or shifting the Aβ42/Aβ40 ratio by increasing the formation of the more aggregate-prone Aβ42 or decreasing the formation of Aβ40 (St George-Hyslop, 2000).

Presenilin mutations, apart from characteristic Aβ phenotypes, cause other biochemical changes, including: increased vulnerability to ER stress, disruption of intracellular calcium homeostasis, disruption of autophagy, disruption of neurotransmitter release and acceleration of the apoptotic processes (http://www.molgen.ua.ac.be/Admutation/) (Duff et al., 1996; Katayama et al., 2004; McCarthy, 2005; Thinakaran and Sisodia, 2006; Zatti et al., 2006; Miyoshi et al., 2009; Zhang et al., 2009; Lee et al., 2010; Parodi et al., 2010).

The cause of sporadic AD is thought to be a combination of genotype and several environmental risk factors, making certain individuals more susceptible to develop AD. Several environmental factors are thought to play a role in predisposing people to develop AD, especially cardiovascular risk factors including midlife cholesterol levels and high blood pressure. Others are age, female gender, head trauma, cerebral infarct, oxidative stress, depression and diabetes mellitus type 2 (Munoz and Feldman, 2000; Veurink et al., 2003). Several genes have been found to associate with AD, including *APOE*, *CLU*, *CR1*, *SORL1*, *PICALM* and *BIN1*, however, the apolipoprotein E (*APOE*) gene remains the most strongly established risk factor for AD (Lambert and Amouyel, 2011). The *APOE* exists in three different alleles ε2, ε3 and ε4. Carriers of *APOE* ε4 have an increased risk (3-10 fold) of developing AD and as well as lowering the age of onset (Corder et al., 1993). APOE is a component of triglyceride-rich lipoprotein complexes carrying cholesterol and triglycerides between cells and in the blood. In the brain, APOE is thought to be particularly important since large lipoproteins do not pass the blood brain barrier (BBB) and the cholesterol metabolism is thought to be separated from the periphery. Twenty-five percent of the total cholesterol in the body resides in the brain. The cholesterol is produced mainly by the astrocytes and then transported by APOE to neurons and other cells in the brain. Cholesterol is an important building block for myelin, plasma membranes and lipid rafts, and thereby essential for brain plasticity and repair (Bjorkhem and Meaney, 2004). It has been suggested that lipid particles containing the ε4 allele contain less cholesterol and, thereby, deliver less cholesterol to the neurons as compared to the ε3 allele (Gong et al., 2002). *APOE* has also been associated with reduced clearance of Aβ since ε4 carriers often have an increased plaque load (Dolev and Michaelson, 2004). Moreover, ε4 carriers display increased hyperphosphorylation of tau, reduced glucose metabolism, exacerbation of medial temporal lobe atrophy, reduced fMRI activity and connectivity as well as greater loss of white matter (Pievani et al., 2011; Canu et al., 2012; Patil et al., 2012). The ε4 protein mediates potentially detrimental effects upon the mitochondria via the lipid- and receptor-binding regions, resulting in mitochondrial dysfunction and neurotoxicity (Chang et al., 2005; Chen et al., 2011). However, the exact pathophysiological mechanism associating isoform ε4 with increased AD-risk are still not clear. Other diseases, like cardiovascular disease and atherosclerosis, are also associated with the ε4 allele (Verghese et al., 2011). *APOE* is located on chromosome 19 in a region of linkage disequilibrium (LD) that includes the genes: translocase of the outer mitochondrial membrane 40 (*TOMM40*), apolipoprotein C1 (*APOC1*) and poliovirus receptor-related 2 (*PVRL2*). Recent genome-wide association studies report that the gene, *TOMM40*, is associated with AD (Roses et al., 2009; Shen et al., 2010). Several polymorphisms have been identified in *TOMM40*, though, which polymorphisms and in what manner each contributes to the disease is unknown. One of these polymorphism, a poly-T repeat, was studied in **paper II** and will be discussed further in the section entitled "Aβ inside the mitochondria" and in the "Results and Discussion" section below.

Interestingly, some factors decrease the risk of developing AD. For instance, a mutation in APP at the β-secretase cleavage site, which reduces BACE1 cleavage, thereby lowering the production of Aβ, has been found to protect against AD and cognitive decline in elderly (Jonsson et al., 2012) (for information about β-secretase cleavage, see the section "APP processing"). Furthermore, postmortem studies on 86-89 year-old individuals that regardless of profound accumulation of plaques and tangles in their brains had remained cognitively intact, point towards the mitochondria. The cognitively intact individuals showed preserved insulin response and no accumulation of Aβ in their mitochondria, unlike AD patients, who showed profound accumulation of Aβ oligomers and deranged insulin signaling (Taglialatela, Poster, AAIC, 2012, Vancouver, Canada).

1.2 THE INVOLVEMENT OF CASPASES AND CELL DEATH IN AD

1.2.1 Apoptosis

Synaptic damage, neuronal network loss and cell death are processes giving rise to the characteristic brain atrophy seen in AD. Apoptosis has been implicated as the main cell death mechanism by which synapses degenerate and neurons are lost in AD (Stadelmann et al., 1999a; Louneva et al., 2008a; Albrecht, S. et al., 2009). Caspases, a group of cysteine proteases cleaving after aspartyl residues, play an essential role in apoptosis. Upon activation, caspases target a broad spectrum of cellular proteins, ultimately leading to disassembly of the cell (Alnemri et al., 1996). In contrast to necrosis, apoptosis is strictly regulated and is characterized by several morphological and biochemical changes, including cell shrinkage, nuclear fragmentation, chromatin condensation, plasma membrane blebbing, exposure of phospatidylserine on the cell surface and, finally, engulfment by phagocytes. Regulation of the apoptotic process and equilibrium between cell division and apoptosis are essential for the organism. Disturbance can result in cancer or degenerative disease depending on either inappropriate suppression or activation of apoptosis. There are two main routes to initiate apoptosis, one involves stimulation of death receptors by external ligands (death receptor pathway) and one arises within the cell (mitochondrial pathway) activated by various forms of cellular stress such as oncogenes, DNA damage, hypoxia, oxidative stress, excitotoxicity or deprivation of survival factor (see Figure 1).

Both these routes activate the caspase cascade. Based on their function, the size of their pro-domain, homology in amino acid sequence and cleavage specificity, caspases can be divided into one inflammatory group (caspase-1, -4, -5, -11, -12, -13 and -14) and one group regulating apoptosis. Those regulating apoptosis are divided into two classes: initiator caspases (caspase -2,-8, -9 and -10) and effector caspases (caspase-3,-6 and -7). These reside in the cells as zymogens (procaspases) and are activated post-translationally. The N-terminal contains the prodomain, which is required for activation. Caspase-2 and -9 contain the caspase recruitment domain (CARD) and caspase-8 and -10 contain the death effector domain (DED). The protein-protein interactions between these two domains are involved in procaspase activation and downstream caspase activation. Mitochondria are central in apoptotic signaling, both by providing ATP that supports the high energy demanding events during apoptosis, and by releasing death proteins from the intramembrane space (e.g. cytochrome c, Omi/HtrA2, Smac/DIABLO) after the mitochondria permeability transitions pore (PTP) opening.

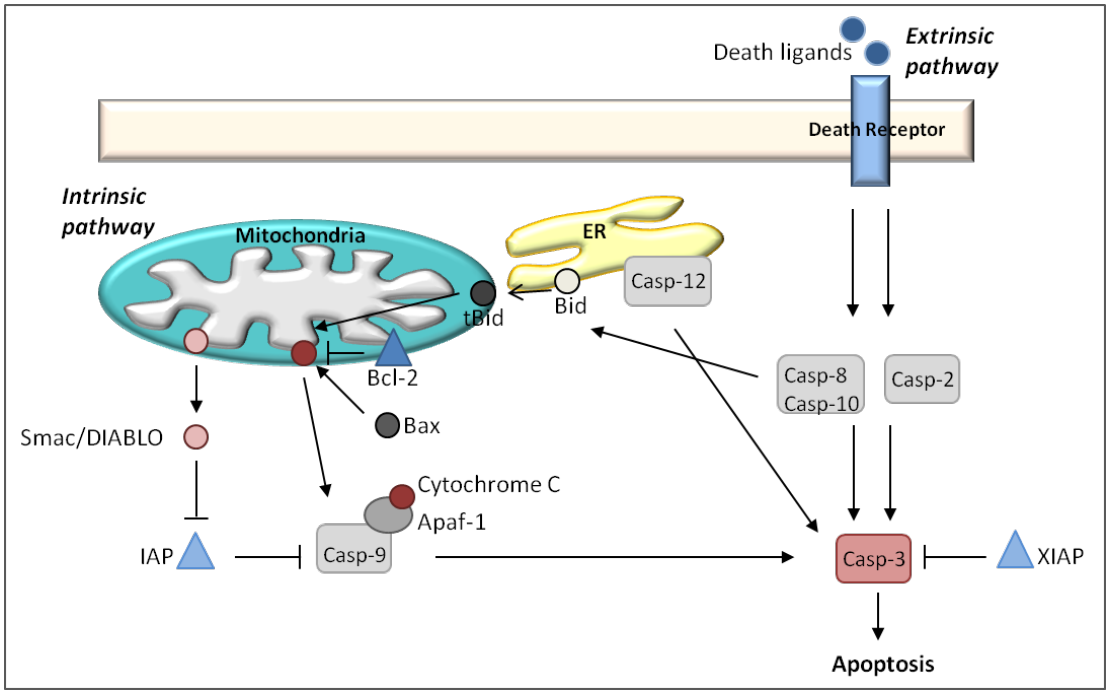

Figure 1: Apoptosis is induced by the extrinsic or the intrinsic pathway. The **intrinsic pathway** is initiated with outer mitochondria membrane permeabilization by upregulation of proapoptotic Bcl-2 proteins (e.g. Bid and Bax), and by the repression of anti-apoptotic Bcl-2 protein and IAPs, which lead to release of apoptotic mediators e.g. Smac/DIABLO and cytochrome C. These factors activate the caspase cascade through caspase-9 leading to cell death. The **extrinsic pathway** is initiated by death ligands activating the caspase cascade through Caspase-2, -8 and -10 leading to caspase-3 activation and cell death.

1.2.2 Caspases in healthy brain

Caspase activation is not only involved in apoptosis but also in other processes including red blood cell development and microglia activation and as recently shown also in remodeling of dendrites, spines and synaptic connections in the healthy brain (Gilman and Mattson, 2002; Lamkanfi et al., 2007; Burguillos et al., 2011; Hyman, 2011). Caspase-3, earlier thought to be activated exclusively during apoptosis, can be activated in a transient fashion mediating neuronal plasticity, including: long-term depression (LTD), long-term potentiation (LTP), synaptic reorganization, and neurite retraction in the healthy brain without completion of the apoptotic program (Mattson et al., 1998; Gilman and Mattson, 2002; Gulyaeva et al., 2003; de Calignon et al., 2010; Li et al., 2010). Furthermore, caspase-3/7 and caspase-8 have been shown to regulate microglia activation, also without completion of the apoptotic program (Burguillos et al., 2011). Therefore, it is suggested that certain cell types (e.g. neurons and microglia) utilize apoptotic signaling pathways to regulate processes such as plasticity or activation. However, since aberrant caspase activation and apoptosis also have been linked to synaptic loss and neurodegeneration in AD (Mattson et al., 1998; Stadelmann et al., 1999b; Louneva et al., 2008b) caspases must maintain activity in a transient fashion to hinder massive caspase activation and ultimate completion of the apoptotic program. Apoptosis might be restricted by trophic factors (Heerssen and Segal, 2002), cell adhesion proteins (Benson et al., 2000) and autophagy. In a rat model of subarachnoid hemorrhage, activation of autophagy was associated with neuroprotection against apoptosis by decreasing bax translocation to the mitochondria, thereby reducing early brain injury (Jing et al., 2012). Caspases are naturally controlled by various inhibitors such as the inhibitor of the apoptosis protein (IAP) family and Bcl-2 family proteins, which inhibit apoptosis by binding to active caspases. Eight members of the IAP

family have been described so far, including X-linked inhibitor of apoptosis (XIAP), neuronal apoptosis inhibitory protein (NAIP), survivin and livin. XIAP has dual functions, both as potent inhibitor of caspase activation and as an E3 ubiquitin ligase targeting caspases for degradation (O'Riordan et al., 2008). Currently, XIAP is the only caspase inhibitor known to inactivate active caspases (Eckelman et al., 2006). In AD, NIAP has been shown to be downregulated and XIAP to be upregulated (Christie et al., 2007). Such upregulation of XIAP might, unfortunately, not be sufficient to adequately regulate caspase activity in the AD brain since a significant amount of XIAP is inactivated by S-nitrosylation (SNO-XIAP). This inactivation, as reported recently, was caused by nitrosative stress inhibiting the E3 ligase and antiapoptotic activity of XIAP and, thereby, could play a role in causing aberrant caspase activation in AD (Nakamura et al., 2010). At present, the exact mechanism and factors that may be involved in keeping caspases active in a transient fashion are not fully understood. Increased understanding of the dysregulated apoptotic processes in AD would be important to gain therapeutic perspective on how to intervene and hinder aberrant caspase activation.

1.2.3 Caspases in AD and their relationship to Aβ

Several lines of evidence indicate excessive caspase activation in the AD brain. Activity of several caspases, especially caspase-3 and -6, has been demonstrated in both postmortem AD brains, postsynaptic densities in AD brains as well as in the cerebrospinal fluid of sporadic and FAD patients (Guo et al., 2004; Louneva et al., 2008b; Albrecht, P. et al., 2009; Albrecht, S. et al., 2009). Moreover, caspase activation and apoptosis have been linked to synaptic loss and neurodegeneration in human AD brain (Stadelmann et al., 1999a; Louneva et al., 2008a; Albrecht, S. et al., 2009; D'Amelio et al., 2011). Studies report that Aβ induces LTD in a caspase-3 dependent manner (Jo et al., 2011). Several lines of evidence suggest a correlation between caspase activation and elevated Aβ production (LeBlanc, 1995; Galli et al., 1998; Tesco et al., 2003; Takuma et al., 2005a; Cicconi et al., 2007; Xie et al., 2007). It has been suggested that caspases induce increased β-secretase cleavage of APP. This phenomenon can be explained by caspase-3 cleavage of GGA3, an adaptor protein involved in BACE trafficking, which thereby stabilize BACE (Tesco et al., 2007). Caspases cleave numerous other substrates, including the AD associated proteins APP, tau, PS1 and PS2. The cleavage of tau induces mitochondrial dysfunction and is required for tangle formation *in vivo* (Quintanilla et al., 2009; de Calignon et al., 2010). Caspase cleavage of APP generates two putative toxic peptides C31 and Jcasp (Park et al., 2009). However, elevated Aβ production observed after caspase activation occurs independently of caspase cleavage of APP (Tesco et al., 2003). The role of PS1 and PS2 in apoptosis has been studied by several groups showing that PS1 and PS2 in most cases accelerate the apoptotic program (Wolozin et al., 1996; Alves da Costa et al., 2002; Alves da Costa et al., 2003; Fluhrer et al., 2004; Cai et al., 2006; Miyoshi et al., 2009). We have recently investigated caspase cleavage of PS1 and found that the elevation of the intracellular Aβ42/Aβ40 ratio can indeed partly be explained by the formation of γ-secretase complexes containing caspase cleaved PS1 (PS1caspCTF) as shown in **Paper I**. This will be further discussed under the subheading “The γ-secretase complex” and in section “Results and Discussion”.

Axonal degeneration has been associated with caspase-6 activation. Caspase-6-knockout neurons have been shown to be protected against excitotoxicity, nerve growth factor deprivation and myelin-induced axonal degeneration (Uribe et al., 2012). However, caspase-6-knockout mice display a hypoactive phenotype with learning deficits, thus indicating that caspase-6 activity is important for neuronal health. Caspase-6 activity must, however, be accurately regulated in order to hinder axonal degeneration (Uribe et al., 2012). Recently, a

ligand for death receptor 6 (DR6) was identified, which downstream activates caspase-6. Interestingly, it is the N-terminal fragment of APP, produced after β-secretase shedding (by BACE1 or meprin β), that binds to DR6 during trophic-factor deprivation and, thereby, induces caspase-6 dependent axonal degeneration (Nikolaev et al., 2009; Jefferson et al., 2011). Thus, transient caspase activity is important for several biological processes, implying that caution should be taken when considering caspase inhibition as a therapy for AD. Nevertheless, modulation might have positive results as demonstrated in the triple-transgenic AD mouse model lacking pathology when overexpressing anti-apoptotic protein Bcl-2 (Rohn et al., 2008).

1.3 Aβ PRODUCTION, FUNCTION AND TOXICITY

1.3.1 Amyloid precursor protein

Amyloid precursor protein (APP) is an integral type I transmembrane protein with a long N-terminal domain and short cytoplasmic C-terminal domain (see Figure 2). Two homologues to APP have been identified, including APP-like proteins 1 and 2 (APLP1 and APLP2), which share sequence similarity to APP, though lacking the Aβ part (Sprecher et al., 1993). Their function is not yet clarified but it has been proposed that the APP family members play a role in neurite outgrowth, synaptic plasticity, neuronal protein trafficking, transmembrane signal transduction, cell adhesion and neuronal survival (Mattson, 2004; Zheng and Koo, 2006; Zhang et al., 2012). Upon cell adhesion, APP together with other APP family members is thought to dimerize into homodimers or heterodimers in the process of cell adhesion (Soba et al., 2005). Double- and triple-knockout studies reveal that, while APP/APLP1-knockout mice survive, other combinations, including APP/APLP2, APLP1/APLP2 and APP/APLP1/APLP2 each individually show early postnatal lethality (Zhang et al., 2012), reduced number of synaptic vesicles and deteriorated presynaptic terminals at birth (Wang et al., 2005). When only knocking down APP, the mice show reduced brain and body weight, reduced grip strength, impaired spatial memory and LTP. This phenotype could be rescued in APP-N-terminal-part knock-in mice (Ring et al., 2007). Overexpression, on the other hand, results in the phenotype resembling Down syndrome (trisomy 21) and in premature death (Moechars et al., 1996). Overexpression is furthermore associated with degeneration of forebrain cholinergic neurons in a mouse model of Down syndrome due to decreased retrograde transport of nerve growth factor (Salehi et al., 2006). APP is ubiquitously expressed throughout the body and exists in three different isoforms 695, 751 and 770. The isoforms 751 and 770 express a Kunitz protease inhibitor (KPI) sequence in their N-terminal domain and are expressed in all cell types except neurons, which instead express the isoform 695. In neurons, the 695 isoform has been found localized to synapses both in post-synaptic densities and in adhesion complexes (Marotta et al., 1992). In AD, a shift in APP isoform in neurons towards KPI-containing isoforms has been detected, a shift that is associated with increased Aβ production (Menendez-Gonzalez et al., 2005; Bordji et al., 2010). Transportation of APP has been described to occur by fast moving (3 μm/s) large tubules and vesicles out in the neurites by microtubules motors (Goldsbury et al., 2006), and carried along axons to synaptic terminals (Lazarov et al., 2002). Many post-translational modifications have been described in the ectodomain of APP, including N- and O-glycosylation, sulfation and phosphorylation (Gandy et al., 1988; Weidemann et al., 1989; Hung and Selkoe, 1994). Upon phosphorylation at T668, APP has been shown to inhibit neuronal calcium oscillations (Santos et al., 2011). Furthermore, the ectodomain purportedly contains several different domains, including a growth factor-like domain; neuroprotective and neurotrophic domains; and copper-, zinc- and heparin-binding site domains (Chasseigneaux and Allinquant, 2012). Several ligands to APP have been suggested, including Aβ, F-spondin, and Netrin-1 (Lorenzo et al., 2000; Ho and Sudhof, 2004). APP is typically transported from ER

through the secretory pathway to the plasma membrane; however, in AD, APP is reported to also accumulate in the mitochondria (Anandatheerthavarada et al., 2003; Devi et al., 2006). APP contains a mitochondrial targeting signal at the N-terminal part consisting of at least the positively charged residues, 40, 44 and 51. Upon import into the mitochondria, APP is arrested at the acidic sequence 220-290, thereby causing APP to be stuck in the mitochondrial import pore, leaving a large C-terminal part (containing the Aβ region) outside in the cytoplasm (Devi et al., 2006). The accumulation of APP in the mitochondria hinders the import of other proteins and causes mitochondrial dysfunction and impaired energy metabolism (Anandatheerthavarada et al., 2003; Devi et al., 2006). Recently, the C-terminal part of APP was shown to become inserted into the OMM where it could be processed by mitochondrial γ-secretase complexes (Pavlov et al., 2011). Overexpression of APP has been shown to cause mitochondrial fragmentation and abnormal mitochondrial distribution, which was suggested to be caused by C99 or Aβ since the phenotype could be rescued by β-secretase inhibitors (Wang et al., 2008). Also Aβ and C99 have been observed to accumulate in the mitochondria at early points during disease course (Lustbader et al., 2004; Caspersen et al., 2005; Manczak et al., 2006; Hansson Petersen et al., 2008; Devi and Ohno, 2012). These data suggest that APP and its metabolites could, indeed, play an active role causing mitochondrial dysfunction. The accumulation of Aβ inside the mitochondria will be discussed further under the subheading "Aβ inside the mitochondria".

1.3.2 APP processing

APP is processed through two pathways: the non-amyloidogenic and the amyloidogenic pathways (see Figure 2), the latter of which accounts for generation of Aβ through sequential cleavage by β- and γ-secretase. The enzymes that are thought to serve as β-secretases, include membrane-bound aspartyl proteases; and so far BACE1 has been described as the main one. However, cathepsin B and Meprin β have also been suggested (Vassar, 2004; Cole and Vassar, 2007; Jefferson et al., 2011; Hook et al., 2012; Kindy et al., 2012). The cleavage of APP by β-secretase generates two fragments: one extracellularly released soluble ectodomain (sAPPβ) and one membrane-anchored fragment (C99). The subsequent γ-secretase processing of C99 results in the generation of Aβ and the APP intracellular domain (AICD) (see Figure 2). γ-Secretase determines the C-terminal of Aβ through sequential cleavage of C99, generating tri- and tetra-peptides from the C99 stub. There are two proposed alternative cleavage-pathways starting at the ε-site that releases AICD and generates either Aβ48 or Aβ49. The subsequent shortening of Aβ48 and Aβ49 are made by carboxypeptidase-like γ-cleavages where Aβ49 are further processed to Aβ46>Aβ43>Aβ40, and Aβ48 gives rise to Aβ45>Aβ42>Aβ38 (Takami et al., 2009; Chavez-Gutierrez et al., 2012). The shortening of the long Aβ decreases the hydrophobicity and increases the probability for it to be released from the membrane. It has been shown that the first cleavage at the ε-site determines which of the two cleavage-pathways will be chosen *in vivo* (Funamoto et al., 2004). FAD mutations are thought to have already affected the ε-site cleavage site, causing a shift towards the Aβ48 production lineage (Chavez-Gutierrez et al., 2012; Golde et al., 2012).

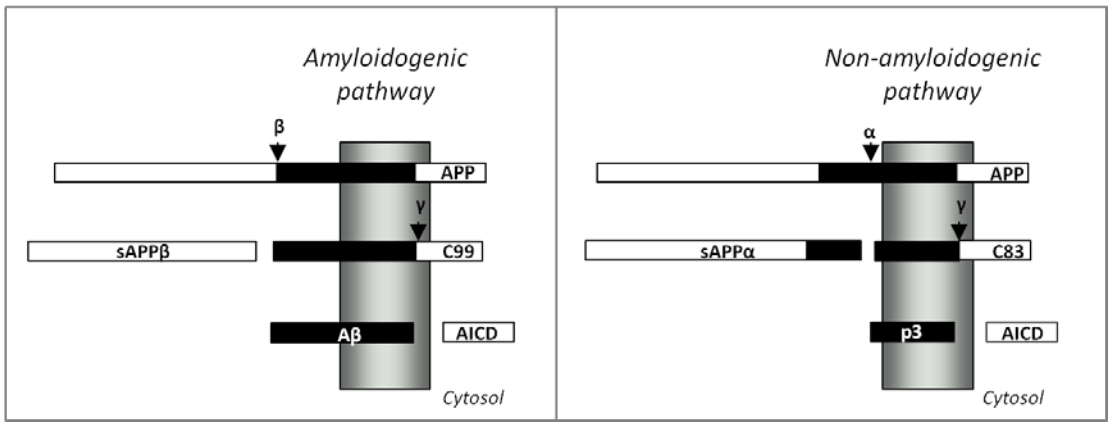

Figure 2. APP processing. APP is processed either by the amyloidogenic pathway by the β- and γ-secretases or by the non-amyloidogenic pathway by α- and γ-secretases. β-secretase cleavage generates C99 that is further processed to Aβ and AICD. α-Secretase cleavage generates C83 which gives rise to p3 and AICD.

The non-amyloidogenic pathway involves cleavage by α-secretase (ADAM10 and ADAM17), which belong to the zinc protease super family (Allinson et al., 2003; Vincent and Govitrapong, 2011) and by γ-secretases. The α-secretase cleaves APP within its Aβ peptide sequence, preventing its production, while generating a longer soluble ectodomain (sAPPα) and one membrane-anchored fragment (C83). The subsequent γ-secretase processing of C83 results in the generation of p3 and the APP intracellular domain (AICD) (see Figure 2). The biological functions of the cleavage products are not fully known, but AICD is thought to translocate to the nucleus where it is involved in transcriptional regulation of different genes (e.g. APP, GSK-3β, KAI1, neprilysin, BACE1, EGFR and LRP1) including genes involved in apoptosis (p53) (Cao and Sudhof, 2004; Kerr and Small, 2005; Pardossi-Piquard et al., 2005; Zhang et al., 2011). AICD also modulates cellular calcium homeostasis and ATP content (Hamid et al., 2007). The physiological role of sAPPα, which differ by only 16 amino acids at its C-terminus as compared to sAPPβ, is purportedly neuroprotective and is far more potent in regulating neuronal activity (Barger and Mattson, 1996; Turner et al., 2003; Turner et al., 2007; Taylor et al., 2008). The products of the amyloidogenic pathway, sAPPβ and C99, have been proposed to possess opposite functions, that is, they activate cell death pathways and cause synaptic and memory deficits (Nikolaev et al., 2009; Tamayev et al., 2012). However, recently, both sAPPα and sAPPβ have been shown to regulate the proliferation and differentiation of neuronal precursor cells deriving from the subgranular zone of the rat hippocampus (Baratchi et al., 2012). Thus, today, the whole understanding of APP metabolites and their role in AD pathogenesis is lacking.

The fate of APP depends on trafficking within the cell. Sortilin-1, a newly discovered risk factor for AD, directs the trafficking of APP from the plasma membrane into the cell were it can be processed by BACE1, preferably in intracellular compartments (Rogaeva et al., 2007). BACE1 has been found along the secretory pathway including trans-Golgi network, endosomes and lysosomes and exhibit its highest activity at acidic pH (Cole and Vassar, 2007). In the brain, BACE1 is heavily expressed in axons and presynaptic terminals of the mossy fiber pathway as well as in the amygdala (Vassar et al., 2009). In sporadic AD patients, BACE1 expression level and activity have been found to be elevated (Yang et al., 2003). This elevation could in some cases be explained by a defect in a microRNA controlling BACE1 expression (Hebert et al., 2008). Apoptotic processes and Aβ have also been suggested to elevate BACE1 expression levels (Xie et al., 2007; Sadleir and Vassar, 2012). BACE1-knockout mice exhibit

hypomyelination and altered neurological behaviors such as reduced grip strength and elevated pain sensitivity (Laird et al., 2005; Hu et al., 2006; Willem et al., 2006; Gersbacher et al., 2010). Inhibition of BACE causes side effects, including: schizophrenic symptoms, increased mortality, epileptic seizures, hyperactivity, anxiety and impaired axon guidance, probably due to the lack of cleavage of its other substrates including neuregulin 1, seizure-protein 6, L1, CHL1 and contactin-2 (Kuhn et al., 2012). The α-secretase, apart from cleaving APP, is implicated in the control of cytokine and growth-factor shedding at the plasma membrane e.g. Notch, tumor necrosis factor-α and epidermal growth factor. This enzyme acts primarily at the plasma membrane where the cleavage of its substrates, including APP, takes place (Zhang et al., 2012). The γ-secretase complex will be discussed separately under the subheading "The γ-secretase complex".

1.3.3 The amyloid β-peptide (Aβ)

In FAD, the mutations in APP, PS1 or PS2 are thought to increase the Aβ42/Aβ40 ratio. In sporadic AD, several events have been shown to increase Aβ production, e.g. ROS production or decreased expression of Aβ-degrading enzymes, including insulin-degrading enzyme (IDE), neprilysin, cathepsin B and PreP (Miners et al., 2008; Alikhani et al., 2011; Leuner et al., 2012). The biophysical and biochemical properties of Aβ vary according to its length, the longer are more prone to aggregate. The two main forms are Aβ40 and Aβ42, the latter of which is the main constituent of amyloid plaques. Also Aβ43 has been detected in plaques (McGowan et al., 2005; Welander et al., 2009). Despite the difference in only two and three amino acids, Aβ42 and Aβ43 are more hydrophobic and their aggregation potential is much higher than that of Aβ40. Several research groups have demonstrated that the relative level of Aβ42 in relationship to Aβ40 is critical for the pathogenesis of the disease, suggesting a central role of Aβ42 in the development of AD. Even a minor increase in the Aβ42/Aβ40 ratio induces formation of toxic oligomers, signifying that Aβ40-monomers could prevent aggregation and toxicity of Aβ42 (Yan and Wang, 2007; Jan et al., 2008; Kuperstein et al., 2010). Studies even suggest that such minor changes of Aβ42 or Aβ43 might trigger a pathogenic cascade and drive the seeding of plaques (Masters and Selkoe, 2012). What drives the transition from normal Aβ production to a pathological state is not fully understood. The complexity of Aβ peptides has become greater still, with the identification of several other enzymatic processes that are involved in modifying the Aβ pool. For example, Aβ peptides can further be modified by aminopeptidase, glutaminylcyclase or isomerase, each generating N-terminally truncated peptides. Further modification by phosphorylation and pyroglutamate reactions result in a mix of more than 20 Aβ variants (De Strooper, 2010; Portelius et al., 2011; Benilova et al., 2012). These might participate differently in normal brain or in oligomerization and fibrillization in AD-afflicted brain. The dominant hypothesis articulating of which Aβ species are most toxic points toward the soluble oligomers that exist between monomeric Aβ and the amyloid Aβ fibrils in the plaques. Whether the polymerization of Aβ occurs intracellularly or extracellularly is not clear. Some studies claim that it mainly occurs in intracellular compartments, in particular, endosomes or lysosomes, where the concentration of Aβ-peptides can be enriched and where the low pH has the capacity to promote the oligomerization. When these are later secreted they become potential seeds for extracellular plaques (Hu et al., 2009). Moreover, Aβ is mainly thought to exert its toxicity intracellularly. More evidence for this was recently found in a study of an autophagy-deficient mouse crossed with a tgAPP mouse. Deficiency in autophagy leads to intracellular Aβ accumulation and severe cognitive deficits. This finding implicates intracellular Aβ as being cytotoxic, thus accounting for its protective role as it, consequently, is secreted into extracellular space (Nilsson, Poster, AAIC, 2012, Vancouver, Canada).

The physiological role of Aβ is not known, but since it normally is continuously and abundantly produced already from embryogenesis, it might not be associated only with neurotoxicity. For instance, Aβ at low concentrations has been shown to be neuroprotective, enhancing the survival of neurons, and to have neurotrophic properties stimulating neuronal (Aβ40) or astrocytic (Aβ42) cell fate of primary neural progenitor cells (Chen and Dong, 2009; Giuffrida et al., 2009). Activity-dependent co-secretion of Aβ, together with neurotransmitters, suggests that Aβ acts as a modulator at the synapse. Aβ depresses excitatory synaptic transmission inhibiting LTP. Therefore Aβ might participate in negative feedback regulating neuronal activity (Hsieh et al., 2006; Hook et al., 2012). Such co-secretion has been found from dense core secretory vesicles containing a variety of neurotransmitters including catecholamine, dopamine, norepinephrine and neuropeptides (Hook et al., 2012). Furthermore, Aβ may have a biological role in lipid metabolism, demonstrating a capacity to reduce synthesis of both cholesterol and sphingomyelin (Grimm et al., 2005). Thus, the production of Aβ might be of physiological importance in the central nervous system. However, dysregulated production, impaired secretion from cells, intracellular accumulation or defective clearance from the brain probably play fundamental roles in the pathophysiology of AD.

1.3.4 The γ-secretase complex

γ-Secretase is a multi-protein complex consisting of at least four subunits: PS1 or PS2, Nicastrin, anterior pharynx defective-1 (Aph-1) and presenilin enhancer-2 (Pen-2) (see Figure 3).

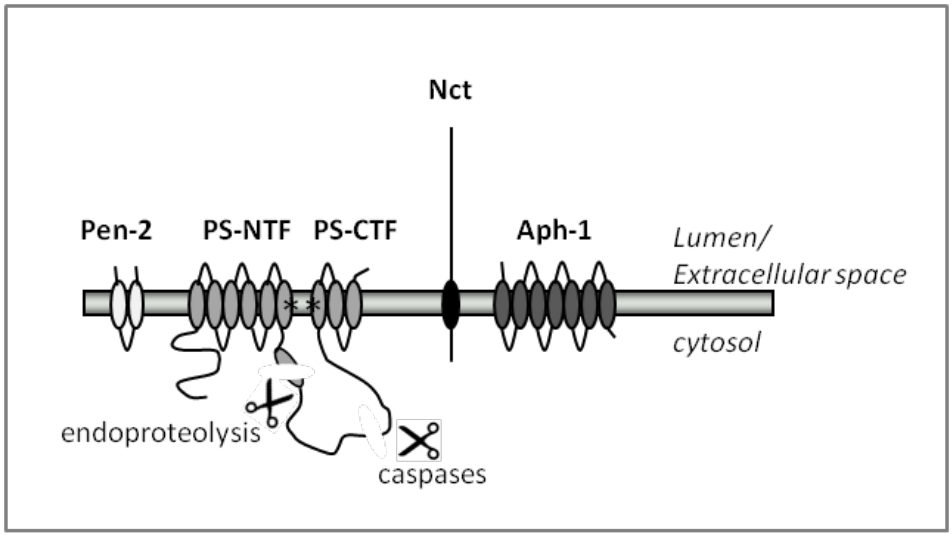

Figure 3. Illustration of the γ-secretase complex. It consists of at least Pen2, PS, Nicastrin (Nct) and Aph-1. PS undergoes endoproteolysis generating a N-terminal fragment (NTF) and a C-terminal fragment (CTF). The CTF can later be cleaved by caspases in the large cytosolic loop. The active site in PS is shown by asterisks.

The assembly of the four subunits takes place within the endoplasmic reticulum (ER) where the γ-secretase complex remains until it is fully assembled (Capell et al., 2005). Membrane protein folding is carefully supervised by the quality control (consisting of e.g. chaperones) within the ER ensuring that misfolded proteins do not leave the ER. Initially, Aph-1 and Nicastrin are assembled together before the remaining components are added (Takasugi et al., 2003). The addition of Pen-2 is thought to result in a conformational change in Nicastrin that promotes endoproteolysis of presenilin (Takasugi et al., 2003). Presenilin undergoes endoproteolytic

cleavage in the large cytosolic loop (Ala299) generating N- and C-terminal fragments (NTF and CTF). The two fragments form a heterodimer within the γ-secretase complex harboring the two highly conserved aspartic acid residues, D257 and D385 in the six and seven transmembrane domains, which form the active site. PS is a multi-membrane-spanning protein with 9 transmembrane regions that, after endoproteolysis in the large cytosolic loop, are separated into NFT and CTF containing 6 and 3 transmembrane regions, respectively (see Figure 3). The CTF contains most of the large cytosolic loop, which is involved in regulating the enzymatic activity of the γ-secretase complex at the γ-cleavage site thereby adjusting Aβ species formed (Deng et al., 2006). This can, for example, be seen in cell lines on a PS-deficient background, and in the absence of the entire loop in the PS1Δexon10 mouse model. These mice exhibit a great reduction of Aβ40, though Aβ42 level is unchanged, which is accompanied by exacerbation of plaque pathology (Deng et al., 2006; Wanngren et al., 2010). Allosteric changes in PS1 conformation have been shown to underlie changes in the Aβ42/Aβ40 ratio (Uemura et al., 2009). This is the case for the FAD mutations in PS1, which cause closer conformation of the γ-secretase complex, favoring the Aβ42 production linage (Uemura et al., 2009). Caspases can cleave PS1 in the CTF-part at Asp333 and Asp345, generating a ~12 kDa C-terminal fragment that is missing a large part of the loop domain (Kim et al., 1997; Grunberg et al., 1998). In our laboratory, it has previously been shown that caspase-cleaved PS1CTF (caspCTF), despite the truncation in the large cytosolic loop, forms active γ-secretase complexes in cells exposed to apoptotic stimuli (Hansson et al., 2006). As we show in **Paper I**, this truncation increases the intracellular Aβ42/Aβ40 ratio and could, therefore, partly account for the elevated Aβ production seen during caspase activation. This finding is further discussed in the section "Results and Discussion". Animal studies have shown that PS1 is essential for embryonic development. PS1 double-knockout is lethal compared to PS2 double-knockout mice that are phenotypically normal (Donoviel et al., 1999; Handler et al., 2000). Furthermore, mice lacking both PS1 and PS2 in the postnatal forebrain exhibit reduced long-term memory formation and shortened neuronal survival, inducing neurodegeneration and memory loss, whereas, mouse models overexpressing Aβ have failed to produce the characteristic AD phenotype (Saura et al., 2005; Shen and Kelleher, 2007).

γ-Secretase is thought to work as an aspartyl-protease with its catalytic activity located inside the lipid bilayer through a process called regulated intramembrane proteolysis (RIP) (Selkoe and Kopan, 2003). The activity of the γ-secretase complex can be modulated by interacting proteins, for example, CD147, TMP21, GPR3 and γ-secretase activating protein (GSAP), which are suggested to thereby regulate Aβ production (Zhou et al., 2005; Chen et al., 2006; Thathiah et al., 2009; He et al., 2010). New proteins that modulate the γ-secretase activity are identified frequently. For instance, two mitochondrial proteins that associate with the γ-secretase and affect Aβ production have recently been reported, including NADH dehydrogenase (ubiquinone) iron-sulfur protein 7 (NDUFS7) and voltage-dependent anion-selective channel (VDAC1) (Frykman et al., 2012; Hur et al., 2012). NDUFS7 is the core subunit of complex I in the electron transport chain in the inner mitochondrial membrane and VDAC1 is an ion channel located at the outer mitochondrial membrane. Another is Erlin-2, which resides in lipid raft like regions on ER (Teranishi et al., 2012).

The γ-secretase complex cleaves type 1 transmembrane proteins. New substrates for the γ-secretase complex are frequently being discovered. To date, more than 90 have been described, including Notch (signaling receptor cell, fate decisions during embryonic development, neuritic growth), CD44 (cell adhesion), E/N-cadherin (cell adhesion), $p75^{NTR}$ (neurotrophin co-

receptor), ErbB-4 (growth-factor-dependent receptor tyrosine kinase), and insulin receptor (glucose metabolism), to name a few (Lleo and Saura, 2011).

The γ-secretase complexes are described as being localized in various compartments of the cell, including: ER/Golgi (De Strooper et al., 1997), nuclear envelope (Kimura et al., 2001), endosomes (Vetrivel et al., 2004), lysosomes (Pasternak et al., 2003), mitochondria (Hansson et al., 2004), plasma membrane (Tarassishin et al., 2004), synaptic vesicles (Frykman et al., 2010) and intercellular contacts known as adherens junctions (Marambaud et al., 2002). γ-Secretase activity is enriched in lipid rafts, which are specialized regions in cellular membranes situated in plasma membrane or intracellular compartments such as the mitochondria-associated ER membrane (MAM) (Urano et al., 2005; Hur et al., 2008; Area-Gomez et al., 2009). Interestingly, one recent study implies that the γ-secretase complex has its highest activity at MAM (Area-Gomez et al., 2009), a highly specialized subregion in ER that enables close contact with the mitochondria. MAM will be discussed separately under the subheading "Mitochondria-associated ER membrane".

Therapeutics aimed at inhibiting γ-secretase activity have been associated with severe side effects, probably due to suppression of cleavage of the other substrates, especially Notch. Therefore, γ-secretase modulators (GSMs) that selectively reduce Aβ production without affecting the other substrates are potentially safer, leading this class to currently be investigated (Tomita, 2009). The GSMs exert their mode of action by binding to either APP or the subunits of the γ-secretase complex, while PS is thought to be the primary target (Jumpertz et al., 2012).

Some researchers postulate that PSs possess other functions apart from being a component in the γ-secretase complex, including regulating calcium homeostasis, autophagy and neurotransmitter release (Zhang et al., 2009; Lee et al., 2010; Zhang et al., 2010). FAD mutations in PS result in deranged calcium signaling and several reports have indicated that associated neuronal calcium disruptions are early events in AD pathogenesis. Studies of fibroblasts lend support to this description, showing that abnormally high amounts of calcium are detected upon inositol-1,4,5-triphosphate (IP3) stimulation (Ito et al., 1994). Similar results were obtained in studies on cortical and hippocampal neurons from PS FAD mutant knock-in mice (Chan et al., 2000). Several explanations to this phenomenon have been postulated, including that PS affects various calcium channels (e.g. ryanodine receptors, IP3R and SERCA pump) and/or functions as a passive ER calcium leak channel controlling the steady state of calcium in ER (Tu et al., 2006; Zhang et al., 2010). In the case of FAD mutations in PS, these functions are disrupted and the loss of such leak function has been postulated to cause ER calcium overload (Zhang et al., 2010). Furthermore, a new function of PS2 has been described that involves regulating ER-to-mitochondria calcium fluxes at the MAM (Zampese et al., 2011).

In autophagy, PS has a fundamental role in targeting v-ATPase to lysosomes, promoting lysosomal acidification and proteolysis during autophagy (Lee et al., 2010). Autophagy is needed for proper degradation of proteins and organelles including the mitochondria. Defective lysosomal proteolysis causes pathogenic protein accumulation and defective mitochondrial turnover, which, indeed, could be implicated in AD pathogenesis. Moreover, defective autophagy has been associated with increased PS expression and γ-secretase activity (Ohta et al., 2010). Thus, it might be important to consider other biological roles of both PS and APP

apart from their involvement in Aβ generation in order to understand the disease mechanisms behind both FAD and sporadic AD.

1.4 MITOCHONDRIA

1.4.1 Mitochondria: more than just a powerhouse

Mitochondria are cytoplasmic organelles originating from invading bacteria 1.5 billion years ago (Wallace, 1982). Some features remain reflecting this origin like double-membrane structures and a circular genome with mitochondrial-specific transcription, translation and protein assembly systems. To adapt to its new environment the mitochondria has reduced its genome to about 16 500 bp encoding 13 polypeptide, 2 mRNA and 22 tRNA genes. Thus, several essential mitochondrial genes have been transferred to the nucleus and approximately 1000 proteins are translated by free ribosomes in the cytosol and then imported into the mitochondria (Schatz, 1996).

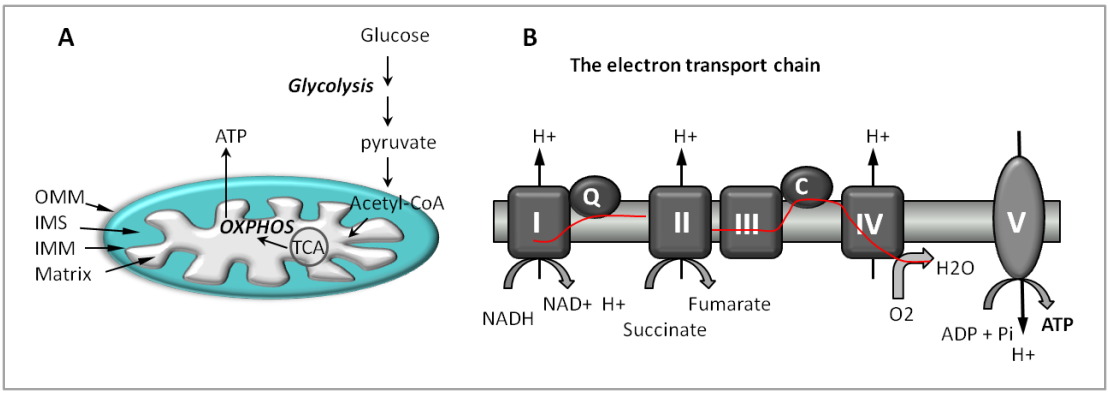

Figure 4. Overview of mitochondria and oxidative phosphorylation. (A) Glucose is metabolized via glycolysis to pyruvate, which enters the mitochondria and converts into Acetyl-CoA, which is used for driving the tricarboxylic acid cycle (TCA) and, subsequently, oxidative phosphorylation (OXPHOS) where ATP is produced. OMM (outer mitochondrial membrane), IMS (intermembrane space) IMM (inner mitochondrial membrane). (B) OXPHOS is conducted by the electron transport chain. The transfer of electron from the donors (NADH and succinate) to the acceptor (O_2) is coupled to proton transfer producing the electrochemical potential, which is used in complex V to produce ATP.

The import machinery consists of TOM and TIM complexes forming pores in the outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM), respectively. The OMM is highly permeable to low-molecular-weight substances. While the IMM provides a barrier through which ions have to be actively transported via specific channels. The IMM houses the electron transport chain complexes (complex I-IV and the ATP synthase) responsible for ATP production via oxidative phosphorylation (OXPHOS) (see Figure 4A). At complex I, III and IV, protons are pumped across the IMM into the intermembrane space (IMS) creating the mitochondrial membrane potential ($\Delta\Psi m$). This electrochemical gradient is crucial for maintaining cellular viability since OXPHOS rely on the energy content of it (see Figure 4B). The brain accounts for only 2% of the body mass but is responsible for 20% of the oxygen consumption via OXPHOS (Papa, 1996). The matrix of mitochondria, house enzymes in the tricarboxylic acid cycle (TCA) and enzymes used to conduct beta-oxidation.

Mitochondria form highly dynamic tubules that continuously fuse and divide so as to control correct distribution, morphology and quality of the mitochondrial pool. The central components that mediate mitochondrial dynamics are the fusion proteins: mitofusin1, mitofusin 2 (Mfn1 and Mfn2) and optic atrophy type 1 (Opa1) and the fission protein, dynamin related protein 1 (Drp1) (Chan, 2006). As described above, mitochondria are key regulators of cell survival and death. Dysfunction of mitochondria leads to insufficient energy metabolism, reduced ATP production, impaired calcium buffering and increased generation of reactive oxygen species (ROS) (Beal, 2005). Neurons are metabolically active cells that consume a lot of energy at locations distant from the cell body. As a result, these cells are particularly dependent on mitochondrial function for energy conversion. Normally, cells contain a mix of healthy and defect mitochondria (heteroplasmy). As we age, the mitochondria pool weakens mainly due to oxidative insult to proteins, lipids and nucleic acids, which is also implicated in driving the aging process (Muller et al., 2010). Mitochondrial turnover via autophagy and fusion/fisson is of great importance as this serves to maintain a healthy cellular pool of mitochondria, especially in post mitotic cells like neurons, where the mitochondrial pool cannot be renewed and limited replacement of damaged cells occur. Since aging is the main risk factor for sporadic AD and mitochondrial dysfunction is implicated in the aging process a role of mitochondria in sporadic AD has been suggested (Reddy, 2007).

1.4.2 Mitochondrial dysfunction in neurons

Several lines of evidence implicate that Aβ exerts its toxicity intracellularly (Wilson et al., 1999; Gouras et al., 2000; Wirths et al., 2004; Aoki et al., 2008) and mitochondria are suspected to have a role in it. Neurons are vulnerable to oxidative insult since they have a high rate of energy and oxygen utilization, poor concentration of classical antioxidants, high levels of redox-active metals and high content of polyunsaturated lipids. Damage to mitochondria in neurons is detrimental since there is limited or no regeneration/replacement capacity of neurons in the brain. Mitochondria are the major source and target of oxidative stress. Reactive oxidative species (ROS) is produced during OXPHOS when electrons passing through the electron transport chain and some electrons leak out at complex I and complex III. These can react with oxygen and yield superoxide anions ($^{\cdot}O_2^-$), which then can be converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). The presence of Fe^{2+} accelerates the decomposition of H_2O_2 to hydroxyl radicals ($^{\cdot}OH$), and nitric oxide ($NO^{\cdot}$) reacts with $^{\cdot}O_2^-$ to produce peroxynitrite ($ONOO^{\cdot}$). The mitochondria are particularly susceptible to oxidative damage. Mitochondrial DNA has a mutation rate estimated to be 10 to 20 times higher than that of nuclear DNA. This sensitivity is due to the lack of protective histones and limited, available repair mechanisms (Brown et al., 1979). For protection, IMM incorporates a number of free radical scavengers, such as vitamin E, ascorbate, catalase and glutathione. There is also enzymatic removal of free radicals by manganese superoxide dismutase (MnSOD) in the mitochondria and by SOD in the cytoplasm. All 13 genes coding for proteins in the mitochondrial genome are essential for execution of normal OXPHOS (Manczak et al., 2005). Complex I is especially susceptible to the aging process since 7 of the 13 genes encoded in the mitochondrial genome code for subunits belonging to complex I. Therefore, complex I deficiency is commonly observed during aging and can trigger neurodegeneration (Abramov et al., 2010). Interestingly, recently, deficiency of complex I and III accompanied with ROS production was shown to be sufficient to induce increased production of Aβ, which in turn triggered a vicious cycle of mitochondrial dysfunction more ROS and Aβ production (Leuner et al., 2012). The triggering event for altered APP processing in sporadic AD is unknown; though hypothetically, this vicious cycle might explain some of the events leading to elevated Aβ

production. This is supported by other studies that found that H_2O_2 and ROS elevate Aβ levels in cells and mouse models (Sun et al., 2006). Several recent findings show that mitochondrial dysfunction is one of the earliest events in AD (Hauptmann et al., 2009; Rhein et al., 2009; Du et al., 2010). Aβ has been detected inside mitochondria in postmortem AD brains, in transgenic mice overexpressing mutant APP and in human brain biopsies from patients with amyloidosis, as well as in neurons exposed to fluorescent Aβ *in vitro* (Lustbader et al., 2004; Caspersen et al., 2005; Hansson Petersen et al., 2008). As described earlier, cognitively intact individuals with profound accumulation of plaques and tangles in their brains could be discriminated from individuals with AD based on absence of Aβ accumulation in their mitochondria (Taglialatela, Poster, AAIC, 2012, Vancouver, Canada).

Early in the pathogenesis, a reduced number of mitochondria is observed in affected neurons (Hirai et al., 2001), brain glucose metabolism is decreased (Mosconi, 2005), the activities of both tricarboxylic acid (TCA) cycle enzymes (Bubber et al., 2005) and cytochrome c oxidase are reduced (Parker et al., 1990; Kish et al., 1992; Parker and Parks, 1995; Cardoso et al., 2004) and mitochondrial gene expression is upregulated perhaps as a compensatory mechanism (Reddy et al., 2004). Furthermore, maternal family history of AD predisposes to reduced brain glucose metabolism (Mosconi et al., 2007). Mitochondrial Aβ accumulation has been shown to occur prior to plaque formation in tgAPP mice, indicating that this is an early event in AD's pathogenesis (Caspersen et al., 2005). Recent findings imply that protein modifications, including carbonyl, 3-nitrotyrosin, 4-hydroxy nonenal (HNE), and S-glutathionyl, could regulate the activity of the metabolic enzymes. Many of these modifications result in significant inhibition of enzyme activity and, therefore, are likely to be involved in the dysregulation of metabolic pathways in AD (Hedskog et al., 2012). Neurons can utilize glucose, lactate, and ketone bodies as energy sources (Izumi et al., 1998; Suzuki et al., 2011). OXPHOS is the main pathway used for energy production in the brain since 15 times more energy is produced from respiration as compared to glycolysis. Cellular energy production is highly regulated. The activities of glycolysis, TCA, and respiration are integrated via feedback, including inhibitory, via ATP (Pasteur effect) and citrate controlling the rate of glycolysis. From the blood, glucose is transported over the BBB (endothelial cells and astrocytes) via GLUT1 transporter and subsequently transported into neurons via GLUT3. During hypometabolism (as is the case in AD brain), the brain cells compensate by increasing the activity of glycolytic proteins, called the Warburg effect, to override transient energy deficits and hypoxic environment (Hedskog et al., 2012). Recently, neuronal cells that utilize the Warburg effect were shown to be resistant to Aβ toxicity (Newington et al., 2011). Although, recent data from Suzuki and colleagues show that glycolysis is not enough for neuronal viability and survival in the long run (Suzuki et al., 2011). This study emphasizes the importance of lactate in synaptic plasticity. Lactate is produced by astrocytes (from glucose or glycogen metabolism) and transported to the neurons via monocarboxylate transporters. The inhibition of these channels, either on the astrocyte or neuronal side, causes impairment of LTP, substantiating the importance of a functional oxidative metabolism for neuronal plasticity (Suzuki et al., 2011). It is possible that the shift from OXPHOS to glycolysis that occurs in AD neurons can temporally provide enough ATP to sustain neuronal function. However, for the formation of LTP and other energy demanding processes a decreased glucose uptake in AD brain in combination with dysfunctional mitochondria may eventually result in synaptic failure and neuronal loss.

Abnormal mitochondrial structure (Baloyannis, 2006) and impaired balance of mitochondrial fusion and fission are found in the AD brain and in AD-animal models (Knott et al., 2008; Wang et al., 2009; Trushina et al., 2012). Exposure of neuronal cells in culture to conditioned

medium from cells stably expressing mutant forms of APP, or exposure to synthetic Aβ, induces mitochondrial fragmentation and abnormal distribution, which results in mitochondrial fission, loss of dendritic spines, and eventually cell death (Rui et al., 2006; Wang et al., 2008). Also, autophagic degradation of mitochondria may be dysfunctional in AD. Lysosomal proteolysis and autophagy require PS1, and AD-related PS1 mutations disrupt this degradation pathway (Lee et al., 2010). Despite the evident alteration in mitochondrial dynamics and metabolism in AD, the underlying mechanisms are unclear. It could result from environmental toxin, Aβ accumulation or acquired or inherited nuclear DNA and mtDNA mutations.

1.4.3 Aβ inside the mitochondria

Inside mitochondria, Aβ could interact with several proteins including complex IV in the electron transport chain (Rhein et al., 2009), Aβ-binding alcohol dehydrogenase (ABAD) (Lustbader et al., 2004), cyclophilin D (CypD) (Du et al., 2008) and the mitochondrial DNA base excision repair (BER) system (Taglialatela, Poster, AAIC, 2012, Vancouver, Canada) (see Figure 5).

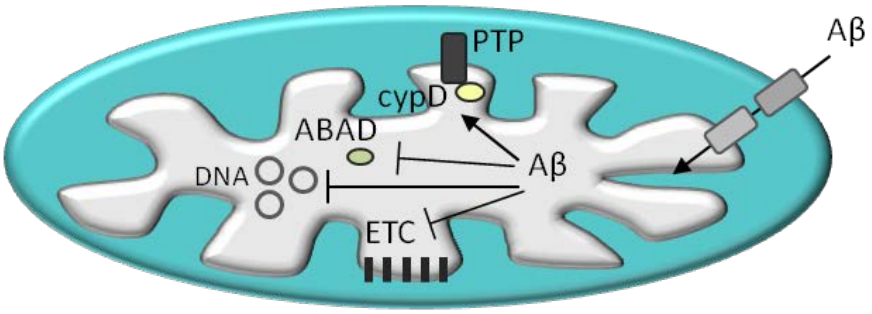

Figure 5. Illustration of Aβ inside the mitochondria. Inside the mitochondria Aβ can cause damage through interactions to various proteins including the mtDNA repair system (BER), complex IV in the electron transport chain (ETC), ABAD, and cypD causing PTP opening.

The binding to complex IV impairs OXPHOS and thereby the energy production, reducing the formation of ATP. ABAD is localized to the mitochondrial matrix and belongs to the short chain dehydrogenase reductase family. It has an essential physiological role in the metabolism of e.g. n-isopropanol, β-estradiol and keton body α-3-hydroxybutyrate and its activity is important to protect against metabolic stresses caused by, for example, ischemia (Du Yan et al., 2000). Inactivation of its counterpart (scully) in Drosophila results in a lethal phenotype with multiple developmental abnormalities. The binding of Aβ to ABAD is thought to disturb its normal function and cause mitochondrial dysfunction, increased ROS production and cell death (Takuma et al., 2005b). Inhibition of the interaction between Aβ and ABAD improves mitochondrial function in a mouse model of AD (Yao et al., 2011). Aβ interacts with CypD, a mitochondrial matrix protein that associates with the inner membrane during the opening of the mitochondrial permeability transition pore (PTP) (Du et al., 2008). Cortical mitochondria from CypD-deficient mice are resistant to Aβ- and calcium-induced mitochondrial swelling and permeability transition. Moreover, Tg mAPP/CypD-null mice exhibit improved learning and memory and synaptic function both in 12- and 24-month-old animals (Du et al., 2008; Du et al., 2011). Inside the mitochondria, presequence protease (PreP), could degrade Aβ (Falkevall et al., 2006). PreP can degrade Aβ40 and Aβ42, as well as Aβ Arctic protein (E22G) at unique cleavage sites, including several sites in the hydrophobic C-terminal Aβ segment that is prone to aggregate. Interestingly, PreP is an organellar functional analogue to the human insulin-

degrading enzyme (IDE). The importance of PreP in AD was recently further emphasized in reports that its activity was decreased in AD brain (Falkevall et al., 2006). The presence of Aβ in mitochondria indicates that it is produced inside mitochondria or taken up from the outside, or both. Most likely, it is taken up from outside, despite finding that both APP (Anandatheerthavarada et al., 2003) and γ-secretase complexes (Hansson et al., 2004) are detected in the mitochondria (Pavlov et al., 2011). Our laboratory has shown that Aβ is imported via the common mitochondrial import pore and thereafter localized to mitochondrial cristae (Hansson Petersen et al., 2008). APP, on the other hand, accumulates in the import pore of AD brain mitochondria, after the import having been arrested due to the acidic sequence described above (see the subheading "Amyloid precursor protein"). A consequence of TOM blockage is dysfunctional import of nuclear encoded proteins important for respiratory chain activity (Devi et al., 2006). Therefore, both Aβ and APP in the mitochondria cause mitochondrial abnormalities, which could result in synapse loss, which are the most robust correlates of AD-associated cognitive deficits (Terry, 2000; Selkoe, 2002). Interestingly, Roses and colleagues recently found that a polymorphic poly-T variant in the *TOMM40* gene could be used to estimate age-of-onset for late-onset AD (LOAD) in ApoE ε3 carriers (Roses et al., 2009). ApoE ε3/4 carriers with long poly-T repeats linked to ApoE ε3 had an age of onset 7 years earlier than individuals with shorter repeats. Whether the long poly-T repeat resulted in a modified TOM40 protein was unknown and needed investigation. In **Paper II**, we investigated this in fibroblast cells from ApoE ε3/4 carriers. However, we could not identify any deficits in mitochondrial functioning or morphology. This will be further discussed in the "Results and Discussion" section.

1.5 MITOCHONDRIA-ASSOCIATED ER MEMBRANE (MAM)

1.5.1 Distribution of mitochondria and ER in neurons

In highly polarized cells such as neurons, bidirectional transport of various cargoes is of great importance. The dynamics have been observed for a variety of different organelles and vesicles including mitochondria, endosomes, proteosomes, lysosomes, peroxisomes, lipid droplets, secretory vesicles, viruses and intermediate filaments (Collins et al., 2002; Welte, 2004),). In a neuron, mitochondria are transported and accumulate where the need for ATP production is especially high. Mitochondrial distribution efficiency is essential for neuronal development and survival. To maintain local energy and meet metabolic demand in all cellular regions, bidirectional motion along the microtubules is governed by growth factor signaling (e.g. NGF) and phosphatidyl-inositol (Chada and Hollenbeck, 2003; De Vos et al., 2003). The neuronal mitochondrial pool is heterogeneous and different properties of isolated synaptic and non-synaptic cortical mitochondria have been described, for example are the synaptic mitochondria more susceptible to calcium overload (Brown et al., 2006). Also the ER moves along the microtubules as both continuous networks and vesicular compartments (Waterman-Storer and Salmon, 1998; Bannai et al., 2004). The ER is a multifunctional organelle involved in protein folding and assembly, disulfide bond formation and calcium storage and to fulfill its many functions, the ER consists of specialized sub regions (Sitia and Meldolesi, 1992). At distal regions of the neurons, in for example spines, ER and mitochondria regulate calcium transients and, thereby, synaptic transmission (Pivovarova et al., 2002). Correct calcium handling by the two organelles and the calcium signaling between them is controlled by microtubules (Mironov et al., 2005). The interaction between ER and mitochondria in the synapse has been postulated to shape intracellular calcium signals and modulate synaptic and integrative neuronal activities (Mironov and Symonchuk, 2006). The specialized region of ER responsible for this interaction

is called the mitochondria-associated ER membrane (MAM) (Rizzuto et al., 1998; Csordas et al., 2006; Hayashi et al., 2009). Extensive investigation of the ER-mitochondrial interplay has been performed in yeast; however, limited research has been conducted on this interaction in cells of the brain. This will further be discussed in the next paragraph.

1.5.2 MAM the control station

Mitochondria and ER, important modulators of several pathways, are connected at specific contact points at a highly specialized region of ER called the mitochondria-associated ER membrane (MAM). At these points the metabolism of glucose, phospholipids, fatty acids, and cholesterol, as well as calcium homeostasis and apoptosis is regulated (Hayashi et al., 2009), (Simmen et al., 2005). Defective processes here could account for abnormalities routinely detected in AD patients, including: elevated cholesterol (Stefani and Liguri, 2009), altered fatty acid (Martin et al.), glucose (Hoyer et al., 1988), phospholipid (Pettegrew et al., 2001) metabolism, and abnormal calcium homeostasis (Bezprozvanny and Mattson, 2008). To date, more than two dozen proteins have been found to be concentrated in MAM (Area-Gomez et al., 2009) including proteins involved in calcium homeostasis (e.g. inositol triphosphate receptor isoform 3 [IP3R3] and Sigma non-opioid intracellular receptor 1 [Sigma1R]), in lipid metabolism (e.g. fatty acid co-A ligase 4 [FACL4] and phosphatidylserin synthase 1 [PSS1]), in intermediate metabolism (e.g. glucose-6-phosphatase), in cholesterol metabolism (e.g. acyl-coenzyme A: cholesterol acyltransferase 1 [ACAT]), and in apoptosis regulation (phosphofurin acidic cluster sorting protein 2 [PACS2]) (Rusinol et al., 1994; Hayashi et al., 2009) (see Figure 6). Increased ER-mitochondrial coupling has been shown to promote mitochondrial respiration and bioenergetics during early phases of ER stress (Bravo et al., 2011).

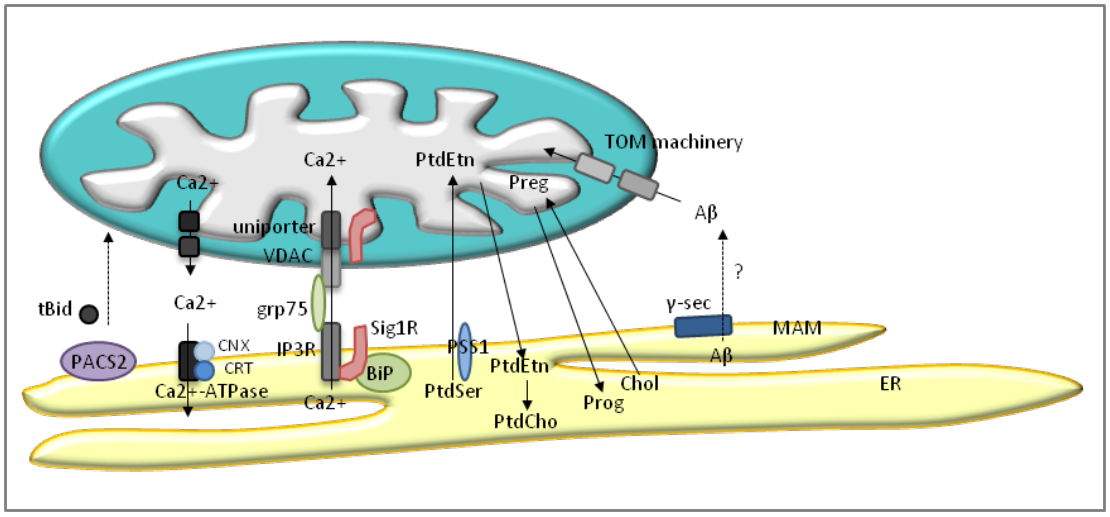

Figure 6. Illustration of signal transduction and metabolism at the MAM region. PACS2 can translocate tBid to the mitochondria and thereby induce cell death. Calcium is transported from ER to mitochondria via IP3R3-grp75-VDAC1 complex. Sigma1R (Sig1R) that normally is bound to BiP regulates the IP3R3 channel. Inside the mitochondria calcium activates enzymes in the TCA cycle leading to enhanced ATP production. Calcium can be transported back to MAM and at MAM be bound to chaperones, e.g. calnexin (CNX), calreticulin (CRT) and Bip. The phospholipid, phosphatidylserine, produced by PSS1 in MAM is transported over to the mitochondria to be converted to phosphatidylethanolamine (PtdEtn), which either is used for cardiolipin synthesis or transported back to MAM for conversion into phosphatidylcholine. Cholesterol is produced in ER and for the synthesis of steroids and neurosteroids, cholesterol is transported to the mitochondria for the conversion to pregnenolone (Preg), which then is transported back for the synthesis of progesterone (Prog). Since the γ-secretase complex has been postulated to exhibit high activity at MAM we speculate that at these tight ER-mitochondria contact points, Aβ is transported over and into the mitochondria.

1.5.3 MAM is connected to the mitochondria by tethering complex/es

The MAM region has been extensively studied in yeast. In this organism MAM is, apart from the above-mentioned processes, also involved in coordinating mitochondrial DNA replication, growth, protein import and maintenance of proper tubular morphology of mitochondria (Kornmann et al., 2009). Moreover, the tethering complex that couples ER to mitochondria has been described and is composed four proteins (Mmm1, Mdm12, Mdm10 and Mdm34). This complex is called the ER-mitochondria encounter structure (ERMES) (Kornmann et al., 2009). Disruption of ERMES impairs phospholipid exchange between ER and mitochondria since the enzymes responsible are located at either the MAM or the mitochondrial side. Recently, another study performed in yeast showed that proteins involved in shaping ER (called lastins in mammals) also play a role in maintaining functional contacts (Voss et al., 2012). Little is known about MAM in mammals and no homologues for ERMES components have yet been found. Several proteins have been suggested to participate in ER-mitochondrial bridging including IP3R, VDAC, grp75, Sigma1R, PACS2 and Mfn2 (Hayashi et al., 2009).

1.5.4 MAM regulates apoptosis

The ER-mitochondrial interface regulates apoptosis by several pathways, including calcium control and Bid translocation by PACS2 (Grimm, 2012). PACS2 is a multifunctional sorting protein that controls ER-mitochondrial communication, membrane traffic and apoptosis (Youker et al., 2009). PACS2 KO by RNAi causes loss of MAM-mitochondrial coupling and mitochondrial fragmentation (Simmen et al., 2005). In response to apoptotic stimuli, PACS2 binds dephophorylated Bid and traffics Bid to mitochondria where Bid is cleaved into its active form, truncated Bid (tBid) (Simmen et al., 2005). At the mitochondria, tBid interacts with Bax, promoting its insertion and oligomerization causing membrane permeabilization and the release of apoptotic molecules (see Figure 1 and Figure 6).

1.5.5 MAM controls calcium homeostasis

Calcium is a highly versatile second messenger that controls critical cellular responses, and which requires tight regulation, especially at the synapse where spatio-temporal patterning of calcium signaling is important for proper transmission. Calcium is essential for the enzymatic activity of many metabolic pathways in the mitochondria. However, excessive amounts can cause calcium overload accompanied by opening of the PTP pore enabling release of apoptotic mediators. Many proteins at MAM control the shuttle of calcium, thereby regulating the metabolic state of the mitochondria. The ATP produced provides energy for the SERCA pump to reload ER with calcium. Calcium is transported into the mitochondria via IP3R3 located at the ER side and the voltage-dependent anion channel (VDAC1) in the OMM (see Figure 6). This calcium signaling regulates mitochondrial metabolic activity since several enzymes in the TCA, require calcium to be active. One protein thought to regulate calcium transport is Sigma1R, which normally binds to the calcium binding chaperone, BiP; and, upon activation after ligand binding or by decreased luminal calcium concentration, BiP and Sigma1R, dissociates and Sigma1R becomes active. The upregulation and redistribution of Sigma1R after ER stress mediates cell protection against apoptosis. Sigma1R has been shown to interact with various ion channels and Ankyrin B (Hayashi and Su, 2001; Maurice and Su, 2009). Recently, Sigma1R has been shown to regulate ER-to-mitochondria calcium fluxes by stabilizing the IP3R3 channel by preventing degradation by the ubiquitin-proteosome system (Hayashi and Su, 2007). IP3R3 is also regulated by anti-apoptotic factors (e.g. Bcl-2), the interaction by which is thought to prevent mitochondrial calcium overload (Hayashi and Su, 2007).

1.5.6 The role of Sigma1R at MAM

Sigma1R has, apart from being involved in calcium signaling, been described as a unique ligand-regulated molecular chaperone, involved in the activity of several neurotransmitter systems (especially cholinergic and glutamatergic pathways), cell survival (Bcl-2 expression), learning processes, memory, mood alteration and regulation of hippocampal dendritic spine formation (Hayashi and Su, 2007; Tsai et al., 2009; Meunier and Hayashi, 2010; van Waarde et al., 2011). It is strongly expressed in both neurons and glia (Hayashi and Su, 2005). The intracellular localization of Sigma1R is mainly in lipid rafts in ER (i.e. MAM) but also nuclear, plasma membrane and mitochondrial localization has been described (Klouz et al., 2002; van Waarde et al., 2011). Sigma1R was first mischaracterized as an opioid receptor but later identified as a unique ligand-regulated molecular chaperone acting primarily at the MAM (Su et al., 1988; Hayashi and Su, 2003; Hayashi and Su, 2007). It has high affinity for diverse classes of psychotropic drugs (e.g. psychotomimetic drugs and neuroleptics), and has been proposed to play a role in a variety of diseases, including addiction, amnesia, pain, depression and stroke (Maurice and Su, 2009). To date, no endogenous ligands have been verified, however, neurosteroids, the hallucinogen, N,N-Dimethyltryptamine (DMT), and shingolipids have been suggested as agonists, and progesterone suggested as an antagonist (Fontanilla et al., 2009; Ramachandran et al., 2009; Johannessen et al., 2011). Activation of Sigma1R has been associated with anti-amnesic and neuroprotective effects and has been shown to protect against various pathological conditions including delirium in AD patients, ischemia, diabetes, inflammation, as well as Aβ toxicity (Marrazzo et al., 2005; Meunier et al., 2006; Furuse and Hashimoto, 2010; Ruscher et al., 2011). This will be discussed more in detail under the subheading “Treatment strategies”.

1.5.7 MAM regulates lipid synthesis

Enzymes in phospholipid and cholesterol synthesis pathways are localized on the MAM side and the mitochondrial side, calling for close contact between MAM and mitochondria for proper synthesis. Phospholipid biosynthesis is carried out as follows; phospatidylserine synthase (PSS1) is located at MAM and catalyzes the formation of phophatidylserine (PtdSer) from serine, which is transported into the mitochondria where it is converted to phosphatidylethanolamine (PtdEth) by phosphatidylserine decarboxylase. This intermediate is either used in the mitochondria to form cardiolipin or transported back to MAM for the conversion into phosphatidylcholine (major component of biological membranes) by the phosphatidylethanolamine methyltransferase-2 located in MAM (Hayashi et al., 2009) (see Figure 6). A reduced rate of lipid exchange, in turn, slows down aminoglycerophospholipid turnover, resulting in impaired cardiolipin synthesis accompanied by decreased mitochondrial membrane maintenance. Synthesis of steroids also depends on shuttling between ER and mitochondria since cholesterol, after its synthesis in ER, is transported to mitochondria for the catalyzed reaction by the cholesterol side-chain cleavage enzyme, forming pregnenolone, which then is transported back to ER for the synthesis of progesterone and several other steroids and neurosteroids (e.g. allopregnenolone, dehydroepiandrosterone) (Baulieu et al., 2001). Mounting evidence also indicates that localized in MAM are enzymes involved in ceramide and sphingolipid metabolism (Rusinol et al., 1994; Bionda et al., 2004).

In AD, a reduction in phospatidylethanolamine (Pettegrew et al., 2001) and alterations in cholesterol metabolism have been detected (Stefani and Liguri, 2009)). The deranged biosynthesis of cholesterol in ER is thought caused by e.g. Seladin-1 downregulation. This in turn lowers the level of cholesterol in lipid rafts, which has been associated with increased Aβ production signifying the importance of functional cholesterol biosynthesis (Bionda et al.,

2004). This is further supported by the finding that the neurosteroid, allopregnenolone, is reduced in the prefrontal cortex of AD patients (Marx et al., 2006). Interestingly, treatment with allopregnanolone reverses cognitive deficits in a triple transgenic mouse model of AD (Marx et al., 2006). Moreover, variations in plasma levels of sphingomyelins and ceramides are early predictor of memory impairment (Mielke et al., 2010). Thus, abnormal lipid metabolism in the brain could impair membrane lipid homeostasis, membrane repair and steroid signaling, and ultimately result in deteriorating synapses and neuronal networks.

1.5.8 Can Aβ be transported into the mitochondria from MAM?

Interestingly, both APP and the γ-secretase complex are enriched in MAM in mouse brain. Furthermore, γ-secretase has been demonstrated to exert its highest activity at MAM (Area-Gomez et al., 2009). This suggests that Aβ might be produced in MAM where the close proximity to the mitochondria might enable Aβ translocation into the mitochondria, and consequenly cause damage (see Figure 6). Thus, we believe that MAM harbors important clues for understanding AD-pathophysiology.

1.6 TREATMENT STRATEGIES

To date, as described above, there are two classes of drugs approved to treat AD, cholinesterase inhibitors (donepezil, rivastigmine, galantamine) and NMDA receptor antagonist (memantine). These drugs provide symptomatic relief but are weak agents modifying the disease pathophysiological process. Lately much effort has been made to find disease-modifying drugs that are targeted to reduce Aβ production (γ-secretase inhibitors/modulators, BACE1 inhibitors), prevent its aggregation or promote its clearance (active/passive immunization); or to affect tau phosphorylation and assembly. Unfortunately, none of these drugs have demonstrated efficacy in Phase III clinical trials. The failures of these clinical trials have raised a number of questions, from who to include in a clinical trial, to questions about our understanding of AD pathophysiology and biology. Hence, AD is increasingly considered as a heterogeneous and multifactorial disease that needs to be treated early with drugs covering multiple targets, the mitochondrion being one of them (Ankarcrona et al., 2010; Mielke et al., 2010). We believe that therapeutic interventions that affect the MAM region or the mitochondria could restore healthy regulation of glucose and lipid metabolism, calcium homeostasis and cell death signaling, each of which is evidently disturbed in AD.

1.6.1 Targeting MAM dysfunction

1.6.1.1 Targeting lipid metabolism

The lipid metabolism, as described earlier, is dysfunctional in AD. This could be due to altered regulation or ineffective transport of lipid intermediates between ER and mitochondria. Membrane cholesterol in lipid raft has been suggested to play a role in Aβ formation (Crameri et al., 2006). Lowered expression of seladin-1, an enzyme involved in cholesterol synthesis in ER, has been detected in AD, and studies in animal models demonstrate that lowering of cholesterol in lipid rafts increases Aβ generation, substantiating the importance of functional cholesterol biosynthesis (Crameri et al., 2006; Stefani and Liguri, 2009). This is further supported by the fact that inhibitors against ACAT, the enzyme converting membrane cholesterol into cytoplasmic cholesterol-ester have been shown to reduce amyloid pathology in an AD mouse model (Puglielli et al., 2001; Hutter-Paier et al., 2004). In addition, treatment

with the neurosteroid, allopregnanolone, has shown promising results in a triple transgenic mouse model of AD (Wang et al., 2010).

1.6.1.2 Targeting Sigma1R

Sigma1R is another promising target at MAM and is implicated in cellular differentiation, neuroplasticity, neuroprotection and cognitive processes of the brain (Marrazzo et al., 2005; Hayashi and Su, 2008). Several drugs show affinity for Sigma-1R, including donepezil, antipsychotics (haloperidol, rimcazole), selective serotonin reuptake inhibitors (fluvoxamine, sertraline) and monoamine oxidase inhibitors (clorgyline) (van Waarde et al., 2011). Depletion of Sigma1R by RNA interference (RNAi) causes inhibition of growth, survival-signaling cascades and induces cell death. The antagonist, rimcazole, produces the same effect. Sigma1R-KO mice display depressive, anxiety-like phenotype and altered motor behaviors. Moreover, the female mice demonstrated reduced levels of 17β estradiol levels and memory deficits associated with age (van Waarde et al., 2011). Treatment of aged rats with Sigma1R agonist (PRE-084) improved the spatial learning deficits that had emerged with age (Maurice, 2001). Furthermore, Sigma1R activation (by the agonist SA4503) stimulates recovery after stroke by enhancing cellular transport of biomolecules required for brain repair, thereby stimulating brain plasticity (Ruscher et al., 2011). Polymorphisms in the sigma1R gene have been linked to frontotemporal lobe dementia (Luty et al., 2010). A positron emission tomography (PET) study of AD patients concluded that the density of Sigma1R is lower early in AD pathogenesis. (Mishina et al., 2008). Similarly, reduced number of Sigma1R-binding sites is found in the CA1 area of the hippocampus in AD, which correlates with pyramidal cell loss in this region (Jansen et al., 1993). The mechanism of action of Sigma1R agonist is not fully understood, however, it is possible that it involves ER-to-mitochondria calcium fluxes via stabilization of IP3R3, thereby activating the calcium sensitive enzymes in the TCA cycle. Increased formation of ATP can, in turn, stimulate neuroprotection and neurite outgrowth leading to improvements in cognition and cerebral metabolism (Hayashi and Su, 2007). As we reported in **Paper III**, Sigma1R is also present in mitochondria, though its function is mostly unknown. However, our data suggest that it can modulate calcium uniporters in the IMM. Indeed, regarding the mode of action, much information points toward a role in regulation of calcium channels and since calcium homeostasis is altered in AD, Sigma1R agonist treatment potentially might aid its restoration. Taking into account the neuromodulatory role of the Sigma1 receptor, agonists can affect a large variety of processes in neurons, and in glia cells (astrocytes and oligodendrocytes), and, thereby, perform neuroprotective actions. Sigma1R agonists might, therefore, be of interest as an AD-therapeutic agent especially targeted to early disease stages where it can increase cellular resistance against various pathogenic processes.

1.6.1.3 Targeting cell death processes

MAM encompasses crucial determinants of life and death, including calcium signaling, Bcl2 family members and PACS2 (Decuypere et al., 2011). Rupture of the OMM plays a crucial role in the intrinsic cell death pathway and causes caspase activation. Drugs that interfere with caspase activation might provide therapeutic effect by, for instance, hindering caspase cleavage of IP3R3 (causes mitochondrial calcium overload) (Decuypere et al., 2011), PS1 (causes elevation of Aβ42/Aβ40 ratio, see **Paper I**) and tau (required for tau hyperphosphorylation) (de Calignon et al., 2010). The drug doxycycline averts Aβ toxicity by preventing Aβ fibrillization by inhibiting caspase 3 activation, as shown in studies using a neuroblastoma cell line and in a Drosophila AD model (Costa et al., 2011). However, many peptides designed to directly inactivate caspases, e.g. z-VAD or z-LEHD-FMK, have shown successful therapeutic effect against neuronal injury after hypoxia and cerebral ischemia injuries *in vivo*, yet have been

found to be ineffective for neuroprotection. Caspase inhibitors disturb the normal homeostasis of the cell and might cause activation of alternative pathways of death. Therefore, caspase inhibition might not be effective as AD treatment. Alternatively, drugs targeting mitochondrial function may be more promising since these could prevent the release of pro-apoptotic factors.

1.6.2 Targeting oxidative stress

Strategies targeting oxidative and nitrosative stress, which are considered important players in AD onset and progression, hold disease-modifying potential (Ankarcrona et al., 2010). Several interventions make it possible to overcome challenges of ineffective uptake over the BBB and limited concentration in the mitochondria (Murphy and Smith, 2007). One strategy is to conjugate the antioxidant to lipophilic cations for example using triphenylphosphonium (TPP^+), which enable rapid BBB and neuronal membrane crossing and to be concentrated (several hundred-fold) in the mitochondria (Murphy and Smith, 2007). Applying this technique, MitoQ and Mito-E_2 were developed. MitoQ is a compound that brings reducing power into the mitochondria in form of coenzyme Q10 (ubiquinone), an endogenous antioxidant and component of the mitochondrial electron transport chain, and which show potential in AD animal models (McManus et al., 2011). Mito-E_2 brings vitamin E into the mitochondria, and has also shown promising results in cell and animal models (Murphy and Smith, 2007; Leo et al., 2008). Moreover, the drug methylene blue (MTC), a tau antiaggregant, has shown promising results in clinical trials (Gura, 2008). Apart from affecting tau aggregation, MTC also has other properties, including function as an antioxidant (Atamna et al., 2008), as an alternative electron carrier that bypasses complex I/III (Wen et al., 2011) and can induce autophagy (Congdon et al., 2012). Interfering with mitochondrial ROS overproduction with mitochondrial-targeted catalase lengthen life span in mice (Schriner et al., 2005) and reduces abnormal APP processing in a AD mouse models (Schriner et al., 2005). Some antioxidants, e.g. trolox or superoxide dismutase, have also been shown to reduce Aβ production by reversing elevation of BACE1 activity apparent after ischemia (Guglielmotto et al., 2009). In the mitochondria, Aβ can interact with ABAD as described earlier. Small molecules that interfere with this interaction have emerged as additional potential drug targets for AD (Xie et al., 2006).

1.6.3 Dimebon

Mitochondrial dysfunction is evident in AD already at early stages in the disease progression. Therefore, rescuing mitochondrial functions is a promising therapeutic strategy since it could have disease modifying effect. The drug, Dimebon (Latrepirdine), which showed pro-cognitive and neuroprotective effects in a Russian Phase II clinical trial (Doody et al., 2008), was a new, hot candidate for AD treatment. Dimebon was originally approved as an antihistamine in the former Soviet Union in the 1980's (Matveeva, 1983) but was withdrawn from the market with the advent of more effective treatments. However, research on Dimebon continued. Around year 2000, it received renewed interest due to discovery of its neuroprotective effects (Lermontova et al., 2000; Bachurin et al., 2001; Wu et al., 2008). Dimebon's mode of action was unknown. It showed low affinity to acetylcholinesterase and NMDA receptors, yet high affinity to histamine, adrenergic, dopaminergic and serotonergic receptors. In our laboratory, we explored its mode of action and concluded that some of its neuroprotective effects could be mediated via stabilizing mitochondrial function (as shown in **Paper IV**). This will be further discussed in the section "Results and Discussion". Unfortunately, recently, two Phase III AD clinical trials failed to meet primary and secondary efficacy endpoints. The CONNECTION study was designed akin to the Russian phase II clinical trial. In this trial that was conducted for one year, mild-to-moderate AD patients were enrolled and received either Dimebon or placebo

for six months. Thereafter, the trial was designed so that also the placebo group where given Dimebon. The results after six-month treatment showed no significantly change from baseline, in either group. The CONCERT study was designed as a double drug-therapy trial, in which Dimebon was an add-on to donecepil. Also in this study, mild-to-moderate AD patients were enrolled. This study was conducted for one year, after which, again, the study failed to meet the endpoint criteria. Thus, Dimebon failed to show improvement of cognition in AD at mild-to-moderate disease stage. Perhaps, treatment provided at an earlier disease stage, prior to the widespread pathology and neuronal loss as would be later manifest could bring greater efficacy. Dimebon will be further discussed in the section “Results and Discussion”.

In summary, targeting MAM and/or mitochondria could potentially reverse failing mitochondrial bioenergetics, including glucose and lipid metabolism, calcium homeostasis, ROS production, intracellular Aβ production and cell survival. Therefore, these targets represent promising strategy for AD therapy.

2 AIMS

The general aim of this thesis was to explore mechanisms underlying mitochondrial dysfunction in AD bringing into focus regulatory mechanisms and caspase activation. Mitochondria are key players in determining cellular fate, including life or death decisions. Already subtle changes in the intracellular Aβ42/Aβ40 ratio are linked to Aβ oligomerization and toxicity, which has been observed during mitochondrial ROS production and activation of caspases.

- **Paper I.** To investigate by what mechanism caspases alter the intracellular Aβ42/Aβ40 ratio and to explore implications of these mechanisms for sporadic AD.

Intracellular Aβ has been found to accumulate in the mitochondria where it causes damage to metabolic pathways, mtDNA repair and increases the likelihood of PTP opening. Aβ might be produced in MAM, subsequently transported over to the mitochondrial side, and imported via the TOM40 pore, the common import machinery through which all nuclear-encoded mitochondrial protein has to be transported. The *TOMM40* gene is in linkage disequilibrium with the *APOE* gene and polymorphisms exist that are associated with AD.

- **Paper II**. To study functional implications of the recently identified poly-T repeat in the *TOMM40* gene linked to lowered age-of-onset of LOAD in ApoE ε3/4 carriers, in regards to mitochondrial function.

MAM regulates vital biological processes, including metabolism of glucose and lipids, calcium homeostasis and regulation of cell death, all of which become dysregulated in AD. Both ER and mitochondria are important players in maintaining proper synaptic function. However, little is known about MAM in neurons, especially as concerns AD perspective.

- **Paper III**. To examine the importance of MAM for neuronal survival, and in relation to AD pathogenesis.

Treatment strategies targeting the mitochondria could have beneficial disease modulatory effects since increasing the mitochondrial resistance towards cellular stressors could strengthen the synapses and thereby hinder dysfunction and loss of synapses.

- **Paper IV**. To investigate mode of action of Dimebon, a potential novel drug for treatment of AD.

3 METHODOLOGICAL CONSIDERATIONS

3.1 ETHICS APPROVAL

Ethical permissions for using animal and human tissues were obtained from the Regional Ethics Committee for Animal Research in Stockholm South and the Regional Ethics Committee of Stockholm.

3.2 MODELS USED IN THE STUDIES

For conducting these studies we have used a variety of models systems including conventional cell lines, human fibroblast cell cultures derived from biopsies, mouse primary neuronal cell cultures, AD-mouse models ($APP_{Swe/Lon}$ and APP_{Arc}) and human postmortem cortical brain tissue.

3.2.1 Cells

The conventional cell lines used included mouse blastocyst (BD8) cells in **Paper I** and neuroblastoma (SH-SY5Y) cells in **Papers I**, **III** and **IV**. Conventional immortalized cell lines are an excellent tool used to study molecular and cellular processes in a simple and controlled environment and do not require ethical permission. However, they are usually poor representations of cells found in intact tissues. The SH-SY5Y cell line, which is a cancer cell line, can be differentiated into neuronal-like cells upon retinoic acid treatment that induce attenuation of proliferation and extension of neuritic processes (**Paper IV)**. This model represents a good substitute to primary neuronal cultures since it does not require sacrificing animals. Nevertheless, for a more comprehensive analysis of processes occurring in neurons, primary cultures are needed. Here, we have used cortical (**Paper IV)** and hippocampal (**Paper III)** primary neurons from mouse embryos. In **Paper II**, we used fibroblast cell cultures generated from skin biopsies from individuals in AD families.

3.2.2 Human postmortem tissue

To study how expression of different proteins is affected in AD, we have used human postmortem cortical tissue (**Paper III**), which is of great value for studying disease mechanisms. However, cautions should be taken since postmortem tissue represents the end stage of the disease and does not give information about progressive mechanisms. Moreover, differences between sample handling and postmortem times can differ substantially and thereby affect protein expressions.

3.2.3 Transgenic mouse models

To be able to follow disease progression transgenic mouse models, $APP_{Swe/Lon}$ and the APP_{Arc}, have been used (**Paper III**). The $APP_{Swe/Lon}$ mouse model overexpresses the Swedish (K670N/M671L) and London (V717I) mutation and, subsequently, accumulates large quantities of dense-core and diffuse amyloid plaques (see Figure 7) (Rockenstein et al., 2001). The APP_{Arc} mouse model overexpresses the Arctic mutation (E693G), which is an intra-Aβ mutation (see Figure 7) that gives rise mainly to diffuse Aβ deposits (Ronnback et al., 2012). The APP_{Arc} model has a milder amyloid pathology affecting primarily the subiculum as opposed to the $APP_{Swe/Lon}$ mouse model that develops mature plaques as early as 3-4 months and exhibits widespread amyloid pathology, affecting neocortex, hippocampus, thalamus and olfactory regions. Studying these two animal models can give valuable information on how

different Aβ pathologies (Aβ-peptides vs. $A\beta_{Arc}$-peptides) affect particular biological processes and pathways at different stages of disease. Nevertheless, the pathology in animal models does only represent some aspects of AD; tau pathology, for instance, is lacking. Therefore, one should be cautious when interpreting data acquired from transgenic animals.

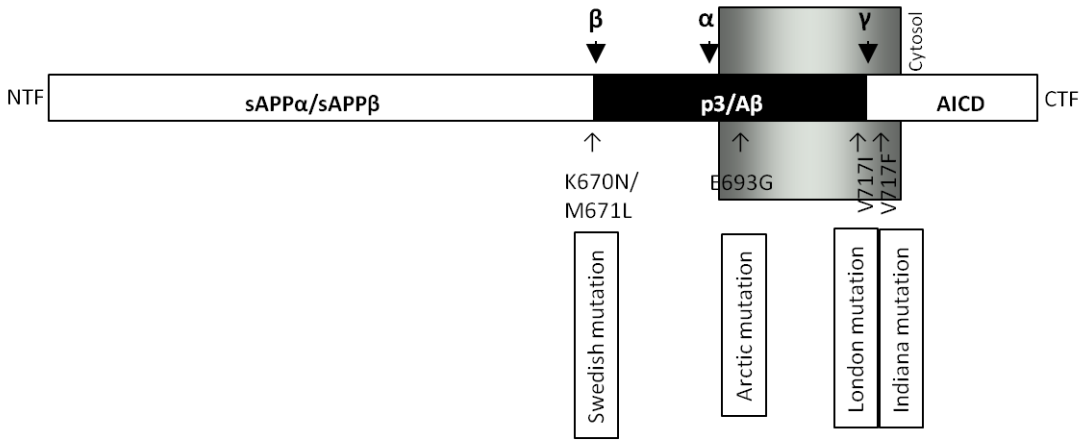

Figure 7. Illustration of APP showing the cleavage sites for α-, β-, and γ-secretases and the position of Swedish, Arctic, London and Indiana mutations.

3.3 QUANTITATIVE MEASUREMENTS OF PROTEIN EXPRESSION

For quantification of protein levels, we used Western blot (**Paper I**, **II and III**) and enzyme-linked immunosorbent assay (ELISA) (**Paper I** and **III**). Both these methods are based on antibody detection. Antibodies have affinity for specific epitopes that at certain circumstances can be hidden in the proteins. This can be caused by for example conformational changes and aggregation. Therefore, this is something that is important to keep in mind when interpreting data generated from antibody-based methods.

A good complement is immunohistochemistry, which was used in **Paper III,** and with which protein expression levels can be visualized and studied in different brain regions. This method is, however, more qualitative than quantitative. Another method used for detection of proteins is liquid chromatography-mass spectrometry (LC-MS), which can be either qualitative or quantitative depending on internal standards. In **Paper I**, an ion-trap mass spectrometer (Agilent 6330) was used to qualitatively analyze Aβ-peptides secreted from cell lines with the aim of identifying long Aβ-peptides (>Aβ42). This was however, not fully successful; though Aβ42 could be detected and this information used to verify our earlier ELISA data.

3.4 SUBCELLULAR FRACTIONATION

Many proteins reside in certain compartments within the cells, and are destined for specific activities. Their intracellular localization can be studied by subcellular fractionation. In order to investigate protein expression at the MAM region and to study ER-mitochondrial contacts in synapses (**in Paper III**) we fractionated mouse brains on percoll gradients according to previously described protocols (Dunkley et al., 2008; Wieckowski et al., 2009). Caution should be taken when interpreting pureness of fractions after subcellular fractiontion since these almost certainly contain contamination from other cellular regions, generating artifacts. Therefore, for comprehensive analysis of pureness, expression of several organelle specific proteins could be studied by Western blot and the fractions could be studied by electron microscopy.

3.5 EXPOSING CELLS TO Aβ

Studying the impact of Aβ on various biological processes is important to understand AD pathogenesis. However, which Aβ species (Aβ40, Aβ42) and assembly forms (monomer, oligomer, fibrils) are the most toxic is highly debated. Currently, soluble oligomers (beta-sheet structures) are thought to have more influence on toxicity than monomers (α-helical or random coil structures) or fibrils (parallel beta-sheet conformation) (Iversen et al., 1995). We chose, in **Paper III**, to use conditioned media from the well characterized CHO7PA2 cell line that overexpresses the Indiana mutation (APPV17F) (see Figure 7), primarily secreting oligomeric Aβ species (Walsh et al., 2002). Our selected concentration for Aβ in our experiments were either 2.5 nM or 4.5 nM (total Aβ40 and Aβ42), which are thought to resembles concentrations that neurons are exposed to in the brain (Cleary et al., 2005).

3.6 STUDY MITOCHONDRIAL FUNCTION

There are several ways to study mitochondrial function. Here, we used techniques that assess different aspects including ΔΨm (**Paper II**, **IV**), complex II activity by MTT-assay (**Paper IV**), ATP production (**Paper IV**) and mitochondrial biogenesis (**Paper II and IV**). MTT assay is a colorimetric technique, in which the color change that occurs when tetrazolium salt is reduced to formazan is measured. This reaction is mainly catalyzed by complex II in the electron transport chain and is, therefore, an indicator of the cells redox activity. Maybe however, other enzymes residing in the cytosol also have this capacity and, therefore, cautions ought to be made when interpreting activity as strictly mitochondrial oriented. ATP levels are studied, as a measure of the coupling of OXPHOS, here, by employing a luminescent method (**Paper IV**). However, a more illuminating view of the bioenergetic state of the mitochondria could be given assessing the ADP/ATP ratio. The ΔΨm is used to drive the phosphorylation of ADP at complex V in order to generate ATP. To monitor alternations in the ΔΨm, the cationic dye TMRM, which accumulates inside the mitochondria in proportion to the ΔΨm, was used. Mitochondrial biogenesis was assessed by one of two different ways, either by quantifying mitochondrial volume density on electron microscopy pictures (**Paper II)** or by quantifying mtDNA copy number by quantitative real time PCR **(Paper IV)**. The measurement using electron microscopy pictures was performed by point counting using a 2 cm and 1 cm square lattice (Weibel 1979).

3.7 VISUALIZATION OF PROTEINS AND ORGANELLES IN FIXED SAMPLES

Confocal microscopy is a widely used tool to study various cellular processes since it enables visualization along one focal plane by employing a spatial pinhole that eliminates out-of-focus light, increasing optical resolution and contrast. Since only one focal plane is visualized, it enables visualization of protein and organelle interactions. Using the Zeiss LSM510 META confocal microscope we imaged chromatin condensation (**Paper I**), distribution of MAM proteins (**Paper III**), and ER- mitochondrial interactions (co-localization) after Aβ treatment (**Paper III**). For this purpose we used 2D co-localization method in the LSM 510 software program. However, care must be taken when attributing significance to the 2D method since signals used in calculations might originate from outside the focal plane of interest. Therefore, 3D methods have gained increased interests since the calculated co-localization originates from volume reconstruction and, therefore, has the capacity to reduce these problems.

Transmission electron tomography (TEM) is a method to image cellular structures with higher magnification. This technique utilizes a beam of electrons to increase the resolution instead of photons, which are used in a regular microscope to create the image. We used this technique to study mitochondrial morphology (**Paper II**) and ER-mitochondrial interactions in synaptosomes (**Paper III**).

3.8 LIVE CELL IMAGING

Live cell imaging is a widely used technique to study dynamic networks in cells. We performed several live cell analyses using the Zeiss LSM510 META confocal microscope, including visualizing of ΔΨm using the dye TMRM (**Paper II**, **IV**), viability of hippocampal neurons after siRNA transfection (**Paper III**), and distribution of mitochondria and ER using Cell-Light probes (**Paper III**). For quantification of changes in ΔΨm, the TMRM intensities were analyzed at different time point using the LSM 510 software program. For this purpose it is important to ensure constant focus, which can be controlled by simultaneous visualization of e.g. nucleus.

3.9 MEASUREMENT OF CALCIUM SIGNALING BETWEEN ER AND MITOCHONDRIA

Spatiotemporal calcium measurements in intracellular compartments including ER, cytosol and mitochondria give valuable insights into biological processes. One method developed for this purpose utilizes aequorin, a protein isolated from a luminescent jellyfish. This protein is an apoenzyme and needs a prosthetic group (cofactor) for its function. The cofactor is coelenterazine, which contains several calcium binding sites. Upon calcium binding the protein undergoes a conformational change and through oxidation converts coelenterazine into the excited coelenteramide, which emits blue light when relaxing to its ground state. This emitted light can be measured by a luminometer. Advantages of using aequorin include easy transfection, that no optical excitation is needed and well tolerability by the cells. One limitation is, however, that the prosthetic group, coelenterazine, requires continuous addition since it is consumed when reacting with calcium. In **Paper III** we used this method to study the calcium signaling between ER and mitochondria.

4 RESULTS AND DISCUSSION

4.1 MITOCHONDRIAL REGULATION AND DYSFUNCTION IN AD

Low glucose metabolism and mitochondrial impairment are observed already at early stages of AD. The triggering events causing altered bioenergetics and mitochondrial dysfunction are not fully understood. We speculate that alterations in MAM functioning may partly underlie these defects. Glucose and lipid metabolism, calcium homeostasis and cell death regulation are processes that are impaired in AD, and all are controlled in MAM. Growing evidence also suggests that Aβ can be produced in MAM. For an illustrational overview of the **Paper I to IV** see Figure 8.

4.2 CASPASE CLEAVAGE OF PS1 INCREASES THE Aβ42/Aβ40 RATIO

The pathology of AD affects the regulation of cell death as demonstrated by aberrant activation of caspases, which is a prominent feature of AD and has been implicated in synaptic degeneration and neuronal loss (Stadelmann et al., 1999a; Louneva et al., 2008b; Albrecht, S. et al., 2009). Active caspases have been detected in cerebrospinal fluid and postmortem brain tissue of sporadic and FAD patients, and in AD-mouse models, and these correlate with elevated Aβ production (Tesco et al., 2003; Albrecht, P. et al., 2009). The mechanism by which caspase activation causes elevated Aβ is not fully understood. Caspases can cleave proteins involved in Aβ generation, including APP, PS1 and PS2; however, the truncation of APP does not seem to influence Aβ production (Tesco et al., 2003). The γ-secretase complex contains either PS1 or PS2. Those incorporating PS1 are more common and also more likely to be involved in APP processing (Franberg et al., 2011). Moreover, the majority of FAD mutations are located in PS1 as compared to PS2 or APP. PS1 can be cleaved at Asp333 and Asp345 in the loop region by several caspases including 1, 3, 6, 7, 8 and 11 causing the loss of half of the loop (Kim et al., 1997; van de Craen et al., 1999) (see Figure 3). The loop region has previously been shown to possess regulatory functions in regards to Aβ production (Deng et al., 2006). Therefore, in **Paper I,** we aimed to investigate caspase cleavage of PS1 more carefully. Previously, in our laboratory we had shown that PS1, despite the loss of half the loop domain after caspase cleavage (caspCTF), is still part of active γ-secretase complexes (Hansson et al., 2006). Thus, we wanted to investigate if this truncation could change the cleavage preferences in APP. To do so, we established stable cell lines either expressing wild type CTF (wtCTF) or caspCTF. As expected, caspCTF formed active γ-secretase complexes together with Nicastrin, Pen-2, Aph-1 and PS1-NTF. When studying the Aβ profile generated from these γ-secretase complexes we identified a shift at the γ-site. The caspCTF-containing γ-secretase complexes had an increased intracellular Aβ42 production, resulting in a significant elevation of Aβ42/Aβ40 ratio as compared to wtCTF-containing γ-secretase complexes. Thus, the increased production of Aβ42 during apoptosis can be at least partly explained by the formation of caspCTF-containing γ-secretase complexes (see Figure 8). Interestingly, activation of caspases is not only a consequence of the pathophysiology of AD but is actually continuously occurring in a transient fashion in normal brain physiology. This is seen, for example, in neurons in which activation of caspase enables synaptic reorganization and neurite retraction. Therefore, activation of caspases is important for neuronal plasticity (Li et al., 2010). Consequently, caspase cleavage of PS1 could occur both under normal plasticity and in pathological conditions, during which caspases are activated. When studying the intracellular localization of caspCTF in healthy mouse brain we identified this truncated form of PS1 in several compartments, however, most abundantly in lysosomes, plasma membrane and, interestingly,

also in MAM (**Paper III**) (see Figure 8). This means that, locally in MAM, the Aβ42/40 ratio could be elevated to influence MAM's many regulatory functions, and at this site, Aβ could be transported over into the mitochondria where damage could trigger mitochondrial dysfunction (see Figure 8).

4.3 *TOMM40* POLYMORPHISMS, IMPLICATIONS FOR AD

Aβ has been detected inside the mitochondria in AD patients, and at an early stage of pathology in AD mouse models. Accumulation of Aβ inside the mitochondria has recently been shown to represent a phenomenon separating individuals with profound AD lesions into two groups one that develops AD and one that remains cognitively intact. The group that developed AD exhibited accumulation of Aβ in their mitochondria whereas the group that remained cognitive intact did not (Taglialatela, Poster, AAIC, 2012 Vancouver, Canada). Inside the mitochondria Aβ interacts with several proteins, e.g. complex IV, cyclophilin D and ABAD, which causes mitochondrial dysfunction. The pathway by which Aβ is transported into the mitochondria has previously been investigated in our laboratory showing that import follows the common rout via the Tom40-import pore, through which all the 1000 nuclear-encoded mitochondrial proteins are imported (Hansson Petersen et al., 2008) (see Figure 8). Several polymorphisms that have been associated with increased risk of late-onset AD have been identified in the *TOMM40* gene. The *TOMM40* gene is located on chromosome 19 in a region of linkage disequilibrium (LD) with *APOE*. Much effort is being brought to identify whether, in addition to APOE, the LD-block contains other risk factors influencing AD. The polymorphism, rs10524523, in *TOMM40,* has received particular attention since one of its variants was shown to lower the age-of-onset of LOAD by 7 years in *APOE* ε3/4 carriers (Roses et al., 2009). Thymidine stretches of varying length in intron 6 comprise rs10524523. The linkage disequilibrium between the *TOMM40* and *APOE* genes ensures that the ε4 allele will always be inherited along with a long poly-T repeat, whereas the ε3 allele is inherited with either a very long or a short poly-T repeat. In **Paper II** we used biochemical methods to investigate rs10524523. We could not detect any functional implications of this polymorphism in regards to (i) expression levels of TOM40 protein and mRNA, (ii) TOM40 mRNA splicing, or (iii) mitochondrial function and morphology. Therefore, we think that this specific polymorphism does not participate in a significant manner in mitochondrial dysfunction. However, several other polymorphisms might be worth investigating (see Figure 8).

4.4 MAM IS MODULATED BY AD PATHOGENESIS

MAM are connected to mitochondria and interaction between ER and mitochondria via its contact points are crucial for proper cell function. In AD, the disturbed glucose, phospholipid and cholesterol metabolism, the altered calcium homeostasis and the dysregulated cell death might indeed be linked to MAM alterations. Having found in the literature a scarcity of knowledge about MAM in the brain and in diseases like AD, we, in **Paper III,** set out to investigate it. We report, that (i) proteins enriched in MAM (e.g. PACS2, PSS1, Sigma-1R, IP3R3) are ubiquitously expressed in neurons and expressed both in soma and at distal parts of the neurites and in synapses, (ii) MAM function is essential for neuronal and astrocytic survival since siRNA treatment against PACS2 and Sigma-1R causes degeneration, and that (iii) the MAM region is altered in the human AD brain, and before the appearance of plaques, at least in the $APP_{Swe/Lon}$ mouse model. In addition, Aβ exposure at nM concentration of hippocampal neurons increased (i) the expression of MAM proteins, (ii) the number of contact points between ER and mitochondria and (iii) the transfer of calcium from ER to mitochondria, modulating especially calcium uptake mechanisms on the mitochondrial side, as shown in SH-SY5Y cells treated with a combination of Aβ and Sigma-1R agonist. Thus, physiological

concentrations of Aβ do modulate ER-mitochondria contact points and calcium shuttling, from the ER to the mitochondria, causing an increase in mitochondrial-calcium concentration, which in the long run may lead to Ca^{2+} overload and neuronal toxicity. Our data indicate that ER-mitochondria contacts could be affected by Aβ during the progress of AD pathogenesis and that MAM modulation may be involved in altered neuronal activity in AD (see Figure 8). This is the first study describing the distribution of MAM in the brain, the subcellular localization of MAM in neurons and the influence of Aβ on MAM protein expression, regulation of contact points and calcium transfer into mitochondria. Moreover, emerging evidence suggests that Aβ actually could be produced in MAM since high activity of the γ-secretase complex has been reported in the MAM region. In concordance, our data further support this theory since we also detected APP, C99 and PS in MAM (**Paper III**). Moreover, preliminary data also indicate that BACE1 is located there (data not shown). Interestingly, we also detected caspCTF in MAM, which locally could change the Aβ42/Aβ40 ratio at these spots. We suggest a novel function of Aβ as a regulator of ER-mitochondria contacts, which may result in altered mitochondrial function both under physiological and pathological conditions, depending on dose and time (see Figure 8). Aβ might regulate (i) calcium uptake into the mitochondria (**Paper III**) and thereby regulate the energetics of mitochondria, (ii) production of both cholesterol and sphingomyelin (Grimm et al., 2005) and (iii) fluidity of the lipid-raft like lipid bilayer that MAM is composed of, and do so by binding to sphingolipids. On the other hand, Aβ can (i) accumulate in the mitochondria causing severe damage, (ii) cause elevation of mitochondrial ROS production (iii) cause release apoptotic mediators from the mitochondria.

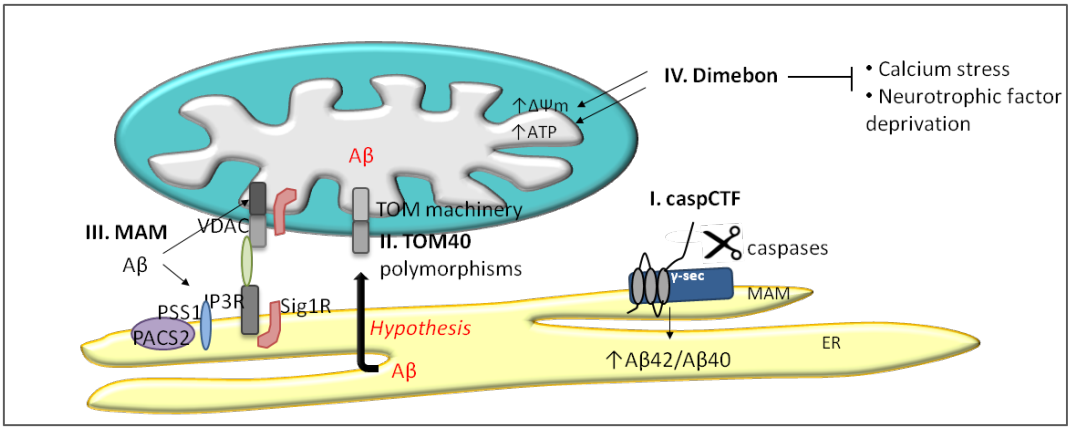

Figure 8. Overview of the studies. In **Paper I** we showed that γ-secretase complexes containing caspCTF increase the intracellular Aβ42/Aβ40 ratio. In **Paper II** we studied a specific polymorphism in the *TOMM40* gene consisting of a poly-T stretch with varying length. Even though no functional implication was found regarding this polymorphism, others could be worth investigating. In **Paper III** we found that the MAM region is altered in AD and the ER-mitochondria contact points and calcium transport increases after Aβ exposure. In the last study, **Paper IV**, we looked at the mode of action of a potential novel AD drug, Dimebon, and found that it enhances mitochondrial function and increases the resistance against various stressors. We hypothesize that Aβ produced at MAM could modulate the MAM region, modulate the amount/distance of ER-mitochondria contact points and be the region where Aβ is transported over to the mitochondria.

4.5 TARGETING MITOCHONDRIAL FUNCTION

The final study, **Paper IV**, concerned the potential novel drug, Dimebon (Latrepiridine), intended for treatment of AD (Doody et al., 2008). We investigated Dimebon's mode of action in two *in vitro* models, primary mouse cortical neurons and SH-SY5Y cells. After one to five days incubation with nM concentrations of Dimebon, increased ΔΨm accompanied with increased cellular ATP levels was observed in both cell systems. Pretreatment with Dimebon made cells more resistant to calcium overload and loss of ΔΨm. Moreover, cells exhibited increased resistance towards induction of cell death after growth factor deprivation (see Figure 8). Thereby, our study suggests that Dimebon affects the bioenergetics of the mitochondria and thereby protects cells against various cell stressors. Notably, resistance towards calcium stress was only observed in pretreated cells. Thus, Dimebon enhanced mitochondrial function, however, for optimal protection and for maintenance of cell viability, pretreatment is preferable. Recent studies on Dimebon have shown that Dimebon can ameliorate Aβ induced mitochondrial impairments (Eckert et al., 2012) and increase cerebral glucose utilization in aged mice (Day et al., 2011). These data further support Dimebon's bioenergetic effects. Other modes of action have also been suggested including modulation of PTP (Bachurin et al., 2003), protection against Aβ toxicity (Bachurin et al., 2001), and decrease aggregation of TDP-43 and γ-synuclein (Yamashita et al., 2009; Bachurin et al., 2012). Interestingly, in three model systems Dimebon treatment was shown to increase the secretion of Aβ. The treatment of APP_{Swe} overexpressing cells and synaptoneurosomes isolated from $APP_{Swe/Ind}$ mice (1-10 min or 6 h with Dimebon) elevated the secretion of Aβ. Injection of Dimebon into the brains of APP_{Swe} mice showed a 40% increase in Aβ40 in interstitial fluid 10 h post-injection (Steele et al., 2009). Increasing Aβ secretion could be beneficial since secretion has been associated with neuroprotection in contrast to intracellular Aβ accumulation, which causes severe damage (Nilsson, Poster, AAIC, 2012, Vancouver, Canada). By strengthening the mitochondria and increasing Aβ secretion from neurons, Dimebon exhibits properties that could be important for generating disease-modifying effects. Unfortunately, as described earlier, Dimebon has recently failed two Phase III AD clinical trials (www.alzforum.org/new/detail.asp?id=2387). Nevertheless, the rich pharmacological profile of Dimebon is a basis to suggest it could be effective against other neurodegenerative diseases such as Huntington disease and neurological diseases like Schizophrenia. A Phase II clinical trial for Huntington disease has shown promising results (Kieburtz et al., 2010). Also a recent Phase II clinical trial for Schizophrenia, in which Dimebon was administered as an add-on to risperidone therapy, showed positive outcome. Several cognitive measures were improved possibly via serotonin receptor (5-HT6) blockage (Morozova et al., 2012).

Drugs restoring the function of mitochondria and/or MAM might have disease-modulatory effect. The importance of a proper interaction between ER and mitochondria is evident as demonstrated in two other neurodegenerative diseases: GM1-gangliosidosis and Charcot-Marie-Tooth neuropathy type 2a. In GM1-gangliosidosis, GM1-ganglioside accumulates in MAM where the interaction with IP3R leads to clustering of IP3R and increased calcium signaling resulting in calcium-dependent mitochondrial apoptosis (Sano et al., 2009). Charcot-Marie-Tooth neuropathy type 2a is a disease caused by missense mutations in Mfn2. The interaction between ER and mitochondria depend on Mfn2 and absence of Mfn2 diminishes these interactions, thereby hampering calcium signals to the mitochondria. It is speculated that the mutations causing the disease might involve similar mechanisms (Zuchner et al., 2004; de Brito and Scorrano, 2008). Hence, the understanding of MAM *per se* and in regards to mitochondrial function is just in the beginning and research in this field might shed light to

several pathophysiological mechanisms involved in AD as well as other neurodegenerative diseases.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In many respects, neurons, to sustain proper function, depend on an ability to form and maintain synapses, an operation which requires adequate energy supply and calcium handling. Mitochondria are the main energy providers in the brain and are the site for numerous biosynthetic and catabolic pathways essential for cell function. Their distribution is thought to be controlled by local energy and metabolic demand. Maintaining sufficient energy and metabolic supply to all cellular regions throughout the cell is a great challenge and becomes exceedingly difficult in polarized cells like neurons. Consequently, efficient control of mitochondrial distribution and expedient transport in response to different needs is essential for neuronal development and survival. Recently, it become clear that the mitochondrion does not act entirely alone; it needs to interact with ER to enable many processes. ER distribution is, as well, heavily regulated to ensure transport to all cellular regions. At the MAM region, ER interacts with the mitochondria, ensuring proper regulation of glucose, phospholipid and cholesterol metabolism, calcium homeostasis and apoptosis. All of these processes are known to be defective in AD. Since it is well established that mitochondrial bioenergetics and function are deranged already at early stages of AD, it is possible that alterations in MAM function may partly underlie these defects.

We have detected MAM in the synapse where calcium homeostasis, energy metabolism and cell death signaling needs to be heavily regulated. With the knowledge that synapse dysfunction and synapse loss are events correlating best with cognitive impairments, we speculate that MAM function plays a pivotal role in synaptic integrity. Both dysfunctional mitochondria and aberrant caspase activation are prominent features of synapse dysfunction (Wei et al., 2010; Zempel et al., 2010; Trushina et al., 2012). Caspase activation is important for synaptic reorganization, LTD, LTP and plasticity in the brain, where caspase cleavage of structural proteins, like the actin skeleton, enables change in morphology. The activity of caspases must, however, be heavily regulated so to not cause aberrant activation and execution of the cell death program. Since the feature of plasticity involves activation of caspases, this means that several proteins during the process of activation can be cleaved, including PS1 and tau. The formation of caspCTF and the consequent elevation in intracellular Aβ42/Aβ40 ratio might be an important factor in plasticity, particularly since Aβ has been reported to regulate AMPA receptor internalization. Also the cleavage of tau might enable microtubule derangement. In any case, caspase activity can, indeed, also cause both Aβ toxicity and tau hyperphosphorylation (Quintanilla et al., 2012). Already, minor changes in intracellular Aβ42/Aβ40 ratio have been shown to be detrimental (Masters and Selkoe, 2012) and both Aβ and caspase-cleaved tau impair mitochondria (Quintanilla et al., 2012). Since the γ-secretase complex, APP and BACE1 are all located to MAM we speculate that Aβ from this location can easily be transported over to the mitochondria and then be imported via the TOM40 pore (Hansson Petersen et al., 2008) (see Figure 8). Moreover, we have detected caspCTF in MAM, which further suggests that the Aβ42/Aβ40 ratio could locally change in this region. Intra-mitochondrial Aβ accumulation is an early phenomenon in the synapses and might indeed be one of the reasons for dysfunction and loss.

There are several polymorphisms in the *TOMM40* gene that associates with increased risk of developing AD. Interestingly, *TOMM40* and *APOE* are in linkage disequilibrium with each

other, making it hard to separate risks associated with either gene. In our study, we did not learn of any functional implications concerning the specific poly-T repeat-polymorphism that had attracted a lot of attention after its claimed association to disease onset of AD. However, there are several other polymorphisms that might affect the mitochondria function and, thereby, increase the susceptibility for developing AD.

Based on our observations we hypothesize that Aβ can interfere with processes on the MAM side as well on the mitochondrial side, affecting bioenergetics, lipid metabolism, calcium signaling and cell death regulation in the vulnerable synapse, which depends on correct distribution and contact between the two organelles. Aβ produced at these spots can, in theory, either be of physiological benefit or be a cause of damage. The drug Dimebon, tested for a variety of neurological disorders, has a wide pharmacological profile enhancing inter alia mitochondrial bioenergetics and Aβ secretion. These characteristics might definitely be of importance for disease-modifying treatments aiming to reestablish synaptic integrity by restoring mitochondrial function and decreasing intracellular Aβ levels. The reason for the failure of Dimebon in the Phase III clinical trials might be due to the fact that treatment at the stage of mild-to-moderate AD is too late. At this stage, restoring synaptic and neuronal function is difficult due to the advanced pathology. In conclusion, we believe that drugs targeting MAM and/or the mitochondria could serve as potential disease-modifying therapies. Drugs that have the capacity to restore mitochondrial glucose and lipid metabolism, calcium homeostasis and cell death signaling could potentially prevent synaptic dysfunction and loss. For efficacy, therapy should be started at the earliest possible disease stage, preferably before the mild-to-moderate stage. However, many important questions remain concerning the nature of communication at MAM in regards to AD, and in what ways it might be possible to therapeutically intervene.

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