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Alzheimer disease associated Aβ and γ-secretase:
Mitochondrial localization and involvement in cell death

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You're only given a little spark of madness.
You mustn't lose it.
Robin Williams 1951

ABSTRACT

In several neurodegenerative disorders, including Alzheimer disease (AD) and Parkinson disease (PD), protein aggregation is accompanied by mitochondrial dysfunction. An increasing number of proteins found in mitochondria have multiple localizations. The Amyloid β-peptide precursor protein (APP), a key protein in AD, has a chimeric targeting signal (endoplasmic reticulum (ER) + mitochondria). APP is subsequently cleaved by the β- and γ-secretase resulting in production of the Amyloid β-peptide (Aβ) of different lengths (e.g. Aβ40 and Aβ42). The γ-secretase complex consists of, presenilin (PS)-1 or -2, Nicastrin (Nct), Aph-1 and Pen-2. Interestingly, both PS and Aβ have been found in mitochondria. The aim of this thesis is to investigate if all the γ-secretase components are present in mitochondria and furthermore, to study the mitochondrial uptake mechanisms for Aβ and to assess the stability and activity of the γ-secretase complex during cell death.

In **paper I** we identified a chimeric targeting signal (ER+ mitochondria) in Nct and showed that active γ-secretase complexes are localized to mitochondria.

In **paper II** Nicastrin was detected in mitochondria independently of PS, PS was detected in mitochondria independently of Nct, and Aph-1 was detected in mitochondria independently of PS or Nct. Further, Nct interacted with the mitochondrial import machinery and ~3% of all Nct and ~1.5% of the active γ-secretase complexes were located to mitochondria. The topology of the mitochondrial γ-secretase complex was similar to the reported topology of the γ-secretase complex in ER.

In **paper III** Aβ40 and Aβ42 were found to be imported through the TOM-complex, translocase of the outer membrane, in the mitochondrial import machinery and found to be localized to the mitochondrial cristae. The same localization pattern was also detected in samples from human cortex with amyloid plaques.

In **paper IV** the stability and activity of γ-secretase complexes during cell death were investigated. γ-Secretase was shown to be active and to contain a caspase cleaved C-terminal of PS1 (PS1-caspCTF). Transient transfections with a caspCTF construct reconstituted the γ-secretase activity in PS null cells (BD8) and apoptotic HEKAPPswe cells showed Aβ40 production.

In **paper V** the Aβ40 and Aβ42 production by γ-secretase complexes containing PS1-caspCTF were investigated in cells stably expressing PS1-caspCTF or PS1-CTF on a PS null background (BD8 cells). Here we demonstrated a significant increase in the intracellular Aβ42/Aβ40 ratio in PS1-caspCTF cells as compared to PS1-wtCTF cells.

We here present data linking some of the central proteins of AD pathogenesis to mitochondria and indicating an altered Aβ production during cell death. Mitochondrial dysfunction may underlie the loss of synapses occurring in AD. Therefore, future drugs targeting mitochondria to strengthen their function may be a novel strategy for treatment of AD.

SAMMANFATTNING PÅ SVENSKA

Proteiner är viktiga beståndsdelar i våra kroppar och verkar som biologiska katalysatorer och som byggstenar i våra celler. Varje cell behöver energi för att tillverka och bryta ner proteiner samt skicka signaler till celler i sin omgivning. Denna energi erhålles genom en process som kallas oxidativ fosforylering och som sker i mitokondrien, cellens kraftverk. I denna process bildas energimolekyler, ATP, som fungerar som uppladdningsbara batterier i kroppen som hela tiden måste fyllas på.

Hjärnan står för ca en femtedel av hela kroppens glukosåtgång och nervcellerna behöver energi för att kunna kommunicera med varandra via kontaktpunkter som kallas synapser. Mitokondrierna producerar inte bara energi utan deltar dessutom i att bibehålla den normala kalciumnivån i cellen och deltar i celldödsmaskineriet. När mitokondrien släpper ut vissa proteiner kan de i sin tur aktivera celldödsmaskineriet genom att aktivera en kaskad där speciella protein, sk caspaser, klyver andra proteiner. Mitokondrier släpper inte bara ut proteiner utan tar även upp en mängd proteiner som den behöver för sina funktioner i cellen. Mitokondrien är omsluten av två membran som inte släpper igenom proteiner. Proteinupptaget behöver därför ske via ett specialiserat maskineri som består av två kanalsystem, translokaser av det yttre och inre mitokondriemembranet (TOM och TIM).

Nervcellerna i hjärnan, neuron, behöver mycket energi och är därför sårbara för förändringar i mitokondrierna. I flera neurondegenerativa sjukdomar såsom, Alzheimers sjukdom (AD) och Parkinsons sjukdom (PD), har man kunnat koppla sjukliga förändringar i mitokondrier till sjukdomsförloppet. Cellen innehåller specialiserade avdelningar, organeller, och dessa innehåller olika proteiner med olika funktioner. Det finns även proteiner som förekommer i mer än en organell. För att proteinerna ska kunna verka normalt är det viktigt att de har rätt form och lokalisering i cellen. I både AD och PD har man kunnat se förändrade sjukdomsrelaterade proteiner som återfunnits i mitokondrierna.

AD är den vanligaste orsaken till demens och är uppkallad efter Alois Alzheimer, en tysk läkare som på tidigt 1900-tal var först med att beskriva de förändringar i hjärnan som sker vid sjukdomen. Typiska kännetecken i hjärnan är inlagring av två olika proteiner, tau och amyloid-beta (Aβ) samt vävnadsdöd. Aβ produceras ständigt i våra celler och förekommer i två olika längder, en kortare form (Aβ40) som är den vanligaste och en längre mer ovanlig form (Aβ42) som lättare kan bilda olika aggregat. Aggregering av Aβ och tau stör cellens funktion, Aβ-aggregaten bildar till slut plack utanför nervcellerna medan tau aggregaten syns inuti nervcellerna. Försämring av

korttidsminnet är ett av de tidigaste symptomen på AD. Så småningom får man även svårt att orientera sig i rummet och att komma ihåg ord, för att sedan få allt sämre minne och intellektuell förmåga. Ett tidigt sätt att undersöka en patient med misstänkt AD är att mäta glukosmetabolismen i hjärnan som är nedsatt hos AD-patienter.

Den mest populära hypotesen som förklarar sjukdomsförloppet i AD är Aβ-hypotesen, vilken bygger på att sjukdomen beror av en ökad mängd Aβ samt mer av den längre formen, Aβ42 som lättare formar aggregat. Det är Aβ42 som bildar de stora aggregaten i hjärnan, sk plack, och som man tror är den giftiga formen av Aβ för nervcellerna. Aβ är en klyvningsprodukt från ett större protein, Amyloid Prekursor Proteinet (APP) och klyvs ut av två enzymer, β- och γ-sekretasen. γ-Sekretaset är ett enzymkomplex som består av fyra proteiner: Nicastrin, presenilin, Aph-1 och Pen-2.

Jag har i mitt avhandlingsarbete studerat lokaliseringen av γ-sekretaset och Aβ proteinet till cellens kraftstationer, mitokondrierna. Dessutom har γ-sekretaset stabilitet och enzymaktivitet under celldöd undersökts.

Studie 1

Bakgrunden till studie 1 är att presenilin lokaliserats till mitokondrien (Ankarcrona et al 2002). Presenilin utgör den aktiva delen av γ-sekretaset och har även visat sig vara viktigt för kalciumbalansen och celldödsmekanismer som är påverkade i AD. Vi gjorde en databassökning (iPSORT) för att leta efter mitokondriesignaler i γ-sekretas komponenternas proteinsekvens. Det enda proteinet som hade en mitokondriesignal var Nicastrin. Hela γ-sekretaskomplexet kunde påvisas i isolerade mitokondrier både som ett komplex och som de individuella proteinerna. Komplexets enzymaktivitet påvisades genom att mäta klyvningsprodukterna från APP.

Studie 2

Efter att vi lokaliserat det aktiva γ-sekretaskomplexet till mitokondrier undersöktes var i mitokondrien som komplexet återfanns samt hur mycket av cellens aktiva γ-sekretas som var mitokondriellt. Våra data indikerar att ~1,5% av γ-sekretaskomplexet finns i mitokondrien och att det är lokaliserat till mitokondriens innermembran. Det finns däremot ~3% Nicastrin i mitokondrien och detta interagerar med mitokondriens importmaskineri. Vidare kunde nicastrin och Aph-1 tas upp av mitokondrien oberoende av presenilin. Dessutom kunde Aph-1 och presenilin tas upp av mitokondrien oberoende av Nicastrin.

Studie 3

Bakgrunden till denna studie är att Aβ har visats orsaka mitokondriestress och en ökad produktion av fria radikaler. Dessutom har APP, det aktiva γ-sekretaskomplexet samt

Aβ lokaliserats till mitokondrier. Då APP visats bli ofullständigt upptaget i mitokondrier, med Aβ-regionen utanför, så är det dock uteslutet att Aβ kan genereras inuti mitokondrier. Istället måste proteinet tas upp utifrån. Vi studerade detta upptag genom att inkubera mitokondrier med Aβ i närvaro av olika inhibitorer för att se om och hur mitokondrierna tog upp Aβ. Här visade vi att Aβ togs upp via TOM-maskineriet, mitokondriens importmaskineri och att Aβ främst ackumulerades i mitokondriens innermembran.

Studie 4

I AD-hjärnan postmortem har man funnit spår av pågående celldöd, såsom aktiva caspaser. Caspaser har visat sig kunna klyva presenilin, som utgör den aktiva delen i γ-sekretaskomplexet, och APP, samt kunna leda till en ökad Aβ-produktion. Här studerade vi γ-sekretaskomplexets stabilitet samt aktivitet i en neuroblastomcellinje som genomgår celldöd. Vi fann att komplexet var aktivt samt att det kunde innehålla caspaseklyvt presenilin och att detta caspaseklyvda γ-sekretaskomplex var aktivt.

Studie 5

För att studera om caspaseklyvt γ-sekretaskomplex påverkar Aβ42/Aβ40-produktionen framställde vi celler som endast uttryckte det caspaseklyvda respektive oklyvda γ-sekretaskomplex och mätte Aβ42/Aβ40-produktionen. Här visade det sig att det caspasklyvda γ-sekretaskomplex hade en ökad Aβ42/Aβ40-produktion i förhållande till oklyvt komplex.

Sammanfattningsvis skulle dessa studier kunna ge ledtrådar till de olika processer som kan orsaka eller bidra till sjukdomsförloppet i AD. PS och γ-sekretaskomplex är involverade i cellens kalciumbalans samt celldöd. De kan dessutom påverka mitokondriens funktion så att dess kapacitet att lagra kalcium försämras. Detta kan bidra till att sätta igång frisläppning av mitokondrieproteiner som sedan startar cellens dödsmaskineri. Mitokondriens upptagsmekanism och lokalisering av Aβ kan förklara hur Aβ orsakar mitokondriestress. I AD-hjärnan försvinner först signaleringsenheterna, synapserna, och det har visats att caspaser är aktiva i synapser hos AD-patienter vilket skulle kunna indikera att det finns caspasklyvt γ-sekretaskomplex i synapsen. Detta i sin tur skulle leda till en ökad Aβ42-produktion samt Aβ42-aggregering som är ett av de typiska kännetecknen för AD. Dessa studier kan öka förståelsen för de patologiska förändringar man sett i glukosmetabolism samt signalering i synapser hos AD-patienter och förhoppningsvis i framtiden bidra till att finna nya angreppspunkter för utveckling av läkemedel.

LIST OF PUBLICATIONS

I. **Hansson CA**, Frykman S, Farmery MR, Tjernberg LO, Nilsberth C, Pursglove SE, Ito A, Winblad B, Cowburn RF, Thyberg J, Ankarcrona M. *Nicastrin, presenilin, APH-1, and PEN-2 form active γ-secretase complexes in mitochondria*
J. Biol Chem (2004) 279: 51654-51660

II. **Hansson Petersen CA,** Wiehager B, Li T, Wong P, Ankarcrona M. *Nicastrin is located to mitochondria independently of presenilin*
Manuscript

III. ***Hansson Petersen CA**, *Alikhani N, Behbahani H, Wiehager B, Pavlov PF, Alafuzoff I, Leinonen V, Ito A, Winblad B, Glaser E, Ankarcrona M. *The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae*
Proc Natl Acad Sci, USA (2008) 105: 13145-13150

IV. **Hansson CA**, Popescu BO, Laudon H, Cedazo-Minguez A, Popescu LM, Winblad B, Ankarcrona M. *Caspase cleaved presenilin-1 is part of active gamma-secretase complexes*
J. Neurochem (2006) 97: 356-64

V. Hedskog L, **Hansson Petersen CA**, Svensson A, Welander H, Bogdanovic N, Tjernberg LO, Karlström H, Ankarcrona M. *Caspase cleaved presenilin-1 in γ-secretase complexes increases the intracellular Aβ42/Aβ40 ratio*
Manuscript

* These authors contributed equally

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Aβ	Amyloid β-peptide
AD	Alzheimer disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
Aph-1	Anterior pharynx defective-1
APLP	APP-like protein
APP	Amyloid β-precursor protein
ApoE	Apolipoprotein E
BACE	β-site APP cleaving enzyme
BD8	Blastocyst-derived embryonic stem cells deficient in PS
caspCTF	Caspase-cleaved CTF
CTF	C-terminal fragment
co-IP	Co-immunoprecipitation
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FAD	Familiar AD
GVP	DNA binding/transactivation domain Gal4/VP16
HES	Hairy/Enhancer of split
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
LC-MS/MS	Liquid chromatography combined with tandem mass spectrometry
LRP	Low-density lipoprotein receptor related protein
MIA	Mitochondrial intermembrane space assembly machinery
MPP	Mitochondrial protein peptidase
Nct	Nicastrin
NICD	Notch intracellular domain
NTF	N-terminal fragment
OMM	Outer mitochondrial membrane
PAM	Presequence translocase-associated motor
Pen-2	Presenilin enhancer-2
PS	Presenilin
PTP	Permeability transition pore
RAGE	The receptor for advanced glycation end products
RIP	Regulated intramembrane proteolysis
ROS	Reactive oxygen species
SAM	Sorting and assembly machinery
SRP	Signal recognition particle
STS	Staurosporine
TIM	Translocase of the inner mitochondrial membrane
TOM	Translocase of the outer mitochondrial membrane
UPR	Unfolded protein response
UPS	Ubiquitin proteasomal system
VDAC	Voltage-dependent anion channel

INTRODUCTION

The eukaryotic cell is highly specialized containing several compartments with specific functions. An increasing number of proteins have been found to be localized to more than one of these subcellular compartments. The rational for this is that several metabolic reactions take place in more than one compartment, for example protein synthesis (cytosol, mitochondria and cytoplasts), fatty acid β-oxidation (peroxisomes and mitochondria) and antioxidant defense (cytosol, mitochondria and peroxisomes). Proteins encoded by a single gene locus, but with functions in more than one intracellular organelle, must use various regulation mechanisms to be targeted to these different organelles. There are several different strategies for a protein to be localized to more than one subcellular organelle, such as post-translational modification, chimeric targeting and protein-protein interactions (Mueller et al., 2004). Every organelle has its own specific machinery for recognition and translocation of precursor proteins (Pfanner and Geissler, 2001).

PROTEIN TARGETING

Secretory and membrane proteins typically have an N-terminal hydrophobic region, including their signal sequence, which is recognized by the signal recognition particle (SRP) in the cytosol. The peptide elongation is subsequently stopped at the ribosome and the ribosome-nascent chain complex is targeted to the endoplasmic reticulum (ER). The complex is transferred into the translocon, which is the protein translocation channel in ER (Sakaguchi, 1997; Johnson and van Waes, 1999; Keenan et al., 2001) where it is fully synthesized, sorted into vesicles and delivered to the Golgi complex. The newly synthesized membrane protein is then going through different posttranslational modifications, usually through the assembly and processing of complex oligosaccharide chains, before it is further transported through the secretory pathway towards the plasma membrane (Mellman and Nelson, 2008).

Protein targeting to mitochondria

Mitochondria, the power plants of the cell, have their own DNA encoding 13 proteins, which are all involved in the respiratory chain. Mitochondria contain approximately 15-20% of all proteins in the cell and approximately 1000 proteins encoded by the nuclear DNA are imported through the mitochondrial import machinery (Neupert and Herrmann, 2007). The import of mitochondrial proteins, are typically described to

occur post-translationally although a co-translational mechanism has been suggested for some mitochondrial precursor proteins (Kellems and Butow, 1972; Kellems and Butow, 1974; Fujiki and Verner, 1991). The proteins translated in the cytosol are often directed to mitochondria by a cleavable N-terminal targeting signal peptide referred to as the preseqeuence. The presequence is usually between 20-50 amino acids long and enriched in basic and hydrophobic amino acid residues, containing very few acidic amino acids (Zhang et al., 2001). Presequences form positively charged amphiphilic α-helices in membranous environments (von Heijne, 1986; von Heijne et al., 1989). However, there are examples of mitochondrial proteins (e.g. metabolic carriers) with internal non-cleavable targeting signals, which can be localized anywhere along the protein (Zhang et al., 2001). Furthermore, there are several mitochondrial membrane proteins, which contain hydrophobic regions like the proteins recognized by the SRP, which still gets targeted to the mitochondria, for example the outer mitochondrial membrane translocases 5 and 20 (Tom5 and Tom20) (Kanaji et al., 2000; Horie et al., 2002; Miyazaki et al., 2005). In conclusion, it seems like the presence of a signal peptide is not required for mitochondrial localization, since proteins without such signals can be targeted to mitochondria (Herrmann and Neupert, 2000).

MITOCHONDRIA

Mitochondria were born approximately two billion years ago when proteobacteria were engulfed by primitive eukaryotic cells and a symbiotic relationship was initiated. The mitochondria have evolved and are highly specialized organelles, which continually divide and fuse to form a dynamic interconnecting network throughout the aerobic eukaryotic cell (Gray et al., 1999; Gabaldon and Huynen, 2004). This mitochondrial network has been shown to be able to interact with other cellular components, especially the cytoskeleton and ER (Rizzuto et al., 1998; Wagner et al., 2003). Every cell contains several hundreds of mitochondria, each containing several copies of their own circular DNA. Mitochondria differ dramatically in oxygen consumption and ATP production between different organisms and also between different tissues. Even though mitochondria can take various forms and sizes (Frey and Mannella, 2000; Perkins and Frey, 2000), they are often characterized as 1-2 μm long and 0.1-0.5 μm wide (Frey and Mannella, 2000). Mitochondria are enclosed by a double membrane, resulting in four different compartments: the matrix (where the mitochondrial DNA is located) (Leist et al., 1999), the profoundly folded inner mitochondrial membrane with its cristaes (the folded invaginations), the intermembrane space and the outer

mitochondrial membrane (Figure 1a). The inner mitochondrial membrane is rich in proteins and impermeable to charged and acidic molecules, whereas the outer membrane is smooth and permeable to ions and molecules between 2-8 kDa. The outer membrane contains the voltage-dependent anion channel (VDAC), which is allowing molecules up to 8 kDa to cross the membrane and is believed to be important for maintaining the homeostasis of small metabolites and ions (Benz, 1994; Baines et al., 2007).

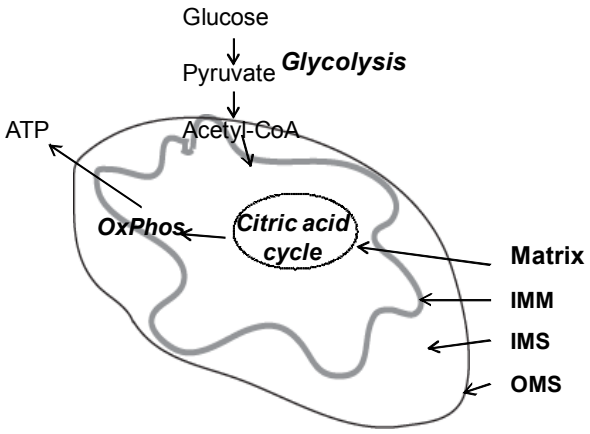

Figure 1a. Organisation of mitochondria.
IMM = Inner mitochondrial membrane, IMS = Intermembrane space, OMM = Outer mitochondrial membrane, OxPhos = Oxidative phosphorylation. The glycolysis produce pyruvate, which then generates Acetyl-CoA, the fuel for the citric acid cycle. Glycolysis produce ca. 2 ATP molecules as compared to the 30-36 ATP molecules formed by the oxidative phosphorylation.

Mitochondria are responsible for approximately 95% of the cells energy production where they play a key role through the electron transport chain and oxidative phosphorylation (Figure 1b) (Rezin et al., 2008). Under normal conditions the electron-transporting complexes I-V, together with co-enzyme Q and cytochrome c, drive the oxidative phosphorylation. Complex I, III and IV couple the transport of electrons with the transport of protons. In this fashion electron transporters build up a proton-motive force over the inner mitochondrial membrane through the mitochondrial electrical potential ($\Delta\psi m$) and the proton concentration gradient (ΔpH). The energy in this potential is then used by complex V, the ATP synthase, in order to synthesize ATP (Lesnefsky and Hoppel, 2006).

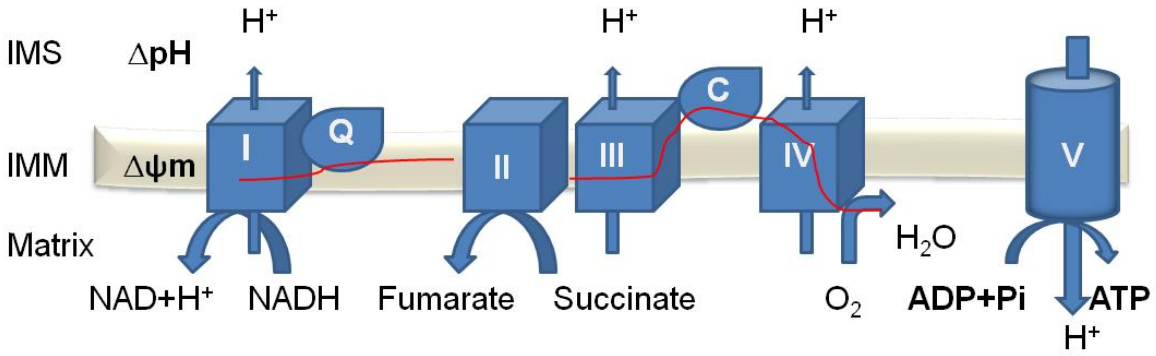

Figure 1b. The electron transport chain in mitochondria is the site of oxidative phosphorylation. As NADH and succinate are oxidized, energy is released, which is used to build up a pH gradient (**ΔpH**) and a electrical potential (**Δψm**) over the inner mitochondrial membrane. This is then used by complex V to synthesize ATP.

The respiration can be uncoupled from the ADP phosphorylation when cytochrome c oxidase is phosphorylated by upstream kinases allowing electron transfer without proton translocation ("proton-slip") (Papa et al., 1997; Kadenbach, 2003). This uncoupling is a mechanism for physiologic fine-tuning of the oxidative phosphorylation. In addition, there are uncoupling proteins, which are involved in thermogenesis releasing the energy as heat rather than ATP or can act to decrease ROS production. Furthermore, there are also several pathological processes, such as oxidative damage to the inner mitochondrial membrane, which can cause uncoupling. This pathological uncoupling of respiration lacks the control of metabolic feedback and can cause a collapse of the electrochemical gradient and then result in mitochondrial depolarization through mitochondrial permeability transition causing cell death (Lesnefsky and Hoppel, 2006).

MITOCHONDRIAL IMPORT

The above described electrochemical proton gradient over the inner mitochondrial membrane is also required for import of most proteins to mitochondria (Lesnefsky and Hoppel, 2006; Krayl et al., 2007). The mitochondrial import machinery is mainly constituted of the translocases of the outer (TOM) and inner mitochondrial membrane (TIM) (Figure 2). Mitochondria have two different TIM complexes, TIM22 and TIM23, but only one TOM. Both TOM and TIM complexes are built up by several protein components, functioning as receptors and forming the pore of the respective translocase. It is believed that the TOM complex is 138 Å wide and contains one or two

voltage-gated and cation-selective pores, with an internal diameter of 20 Å (Hill et al., 1998; Kunkele et al., 1998).

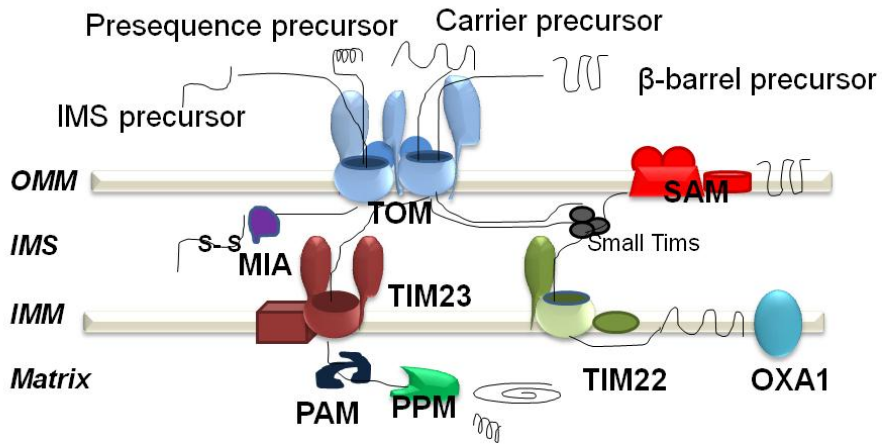

Figure 2. Illustration of the different mitochondrial import pathways.
Depending on the targeting signal proteins can follow different import pathways. The TOM and SAM machineries in the outer mitochondrial membrane (OMM). The MIA machinery in the intermembrane space (IMS). The TIM23,TIM22 and OXA1 machineries in the inner mitochondrial membrane (IMM).The PAM machinery in the matrix

The TOM complex has three different receptors, Tom20, Tom22 and Tom70, which recognize the mitochondrial targeting signals. Tom20 binds the presequence before it gets relocated to the Tom22. Tom70 is the chief receptor for precursor proteins containing an internal targeting signal (Ahting et al., 2001). Although Tom70 and Tom20 contrast in their substrate specificity, both receptors overlap in function and can partly substitute for each other (Neupert and Herrmann, 2007). The pore is formed by Tom40, which forms the channel of the TOM complex supported by the small Toms (Ahting et al., 2001). In brief, the newly translated precursor protein must be kept in an unfolded and import-competent state by cytosolic chaperones before it can be recognized by the TOM receptors, and subsequently imported through the Tom40 pore. Depending on the targeting signal the protein can follow different import pathways (Figure 2).

Proteins targeted to the outer mitochondrial membrane

Proteins targeted to the outer mitochondrial membrane can use different strategies to be incorporated. Most of the proteins first pass through the TOM-complex. The mechanisms responsible for inserting proteins containing membrane-spanning α-helices or association of peripherally attached proteins with the outer membrane are poorly understood. However, several of the proteins of the outer membrane forms β-barrels

and pass through TOM before reaching the sorting and assembly machinery (SAM complex), where they get incorporated into the membrane. The SAM complex is believed to be formed by Sam35, 37 and 50, but their exact function in the complex is not fully known (Pfanner et al., 2004; Becker et al., 2008).

Proteins targeted to the intermembrane space

Most soluble intermembrane space proteins are imported without help of the TIM complexes and are typically lacking N-terminal targeting signals. These proteins are sorted in the intermembrane space by the mitochondrial intermembrane space assembly machinery (MIA). MIA is not only directing this pathway but also catalyze the formation of disulfide bonds to the precursor proteins (Herrmann and Hell, 2005; Terziyska et al., 2005).

Proteins targeted to the matrix and inner mitochondrial membrane

After passing the TOM machinery these precursor proteins are further transported through the mitochondrial intermembrane space, supported by chaperones, before they interact with the import receptors of the TIM complex. Thereafter, the presequence translocase-associated motor (PAM) complete the protein translocation to the matrix, where the presequences are cut off by the mitochondrial processing peptidase (MPP) (Pfanner and Geissler, 2001). The different TIMs, TIM23 and TIM22, represent two main protein import pathways. In the first, the presequences directed protein is imported through TOM via TIM23 and motor PAM to reach the matrix. In this pathway the inner mitochondrial membrane proteins, with cleavable presequence are laterally released from the TIM23. In the second, the carrier proteins with internal targeting signal are recognized by Tom70 and subsequently translocated by TOM. Tim9- 10 chaperones in the intermembrane space direct the protein towards the TIM22 complex. The TIM22 complex then promotes the insertion of the carrier protein into the inner membrane. In addition, the inner mitochondrial membrane also harbours OXA1 complex that can facilitate the insertion of precursor proteins from the matrix side to the inner mitochondrial membrane (Jensen and Dunn, 2002).

In summary, the mitochondrial import machinery is built up by several different complexes and pathways, directing the import of the proteins required for maintaining mitochondrial functions. Functional mitochondria are crucial for cell survival as they produce most of the cellular energy. In addition, mitochondria are the main cellular source of free radicals and involved in regulation and initiation of cell death pathways (Horn and Barrientos, 2008; Rezin et al., 2008).

CELL DEATH

There are several different forms of cell death but the two main types are necrosis and apoptosis. Necrosis is often described as an uncontrolled pathogenic type of cell death where the cells lose their ionic balance, absorb water and finally lyse. In contrast, apoptosis or programmed cell death is a highly regulated and energy demanding process. Morphological changes occurring during apoptosis are nuclear condensation and fragmentation, cytoplasmic shrinkage, plasma membrane blebbing and exposure of phosphatidylserine on the cell surface (Lockshin and Zakeri, 2004). Eventually the dead cells fragment into membrane bound apoptotic bodies, which are phagocytosed by macrophages without inducing an inflammatory response (Nicotera and Melino, 2004). There are two main pathways resulting in apoptosis, the intrinsic (mitochondrial) and the extrinsic (receptor mediated) pathway (Figure 3) (Bredesen et al., 2006). Caspases, a group of cysteine proteases cleaving after aspartic acid (Alnemri et al., 1996), are activated in both the intrinsic and extrinsic pathway and cleave several substrates important for apoptotic signalling (Nicotera and Melino, 2004).

Table 1. Classification of caspases

A. INFLAMMATORY CASPASES	B. APOPTOTIC CASPASES	
-1,-4,-5,-11,-12,-13,-14	INITIATOR	EFFECTOR
	-2,-8,-9,-10	-3,-6,-7

Caspases can be divided into a set of inflammatory caspases and a set of apoptotic caspases (Table 1). The initiator capsases-2 and -9 contains the caspase recruitment domain and the caspase-8 and -10 contain the death effector domain. These domains allow recruitment of other signalling molecules, forming a large protein complex capable of initiating apoptosis (Shi, 2004).

Mitochondria play a vital part in the cell death route operating as a switch between necrosis and apoptosis depending on the levels of ATP available (Leist et al., 1999). The mitochondria harbour several potential harmful proteins (e.g. cytochrome c, Omi/HtrA2, SMAC/Diablo). During the initiating phase of apoptosis the action of death agonist, such as Bax, renders the outer mitochondrial membrane permeable, allowing harmful proteins to leak out into the cytosol (Kluck et al., 1999; Green and Kroemer, 2004). When cytochrome c is released in the cytosol it forms a complex (the apoptosome) together with Apaf-1, caspase-9 and ATP, resulting in caspase-3 activation and apoptosis (Riedl and Salvesen, 2007). Permeablilization of the outer

mitochondrial membrane is an irreversible step in cell death initiation, since the mitochondria will not be able to function properly (Kroemer and Martin, 2005). Similar pathways are also activated by opening of the mitochondrial permeability transition pore (PTP), a mitochondrial megachannel located in close contact with the outer and inner mitochondrial membranes. The composition of this pore is still not fully known, but cyclophilin D, a mitochondrial matrix prolyl cis-trans isomeras, has been shown to regulate the calcium- and reactive oxygen species (ROS)-dependent mitochondrial permeability transition. This opening of the PTP can have several cellular outcomes and takes place under circumstances of oxidative stress or electron transport chain inhibition, leading to a collapse of the mitochondrial membrane potential (Baines et al., 2005; Baines et al., 2007).

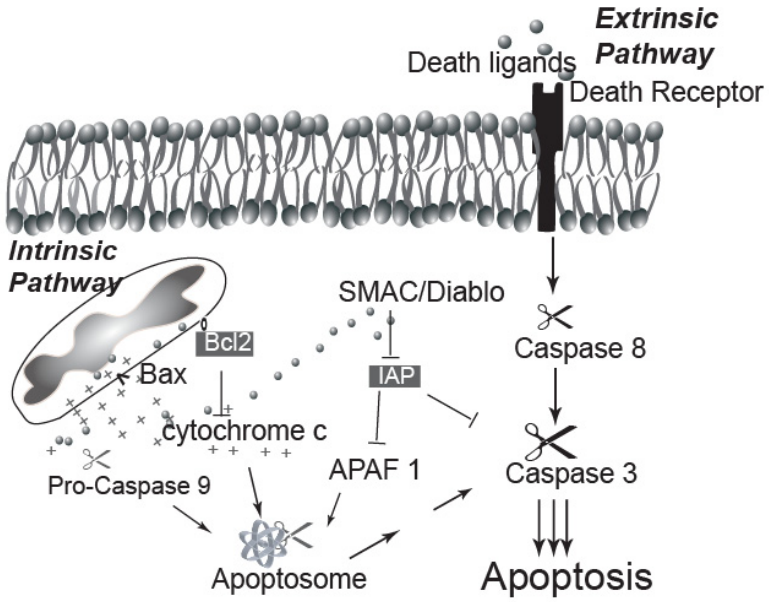

Figure 3. The intrinsic respectively extrinsic apoptotic pathways.
The intrinsic patway is initiated by an imbalance between apoptotic antagonist and agonist in the Bcl2-family. When the agonist dominates the mitochondrial outer membrane gets permeabilized and release harmfull proteins (cytochrome c, SMAC/Diablo) into the cytosol, resulting in caspase activation and cell death.
The extrinsic pathway is activated when death ligand binds to the death receptor, activating caspase and resulting in cell death.

The dynamic processes of fusion and fission, necessary for maintenance of mitochondrial integrity are also affected during cell death. Whereas mitochondrial fission permits for renewal, proliferation and redistribution of mitochondria, the mitochondrial fusion, allows mitochondria to interact and exchange contents with each other. The mitochondrial network falls apart during apoptosis, before caspase activation at the time of cytochrome c release, yielding more numerous and smaller mitochondria

(Karbowski et al., 2004). Newly published data shows that the proteins, which are involved in the fission and fusion also actively, participate in the initiation of apoptosis. For example, the fission regulating protein dynamin related protein 1 (DRP1) has been reported to interact with Bax, resulting in a more stable association of DRP1 to the mitochondrial membrane (Wasiak et al., 2007). Furthermore, the fusion protein mitofusin 2 (Mfn2) and the mitochondrial membrane shaping endophilin B1 have been shown to interact with Bax (Karbowski et al., 2002; Takahashi et al., 2005). Interestingly, mitochondrial fission seems to be important for the cell to be able to meet the energy demands of various subcellular compartments, like the synapse in the neuron. Mitochondria can be recruited by the synapse to meet the energy demands of signal transmission. Moreover, it has been shown that a defective fission/fusion apparatus can result in atrophy of synapses and dendritic spines (Li et al., 2004; Frank, 2006). Some reports have also shown that apoptosis may take place locally in the synapse and the processes activated during apoptosis in neurons (e.g. caspase-3 activation, mitochondrial calcium uptake, ROS accumulation and mitochondrial membrane depolarization) also can be activated in isolated synaptosomes (Mattson et al., 1998). Another indication of synaptic apoptosis is the presence of the apoptotic agonist p53 to synaptic terminals. During oxidative stress p53 has been shown to cause mitochondrial dysfunction and ROS production (Gilman et al., 2003).

NEURODEGENERATION AND MITOCHONDRIAL DYSFUNCTION

Mitochondrial dysfunction is implicated in several disorders and neurodegenerative diseases; e.g. Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), amyotrophic lateral sclerosis, multiple sclerosis, Wilson disease, Friedreich ataxia (Sas et al., 2007).

The brain account for only 2% of the body mass but is responsible for 20% of the oxygen consumption (Papa, 1996). In the processes of oxidative phosphorylation, a necessity for meeting the brain's high requirement of ATP molecules, approximately 1-5% of the electrons escape the respiratory chain and form oxidative species, ROS. These are highly reactive and unstable species constantly produced in living cells but controlled by the cellular redox defense systems (Pratico and Delanty, 2000; Reddy and Beal, 2005). The brain is particularly vulnerable to ROS since it has a high energy demand and contains a high percent of lipids that are susceptible to oxidative damage. Mitochondria have their own redox defense systems, e.g. the Mn superoxide dismutase converting the superoxide anion (O_2^-) to hydrogen peroxide (H_2O_2), which then can be

converted into water (H_2O) by the glutathione peroxidase (Reddy and Beal, 2005). Given that mitochondria is the prime site for the formation of oxidative species it is likely that oxidative damage to proteins and DNA in mitochondria may contribute to the pathology in neurodegenerative disorders (Lin and Beal, 2006).

Oxidative stress has been suggested to increase with age and further to be caused by mitochondrial dysfunction. Interestingly, it has been shown that there is an age-related decline in mitochondrial activities in the brain, which may be associated with the accumulation of somatic mitochondrial DNA defects (Reddy and Beal, 2005). The excitatory neurotransmitter glutamate can lead to exitotoxicity, increasing the intracellular calcium levels. This can then result in a calcium overload in mitochondria, leading to mitochondrial injury (Nicholls et al., 2003) and reduced ATP production (Jekabsons and Nicholls, 2004). On the other hand, mitochondrial energy metabolism dysfunction can sensitize neurons to normal glutamate levels (Beal et al., 1993) and also compromise the glutamate transport, increasing the extracellular levels of glutamate, leading to a pathological stimulation of glutamate receptors (Jabaudon et al., 2000).

The pathophysiology of several neurodegenerative disorders (e.g. AD, HD and prion disease) include characteristics of misfolded, aggregated proteins and amyloid deposits, which are composed of fibrils formed by specific peptides or proteins. These fibrils share the structural motif of cross-β structure. The misfolding and aggregation of proteins can be due to dysfunctional ER or proteasomal systems, which are believed to cause toxic protein aggregation that potentially can cause deposits. Cells have developed adaptive mechanisms to avoid accumulation of incorrectly folded proteins. These engage protein folding by chaperones and the ubiquitin proteasomal system (UPS), which degrades misfolded, proteins (Paul, 2008). This protein quality control system is also complemented by a number of signalling pathways called the unfolded protein response (UPR), which is recruited after ER-stress. Briefly, this program decreases protein synthesis, promotes degradation and enhances expression of ER-chaperones (Matus et al., 2008). In addition, mitochondrial dysfunction has been suggested to contribute to overloading the UPS, thus resulting in accumulation of misfolded and damaged proteins (Paul, 2008).

In AD brain both the amyloid precursor protein (APP) and amyloid β-peptide (Aβ) are localized to mitochondria, APP is blocking the import pore (Anandatheerthavarada et al., 2003; Caspersen et al., 2005) and Aβ cytotoxicity has

been shown to require a functional electron transport chain (Cardoso et al., 2001). Recently it was shown that it was the oligomeric rather than fibrillar Aβ42 species, which could cause a lowering of mitochondrial potential in cortical brain cells in a frontotemperal dementia mice model, i.e. the P301L transgenic tau mice. In addition, a significant reduction of complex III respiration was shown after treatment with oligomeric Aβ42 (Eckert et al., 2008). In patients with PD, α-synuclein is accumulated in mitochondria (Devi et al., 2008) and in patients with HD, huntingtin with its increased number of CAG repeats disturb the mitochondrial function (Orr et al., 2008). Thus, the mitochondrial dysfunction seen in these disorders can be due to either protein aggregation or protein mislocalization.

ALZHEIMER DISEASE

AD is a progressive neurodegenerative disorder and the most common form of dementia. The prevalence of the disease increase with age, which is the most important risk factor for AD. The disease is named after the German psychiatrist Alois Alzheimer who in 1907 described the pathological changes in post-mortem brain tissue from his deceased demented patient Auguste D (Alzheimer, 1907). Early symptoms of AD are failure in short term memory and problems to learn and process new information, followed by difficulties with speech and a general decline in cognition. As the disease progress the patient lose their ability to function in daily life and needs more and more care (Hodges, 2006). Five to ten years after receiving the diagnosis of AD the patient often dies from infections, such as pneumonia. To establish a definite diagnosis of AD a post-mortem analysis is necessary. For diagnosis the patient needs to undergo several physiological evaluations and cognitive tests, sometimes followed up by an assessment by some brain imaging techniques. AD is a family tragedy, slowly destroying the personality of demented individuals by robbing them of their memory, insight, and language. At present there is no cure for AD and to this point only symptomatic treatment is available.

PATHOLOGY

In AD, synapses and neurons are lost in cerebral cortex and specific subcortical regions. The greatest loss can be seen in the temporal lobe, parietal lobe, areas in frontal lobe and in cingulate gyrus. There is a striking decline in the cholinergic innervation in cortex and hippocampus due to the loss of neurons in the basal forebrain (Selkoe, 1991). The two major pathological hallmarks of AD, first described by Alois

Alzheimer (Alzheimer, 1907), are extracellular plaques and intracellular tangles. Plaques are built up by aggregates of Aβ (Glenner and Wong, 1984; Masters et al., 1985) and tangles are built up by aggregates of hyperphosphorylated tau (tau-p) (Nukina and Ihara, 1986) (Figure 4). Aβ aggregates into oligomers and fibrils, which can be localized both intracellular and extracellular, whereas aggregates of tau-p, neurofibrillary tangles (NFT), are explicitly found within the neurons (Selkoe, 1991). In addition, Aβ deposits are also frequently found in the walls of blood vessels (Hart et al., 1988). Aβ-derived diffusible ligands (ADDLs) including Aβ dimers and trimers have been shown to induce progressive loss of hippocampal synapses and induce neuronal cell death (Lambert et al., 1998). Furthermore, the intracellular Aβ oligomers are now believed to be responsible for most of the neuronal toxicity seen in AD (Walsh et al., 2000; Takahashi et al., 2004). The physiological role of Aβ is not fully known (Chiang et al., 2008). Tau is a microtubule-associated protein and its dissociation from microtubules results in impaired axonal transport and finally synapse loss and retrograde degeneration (Iqbal and Grundke-Iqbal, 2005). Tau has more than 30 known phosphorylation sites and there are several different kinases responsible for phosphorylating these (Wang et al., 2007). Tau pathology typically starts in the enthorinal cortex, spreads to hippocampus and amygdala before reaching the neocortex (Braak and Braak, 1991). The plaques distribute in a different anatomical pattern, starting in the orbitofrontal and temporal cortices and further spread to the parietal cortex and neocortex.

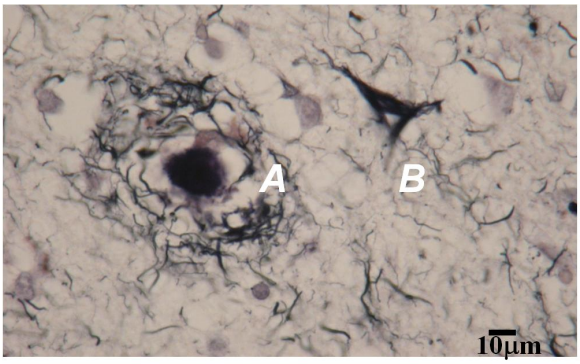

Figure 4. Amyloid plaque and tangle detected by Gallyas method.
A= Amyloid Plaque, *B*= neurofibrillary tangle.
Courtesy of Dr. Nenad Bogdanovic

The degree of dementia is better correlated to the level of soluble Aβ and the number of tangles, than to the number of plaques (Arriagada et al., 1992; Naslund et al., 2000). Synapse loss however gives the best correlation with the degree of cognitive decline in AD (Terry et al., 1991; Terry, 2000).

GENES AND RISKFACTORS

Most AD cases occur sporadically and less than 1% are inherited as familial forms with known mutations (FAD) (Campion et al., 1999). The FAD cases are caused by

dominant missense mutation in genes encoding either the APP (Goate et al., 1991), presenilin 1 (PS1) (Sherrington et al., 1995) or presenilin 2 (PS2) proteins (Levy-Lahad et al., 1995). Mutations in *PSEN1* and *PSEN2* trigger a shift in the generation of Aβ species, favoring the longer species, whereas mutations in *APP* can either cause an increase of total Aβ levels or have a similar effect as the *PSEN*-mutations (St George-Hyslop, 2000). Studying these causative genes for AD and their corresponding proteins, which are implicated in the pathogenic process, have greatly increased the knowledge of the disease.

There are also several known risk factors for AD, of which old age and apolipoprotein E epsilon-4 (*APOE-ε4*) are the most important. *APOE* shows several polymorphisms including the three different epsilon alleles (ε2,-3 and -4), where carriers of *APOE-ε4* have an increased risk for AD (Corder et al., 1993). ApoE is engaged in the delivery and metabolism of cholesterol and triglycerides and the ApoE-4 is implicated in both Aβ deposition and tangle formation. In addition, ApoE-4 have the highest affinity for Aβ, is found to be associated with senile plaques and is believed to accelerate fibrillogenesis (Wisniewski et al., 1994). Gene polymorphisms increasing the risk for AD have also been shown in the gene encoding sortilin 1. Sortilin 1 is a sorting receptor for APP, and in the absence of sortilin 1 APP is directed towards the late endosomes and subsequently cleaved by β-secretase and γ-secretase resulting in an increased Aβ production (Rogaeva et al., 2007). Furthermore, cardiovascular risk factors, such as midlife cholesterol levels and high blood pressure have been shown to be a risk factor for AD (Kivipelto et al., 2002). To conclude, there are several suggested risk factors for AD, but only a few that are considered to be well recognized, like old age, *APOE-ε4*, hypertension, high cholesterol, head trauma and female gender (Munoz and Feldman, 2000).

APP

APP was discovered in 1987 by Kang (Kang et al., 1987) and is a glycosylated type I transmembrane protein with a large extracellular domain and a short cytosolic domain . APP is ubiquitously expressed throughout the body (Dyrks et al., 1988). APP mRNA is alternatively spliced giving rise to three different isoforms: APPP695, 751 and 770, of which APP695 is the predominant form in brain (Weidemann et al., 1989). The longer splice versions contain a KPI domain, Kunitz family of serine protease inhibitors. Furthermore, APP has been argued to have the general properties of a cell surface receptor (Kang et al., 1987; Tanzi et al., 1988) , but the cellular function of APP is still

not known. APP has two protein homologs: APP like protein 1 (APLP) and 2. APLP2 deficient mice show no clear abnormalities. However, when this mouse was crossed with a mouse lacking APP the offspring was early postnatally lethal and the same happened with the combined APLP1 and APLP2 deficient mice, indicating a functional redundancy (Zheng et al., 1995; von Koch et al., 1997). In addition, the APLP2 and APP deficient mice showed an impaired synaptic structure and defective synaptic transmission, indicating that the APP family may be involved in the formation or differentiation of neuromuscular synapses (Wang et al., 2005).

APP processing

APP is typically transported through the secretory pathway to the plasma membrane, during which it is N- and O-glycosylated and tyrosine-sulfated (Weidemann et al., 1989). In addition, APP is also phosphorylated on its ectodomain and intracellular domain (Gandy et al., 1988; Hung and Selkoe, 1994). Interestingly, APP has been shown to contain a chimeric targeting signal and been found to accumulate in mitochondria from AD brains (Anandatheerthavarada et al., 2003; Devi et al., 2006). Furthermore, an import assay with APP and isolated mitochondria has shown an arrested import through TOM. This can be explained by the presence of an acidic region between amino acids 220 and 290 in APP, which can act as a stop translocation signal during import. This implies that only 220 amino acids of the N-terminal domain of APP are inside mitochondria, leaving most of the protein in the cytosol. In addition, it was shown that APP blocks the mitochondrial import pore in brain from AD patients, restraining protein import (Anandatheerthavarada et al., 2003; Devi et al., 2006). Overexpression of APP has recently been reported to cause mitochondrial fragmentation and abnormal mitochondrial distribution in both neuroblastoma cells and rat primary hippocampal neurons. This phenotype could be rescued by a β-secretase inhibitor, indicating that the fragmentation was caused by Aβ (Wang et al., 2008) or C99.

APP is subjected to several proteolytic events, including some performed by caspases. Three cleavage sites have been recognized, one at the C-terminus (Asp720) and two at the N-terminus (Asp197 and Asp219) (Tesco et al., 2003). Interestingly, caspase activity has been shown to increase Aβ secretion (Gervais et al., 1999). However, the two main pathways of APP processing are the non-amyloidogenic and the amyloidogenic pathway. The majority of APP is cleaved by α-secretase (Anderson et al., 1991) in the non-amyloidogenic pathway, cleaving APP inside the Aβ sequence

resulting in a soluble αAPP and C-terminal fragment, C83. C83 is subsequently cleaved by the γ-secretase complex generating a 3 kDa peptide (p3) and APP intracellular C-terminal domain (AICD). In the amyloidogenic pathway β-secretase cleaves APP generating a soluble βAPP and a C99 peptide, which is subsequently cleaved by γ-secretase generating Aβ and AICD (Figure 5) (Kume and Kametani, 2006). There are several different candidates for α-secretase including the disintegrin and metalloprotease (ADAM) 9, 10 and 17 (Asai et al., 2003).

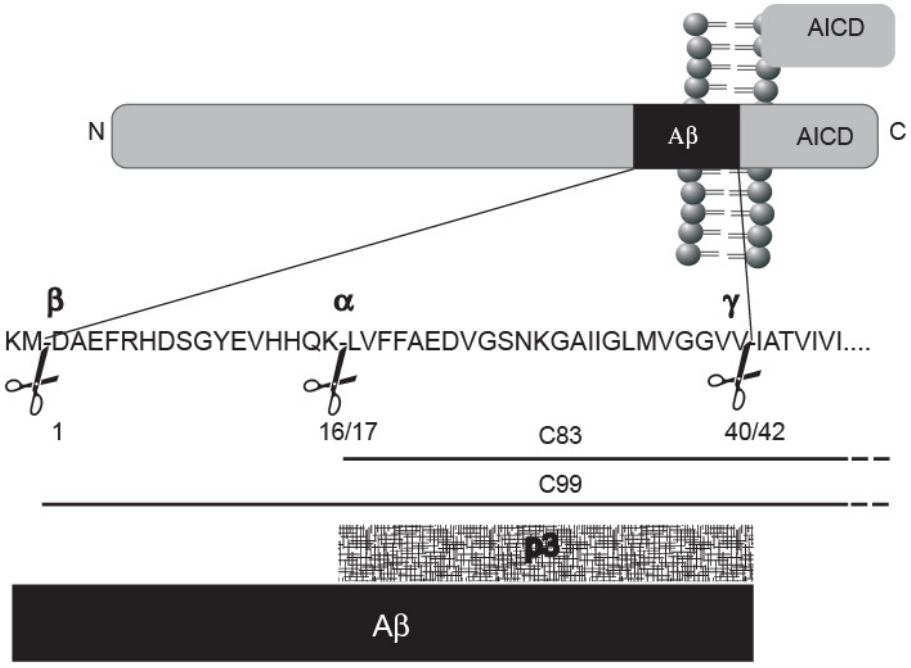

Figure 5. The non-amyloidogenic and amyloidogenic pathways.
APP is cleaved by the α-secretase resulting in a C83 peptide, subsequently cleaved by γ-secretase giving a p3 and a AICD fragment. In the amyloidogenic pathway APP is instead cleaved by the β-secretase resulting in a C99 peptide, subsequently cleaved by γ-secretase giving a Aβ and a AICD fragment.

β-Secretase has been identified as beta-site APP cleaving enzyme 1 (BACE1) also referred to as memapsin 2 and is an aspartic protease (Hussain et al., 1999; Vassar et al., 1999). In addition, the trafficking of APP is important for its proteolytic faith. The sortilin 1, a newly discovered risk factor for AD, directs trafficking of APP from the plasma membrane, so that it is either processed by the α- secretase or the β-secretase (Rogaeva et al., 2007).

γ-SECRETASE AND RIP

γ-Secretase is an enzyme complex comprising at least four proteins; Nicastrin (Nct), PS1 or PS2, anterior pharynx defective 1 (Aph-1) and PS enhancer-2 (Pen-2) (Figure 6) (Yu et al., 2000; Zhang et al., 2000; Chung and Struhl, 2001; Francis et al., 2002; Goutte et al., 2002). Recently, two proteins with proposed γ-secretase modulatory functions have emerged, CD147 and TMP21, which interact with the γ-secretase complex and possibly regulate the Aβ production (Zhou et al., 2005; Chen et al., 2006). However, the CD147 effect on Aβ levels has recently been shown to be caused by an increased degradation of extracellular Aβ, by proteases induced by CD147, indicating that this protein may regulate Aβ levels in a γ-secretase independent manner (Vetrivel et al., 2008).

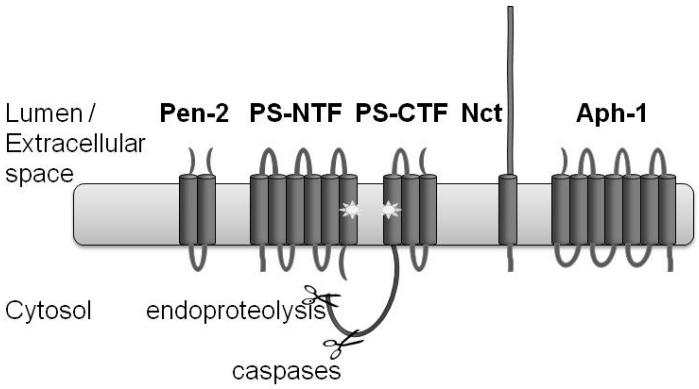

Figure 6. Illustration of the γ-secretase.
Showing the sites for endoproteolysis and caspase cleavage. The stars mark the active site, Asp257 and Asp385.

The γ-secretase complex is located in the membrane and cleaves type 1 transmembrane proteins through regulated intramembrane proteolysis (RIP). RIP is believed to initiate signalling events in the cell and to be a highly regulated process. In addition to the γ-secretase complex, there are three other groups of RIP enzymes: site-2 protease, signal peptide peptidases and rhomboid (Urban and Freeman, 2002). If any of the proteins required for γ-secretase assembly and activity (PS, Nct, Aph-1 or Pen-2) are knocked out in an animal model it will result in prenatal lethality. This is mainly due to problems with Notch signalling (Donoviel et al., 1999; Francis et al., 2002; Li et al., 2003; Ma et al., 2005). Many γ-secretase substrates have been identified and Notch, a cell surface receptor, which is involved in cell faith decision (Jarriault et al., 1995) was among the first to be discovered (Schroeter et al., 1998).

Presenilin

PS exist as two different proteins encoded on different chromosomes, PS1 on chromosome 14 and PS2 on chromosome 1. PS1 and PS2 share approximately 67% of the amino acid sequence and both were identified in 1995 (Levy-Lahad et al., 1995; Sherrington et al., 1995). PS harbours the active site of γ-secretase, Asp257 and Asp385 (Wolfe et al., 1999) and a substrate docking site where the transmembrane region of the substrate binds before reaching the active site (Kornilova et al., 2005). PS have nine transmembrane domains, with the N-terminus facing the cytosol and the C-terminus facing the lumen (Laudon et al., 2005). Between transmembrane domain 6 and 7 there is a large cytosolic hydrophilic loop, which can be endoproteolytically cleaved at amino acid 298 through autoproteolysis or by an unknown protease (Wolfe et al., 1999; Campbell et al., 2003). This cleavage generates an N-terminal and a C-terminal fragment (NTF and CTF), which form a functional heterodimer generally believed to be the biologically active form of PS (Campbell et al., 2003). The cytosolic loop can also be processed by caspases, where caspase-6, -8 and -11 cleave the loop at residue 329 and caspase-1, -3 and -7 cleave at residue 341 or 345 (Kim et al., 1997; van de Craen et al., 1999).

Nicastrin

Nct is a 709-residue type 1 transmembrane glycoprotein with a large luminal/extracellular ectodomain and a short cytoplasmic tail (Yu et al., 2000). The ectodomain is modified by glycosylation in the Golgi apparatus and by sialylation in the trans-Golgi network (Herreman et al., 2003). The function of Nct is still unknown, but it has been suggested to be essential for the stability of the γ-secretase complex and to function as a receptor in the γ-secretase complex. A conserved domain consisting of amino acids X-Y (DYIGS) has been shown to be essential for Aβ production and has been proposed to be involved in substrate recognition (Yu et al., 2000). Recently the role of this domain as a receptor has been questioned, since modulation through mutation rather affected complex formation than substrate binding (Chavez-Gutierrez et al., 2008). Nct is expressed ubiquitously and the highest levels of Nct are found in skeletal muscle and brain. In contrast, the PS levels in skeletal muscle are low, indicating the presence of Nct outside the γ-secretase complex (Ilaya et al., 2004). Nct needs PS1 for its glycosylation and maturation (Edbauer et al., 2002). However, this glycosylation is not needed for γ-secretase activity (Herreman et al., 2003).

Aph-1 and Pen-2

Aph-1 is named after the resulting phenotyp in Aph-1 deficient *C. elegans*, i.e. lack of the anterior pharynx (Goutte et al., 2002). There are two different Aph-1 homologs in humans (Aph-1a and Aph-1b) and three different homologs in mice (Aph-1a, b and c), and these have been shown to form different subcomplexes of γ-secretase (Shirotani et al., 2004). Aph-1 has seven transmembrane domains and is highly hydrophobic. The Aph-1 N-terminus and even-numbered loops are facing the lumen, whereas the C-terminus and odd-numbered loops reside in the cytosol (Fortna et al., 2004). Pen-2 has been shown to be dependent on PS and Nct expression and is believed to be involved in the regulation of the endoproteolysis of PS (Steiner et al., 2002; Luo et al., 2003; Bergman et al., 2004). Pen-2 is believed to contain two transmembrane domains with both its N- and C-terminal parts located in the ER lumen/extracellular space and the loop thus facing the cytosol (Crystal et al., 2003).

γ-SECRETASE SUBSTRATES AND SIGNALLING

γ-Secretase has a very loose cleavage specificity and the list of identified γ-secretase substrates is constantly growing. γ-Secretase has been proposed to act as a "cleaning lady" cleaning the membrane from proteins domains. However, for some substrates γ-secretase may play a key role in their signalling function. The γ-secretase cleavage may initiate signalling events by allowing the translocation of the intracellular domain to the nucleus. In addition, γ-secretase cleavage may also terminate signalling when the transmembrane domain responsible for the signalling is cleared by the cleavage. This could be a possible mechanism for receptors, which transmit their signal from the membrane, to down-regulated their receptor activity after ligand binding and ectodomain shedding. Many of the intracellular domains released by γ-secretase have been shown to contain different forms of bioactive domains, such as transcription-activation domains, protein-interaction domains and recognition sites for protein modifications. The γ-secretase cleavage is thus involved in several important biological processes (such as cellular adhesion and development) (Kopan and Ilagan, 2004).

The Notch receptor was the second γ-secretase substrate to be recognized after APP, and is probably the most evident example of how γ-secretase cleavage can be involved in signalling pathways. The Notch receptor is after ligand binding processed by α-secretase (S2-cleavage) and then subsequently by γ-secretase (S3-cleavage) generating the Notch intracellular domain (NICD) (Schroeter et al., 1998). NICD is

then translocated to the nucleus where it is involved in transactivation of *HES* (hairy/enhancer of split) genes. *HES* genes are involved in non-neuronal cell fate decision and are critical for the development of several tissues and organs (Jarriault et al., 1995). In addition, the intracellular domains of Jagged, APP, APLP1 and APLP2 have been shown to be translocated to the nucleus (Cao and Sudhof, 2001; LaVoie and Selkoe, 2003; Walsh et al., 2003). Furthermore, PS is believed to affect the cell calcium signalling and PS FAD mutations have been shown to cause calcium dyshomeostasis and calcium buffering impairment (Begley et al., 1999). This has been suggested to be caused by an increased capacity for ER to release calcium in response to IP_3, and increased calcium influx through plasma membrane channels (Stutzmann et al., 2006).

Aβ

Aβ is a peptide of 39-49 amino acids (Qi-Takahara et al., 2005; Zhao et al., 2005), where Aβ40 is the most abundant specie followed by Aβ42 (Wang et al., 1996). The longer Aβ species (Aβ42) are more hydrophobic and have an increased tendency to form aggregates. The physiological function of Aβ is not known but especially the aggregated longer forms (e.g. Aβ42) have been suggested to be cytotoxic (Chiang et al., 2008). However, the peptide has also been suggested to act as a neurotrophin or antioxidant (Atwood et al., 2003). Aβ is secreted from the cell and can either be endocytosed by the synapse, phagocytosed by microglia or astrocytes, degraded or transported out from the brain via the low-density lipoprotein receptor related protein (LRP)-1 mediated transport (Yamada et al., 2008). In addition, Aβ can also enter the brain from the systemic circulation by binding to the receptor for advanced glycation end products (RAGE). Interestingly, LRP has been reported to be down-regulated whereas RAGE was found to be up-regulated in AD brains (Lue et al., 2001; Deane et al., 2004).

It is the longer versions of Aβ that have the highest inclination to cluster into oligomers and fibrils, and eventually deposit into amyloid plaques. Monomeric Aβ molecules typically have a random coil structure whereas the fibrils have a high percentage of cross β-sheet conformation, which is the reason why they can be stained with Congo red and thioflavin T (Nilsson, 2004; Honson et al., 2007). The amyloid plaques are believed to be less toxic as compared to oligomers, but both could be involved in the local inflammation seen in AD. Electrophysiology data also suggest that a trimeric form of Aβ inhibits long-term potentiation (LTP) and the synaptic plasticity of hippocampal neurons in wild type mice (Townsend et al., 2006). Furthermore,

aggregation of Aβ is believed to cause hyperphosphorylation of tau, leading to formation of intracellular tangles causing dysfunctioning neurons and degeneration (Hoozemans et al., 2006). This process is described in one of the best known theories explaining the cause of AD, the amyloid cascade hypothesis, which was first proposed by Hardy in 1992 (Hardy and Higgins, 1992; Hardy and Selkoe, 2002).

AD HYPOTHESES

AD is a multifactorial disease and several different hypotheses explaining the pathophysiology behind the neurodegeneration have been proposed. One of the first hypotheses presented was the *cholinergic hypothesis*, which is built on the fact that there is selective vulnerability and disruption of the cholinergic system in AD. The nucleus basalis of Meynert, a specific population of neurons in the basal forebrain, which is a major contributor of the cholinergic innervations of the cerebral cortex, was shown to be selectively degenerated in AD (Davies and Maloney, 1976; Whitehouse et al., 1982). However, ever since the *amyloid cascade hypothesis* was presented in 1992, it has been the predominant hypothesis explaining the cause and pathogenesis behind AD. Initially it was believed that the amyloid plaques were the pathogen causing the disease, but now the focus has changed towards the Aβ oligomers and the amyloid cascade hypothesis has been updated (Hardy and Selkoe, 2002). In short, this hypothesis claims that an altered Aβ metabolism and subsequent aggregation initiates a vicious cycle of synaptic deficits, neurofibrillar tangles, inflammatory processes, ROS production, cell death, eventually resulting in dementia and AD (Hardy and Higgins, 1992). The main findings supporting this hypothesis are:

- Humans with trisomi 21, Down's syndrome, have an extra allele of the chromosome 21 (where the gene encoding APP is situated) and develops AD-like dementia and pathology (Olson and Shaw, 1969).
- FAD mutations in *APP* and *PSEN* genes result in an increased ratio of Aβ42/ Aβ40 (St George-Hyslop, 2000)
- Aβ peptides are toxic to neurons and synapses (Selkoe, 2002)

However, the reported APP mutations, resulting in an increased production of Aβ, are responsible for just a few of the known FAD mutations, whereas the *PSEN* harbours the majority of the FAD mutations. This is the background for the *PS hypothesis,* which claims that it is PS dysfunction that is the prime event in AD. Moreover, the mouse models over-expressing mutant human APP have frequently

failed to reproduce neuronal loss and neurodegeneration (Irizarry et al., 1997; Dodart et al., 2002), whereas a conditional PS knockout mice have been shown to develop neurodegeneration and to have deficits in cognition similar to the typical features of AD (Saura et al., 2004; Shen and Kelleher, 2007). Furthermore, several *PSEN* mutations have been shown to disturb the calcium homeostasis in the cell. Tu *et al* has shown that wild type PS, but not FAD-mutated PS are able to form low-conductance divalent-cation-permeable ion channels in lipid bilayers. It is possible that mutated PS would then cause an overload of calcium in ER due to dysfunctioning of the PS-dependent calcium leak channels. In addition, they showed that the ER calcium levels are elevated in PS deficient cells compared to wild type cells (Tu et al., 2006).

Interestingly, there are several experimental studies showing alterations in calcium signalling both in sporadic and in familial cases of AD supporting yet another hypothesis - the *calcium hypothesis* (LaFerla, 2002; Bojarski et al., 2008). The calcium hypothesis was first described by Khachaturian, who suggested that an altered intracellular calcium level was responsible for many of the AD-associated neuronal dysfunctions (Khachaturian, 1994). Pierrot *et al* has recently shown that an increased concentration of cytosolic calcium result in phosphorylation of APP and tau, leading to an increased production of intracellular Aβ (Pierrot et al., 2004; Pierrot et al., 2006). In addition, oligomeric Aβ42 has been shown to cause a massive entry of calcium in cultured neurons causing a mitochondrial calcium overload (Sanz-Blasco et al., 2008).

The *oxidative stress hypothesis* is based on the increased level of oxidative stress associated with ageing and AD. Several antioxidant and redox-defense systems weaken with age, leading to a situation where generation of ROS exceed the ability of the antioxidant defense system, resulting in an imbalance causing oxidative damage (Pratico and Delanty, 2000). The oxidation of proteins can lead to their degradation but also cause protein aggregation (Bota and Davies, 2002; Grune et al., 2004). Increased levels of oxidized proteins and decreased antioxidant enzyme activities have been found in vulnerable brain areas of AD patients and aged controls (Smith et al., 1991; Keller et al., 1997). Mitochondria are the prime site for production of oxidative species and are also an early target for ROS. A number of studies indicate that mitochondria dysfunction may be a common theme for several neurodegenerative disorders including AD, presenting the background for the *mitochondrial hypothesis*. (Lenaz, 1998).

Enzymes involved in both the citric acid cycle (the pyruvate and α-ketoglutarate dehydrogenase) and the electron transport chain (cytochrome c oxidase) have been

suggested to be dysfunctioning in AD (Sorbi et al., 1983; Gibson et al., 2000). If some of the respiratory chain complexes are damaged in AD production of free radicals will increase, causing oxidative damage to mitochondrial proteins, which in the end can initiate apoptosis through mitochondrial permeability transition. Aβ has been suggested to be able to inhibit the electron transport chain and Aβ42 has been demonstrated to inhibit cytochrome c oxidase activity (complex IV) *in vitro*, through a copper-dependent mechanism (Crouch et al., 2005). In addition, APP and Aβ have been detected in mitochondria from AD brain (Anandatheerthavarada et al., 2003; Caspersen et al., 2005), and Aβ has been shown to bind to both the alcohol dehydrogenase ABAD in the matrix, and to cyclophilin D located at the inner membrane of mitochondria (Lustbader et al., 2004; Du et al., 2008).

Reduced glucose metabolism is a well known feature of AD and may even be one of the best documented abnormalities of the disease. Cerebral metabolic rate deficits appear before any evidence of functional impairment by neuropsychological testing or of brain degeneration by imaging (Blass, 2000). To summerize, the mitochondrial key functions in energy production, cell death initiation and calcium homeostasis may render specific subsets of neurons vulnerable for cellular aging and stress, increasing the risk for neurodegenerative disease (Mancuso et al., 2007).

The hypotheses presented above show some degree of overlap probably due to the interconnected features of the disease. Since AD is a multifaceted disease, it is possible that the background and interest of the independent researcher will influence the interpretation of data. Both APP and Aβ is believed to be located in the synapse. Interestingly, synaptic alterations are believed to be predominant among the pathophysiological mechanisms in several neurodegenerative diseases, such as PD, HD and Prion disease. These diseases are characterized by aggregates of specific amyloidogenic proteins, which are localized to the synapse (DiFiglia et al., 1995; Fortin et al., 2004; Kovacs et al., 2005; Fournier, 2008). Hopefully, understanding the mechanisms behind one of these disorders will provide valuable clues on to how to solve a whole range of these neurodegenerative diseases.

TREATMENTS OF TODAY -AND TOMORROW?

Even though the knowledge of the neurobiology of AD has greatly increased during the past years, only cholinesterase inhibitors and the N-methyl-D-aspartic acid (NMDA)-receptor antagonist, memantine, are approved drugs for treating AD. The cholinesterase inhibitors increase the levels of acetylcholine available, and thus the neurotransmission,

by inhibiting the degradation of the neurotransmitter at the synaptic cleft. This strategy is based on the cholinergic hypothesis. Memantine is based on the belief that there is an abnormally high level of glutamate causing the cellular dysfunction and cell death seen in AD. Furthermore, memantine reduce the abnormal excitatory signalling by resetting the NMDA receptor activity allowing physiological transmission to take place at a normal level (Chohan and Iqbal, 2006). Several potential therapeutic agents have been tested in clinical trials (aspirin, non-steroidal anti-inflammatory drugs (NSAIDs), omega-3 fatty acids, estrogen, neurotrophic factors etc). Most have however failed due to lack in safety or therapeutic benefit (Carlsson, 2008).

Target the pathological hallmark: Aβ

There have been several attempts to inhibit γ-secretase, but so far they have all failed due to problems with efficacy and side effects (Citron, 2004). A partial inhibition by lowering the dose of γ-secretase inhibitor is not a good strategy, since lower doses of inhibitor have shown to increase Aβ42 production (Shen and Kelleher, 2007). Still there are modulators of the γ-secretase activity, which could prove to be a better strategy, giving less Aβ42 production without completely inhibiting γ-secretase activity (Citron, 2004; Gura, 2008).

β-Secretase inhibitors have been another promising target. However, as new substrates for β-secretase emerges together with problems due to the large active site of the enzyme, β-secretase has also become a dubious target for drug intervention (Citron, 2004).

Vaccine therapy strategies were developed after Schenk *et al* reported that Aβ42 vaccination in a transgenic mouse model of AD caused a reduced amount of Aβ deposition (Schenk et al., 1999). The first clinical trial of active vaccination for AD patients was halted due to problems with meningoencephalitis in some patients (Citron, 2004). New active and passive vaccines (antibodies raised against Aβ) have been developed, some of which are now undergoing clinical trials in Europe and USA (Gura, 2008).

Inhibiting Aβ aggregation with peptides or small molecules is another possible strategy for drug development. The idea is to find brain-penetrable small molecule drugs that interfere with Aβ–Aβ interactions and thus inhibit the formation of toxic oligomers. However only a few aggregation inhibitors have moved into clinical trials (Citron, 2004). Overall drugs that targets APP processing and Aβ have shown little positive effect in clinical trials so far (Gura, 2008).

Target ROS and mitochondrial function

It has been suggested that calorie restriction and antioxidant treatments can decrease Aβ production in a AD-transgenic mouse, as well as reduce defects in the electron transport chain, decrease mitochondrial ROS and decrease mitochondrial damage in neurons from human brain (Reddy and Beal, 2008). Furthermore, agents and antioxidants affecting mitochondrial function, some directly targeted to the mitochondria, have been developed, e.g. creatine, MitoQ (mitochondrial targeted Co-enzyme Q), MitoVitE (mitochondrial targeted vitamin E) and Szeto-Schiller peptides (SS-peptides). A numberf of these, including creatine and MitoQ are presently in clinical trials for different neurodegenerative disorders (AD, HD and PD). Antioxidant targeting of mitochondria may prove to be a promising strategy to treat AD (Chaturvedi and Beal, 2008; Reddy and Beal, 2008).

In our laboratory we are currently trying to identify the mechanism of action of a drug called Dimebon in collaboration with Medivation, Inc (San Francisco, USA). Dimebon is an old antihistamine drug developed in Russia with weak inhibition ability toward acetylcholinesterase and NMDA receptors. Dimebon has been shown to have a positive effect and may even decrease the progression rate of AD (Doody et al., 2008). A Dimebon trial involving 183 subjects with mild to moderate AD in Russia has shown that the subjects given Dimebon performed significantly better on cognitive and behavioural tests compared with those given placebo. The exact function of this drug is not fully known but it is believed to be able to save neurons by protecting their mitochondrial function (Doody et al., 2008; Gura, 2008).

THESIS AIM

Mitochondria play a central role for survival and death of synapses and nerve cells. In AD there is evidence that both mitochondrial dysfunction and cell death are involved in the development and progression of the disease. The work presented in this thesis aimed to investigate a mitochondrial localization of γ-secretase complex and Aβ. In addition, the stability and activity of γ-secretase during cell death was investigated.

Specific Aims

γ-secretase complex in mitochondria:

To investigate if all the γ-secretase components are localized to mitochondria.

To study the assembly and activity of the mitochondrial γ-secretase complex.

To determine the level of γ-secretase complex localized to mitochondria.

Aβ localization to mitochondria:

To investigate if Aβ was localized to mitochondria and further elucidate the uptake mechanism of mitochondrial Aβ.

To study the mitochondrial sublocalization of Aβ.

γ-secretase complex activity and stability during cell-death:

To investigate the stability of γ-secretase complex during cell death, with focus on the caspase cleavage of PS.

To analyze the activity of γ-secretase complex during cell death.

To elucidate the effect of caspase-cleaved PS1 on γ-secretase actitivity.

COMMENTS ON METHODOLOGIES

In this part of the thesis some of the methods and material used during this thesis work will be described and discussed. Furthermore, some of the dilemmas and benefits with different models and techniques will be mentioned. More detailed descriptions of the different methods are provided in paper I-V.

MODEL SYSTEMS

AD is probably caused by multiple pathogenic processes, which may develop years before symptoms occur. This, together with the problem with availability of human brain tissue samples makes AD studies challenging. Post-mortem brain samples sometimes bear the problem with long post-mortem times, which puts a limit to the processes that can be studied in this material. There are no true animal models of AD since no species, except humans, develop the disease. Immortalised cell lines have the benefit of less ethical considerations and the unlimited number. In addition, they are also very susceptible for vast modifications, such as over-expression of proteins, siRNA knock-down of proteins etc. Nevertheless, cell lines lack complexity, making it hard to extrapolate the data to the human disease. The lack of complexity can however make it possible to study a mechanism of interest in a more simple and controlled environment. In this thesis, tissue samples from human brain, rodent brain and liver and various cell lines have been used. The use of human and animal tissues has been approved by the human ethical committee of Stockholm and the animal ethical committee of south Stockholm.

Human cortical specimens and post-mortem material from AD brain

One problem with using frozen samples from post-mortem AD brain to study the localization of AD-associated proteins in mitochondria has been the broken state of the mitochondria in these samples. It has not been possible to isolate pure mitochondria from the post-mortem AD brain samples available to us and when analysing the morphology by electron microscopy the quality of the tissue has not been sufficient to discriminate any mitochondrial structures. This is probably due to long post-mortem times, often exceeding 20 hours. To be able to study the Aβ localization in human brain we have instead used specimens obtained from surgery of patients with suspected normal-pressure hydrocephalus. These samples are very valuable and limited in size, why immunoelectron microscopy is a good technique to study the Aβ localization to

mitochondria. The samples were also analyzed for amyloid plaques and were de-identified.

Material for localization studies of γ-secretase and Aβ

The starting material for the subcellular fractionation has been cells in culture or brain and liver tissue from male Sprague-Dawley rats or C57BL/6 mice. Cells and tissues were homogenized before the crude mitochondrial fraction was obtained by centrifugation. This mitochondrial fraction is not pure, but represents a good starting material for the mitochondrial import (paper III) since this procedure of obtaining mitochondria is fast and maintains their import capacity. For our studies on the localization of γ-secretase complex (paper I and II) percoll-purified mitochondria were used.

Cell culture

Four different cell lines were used, SH-SY5Y neuroblastoma cells, blastocyst derived BD8 cells (PS deficient or transfected with PS), mouse embryonic fibroblasts (MEF) (Nct deficient), and HEK293 human embryonic kidney (over-expressing APPswedish mutation) cells.

γ-SECRETASE ACTIVITY ASSAYS

We have used three different methods to assess γ-secretase activity; the cell-membrane based γ-secretase activity assay measuring AICD formation (paper I, IV and V), the pull down by γ-secretase inhibitor with a hydrophilic long linker and a cleavable biotin group (GCB-pull down), measuring the total γ-secretase activity (paper II and V) and the luciferase based reporter assay measuring γ-secretase activity towards C99 and Notch (paper IV).

In the membrane based γ-secretase activity assay, membranes from whole cell or mitochondrial homogenates were solubilized and incubated at 37^{o}C for 16 hours with or without γ-secretase inhibitor. Since the endogenous substrate for γ-secretase complex in mitochondria is unknown, for these reactions we supplied solubilized membranes from BD8 cells or recombinant C100-Flag (C-terminal fragment after β-cleavage of APP), which were used as substrates. BD8 cells are PS deficient and thus accumulate γ-secretase substrates, e.g. C83 and C99. The production of AICD was then detected on Western blot.

The luciferase based reporter assay was used to study the γ-secretase activity towards C99 and Notch by transfecting cells for 24 hours with different PS and reporter

DNA constructs. γ-Secretase cleavage of the hybrid proteins C99-GVP and NΔE-GVP, liberates the C-terminal region containing the GVP moiety, which then translocates to the nucleus due to the nuclear localization signals in GVP. The GVP exclusively signals via activation of a UAS-luciferase reporter gene through the specific binding to a UAS promoter (Figure 7). The luciferase based reporter assay has both high specificity and sensitivity (Karlstrom et al., 2002).

Pull down of PS1 with GCB is an indirect measurement of γ-secretase activity and was used in paper III to analyze the amount of active γ-secretase complexes in mitochondria compared to whole cell homogenate by detecting levels of PS1 on Western blot and in paper V to pull down active γ-secretase complexes.

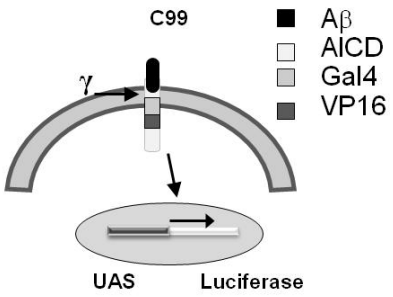

Figure 7. The luciferase based reporter assay. Upon γ-cleavage the intracellular domain is released and activates transcription of luciferase gene via the upstream activation sequence (UAS).

STUDYING PROTEIN INTERACTION AND γ-SECRETASE COMPLEX

Protein-protein interaction can be sensitive to detergents, which make the studies of membrane protein assembly challenging. In these studies two mild detergents (DDM and CHAPSO) were used to study protein-protein interactions and γ-secretase complex assembly. SDS-PAGE and blue native-PAGE (BN-PAGE) were used to separate proteins. Since SDS-PAGE is a method where the strong detergent SDS is used to reduce the sample, almost all protein complexes are destroyed. In contrast, BN-PAGE (used in paper I) uses non-reducing agents and thus whole protein complexes can be detected. Both methods separate proteins according to size.

Co-immunoprecipitations (co-IP) is another method to study protein complexes and protein-protein interaction. All co-IPs were performed using supernatant isolated from the different membrane fractions, solubilized in 1% CHAPSO. An advantage with co-IP, as compared to BN-PAGE, is that it is easier to include a negative control.

DETECTION AND QUANTIFICATION OF Aβ

In paper V we used two different methods to analyze Aβ production:

Enzyme-linked immunosorbent assays

Enzyme-linked immunosorbent assays (ELISA) was used in paper V to quantitatively measure the intracellular level of Aβ42/Aβ40, in different cell lines. The cells were cultured in OptiMEM, supplemented with 0.35% DMSO, using these serum-free conditions to decrease the risk of degradation and aggregation of Aβ. As another precaution the lysing buffer was supplemented with 0.1% SDS and to avoid Aβ interaction with plastic surface special siliconized plastic tubes from Sigma were used. Nevertheless, it cannot be exclude that some of the Aβ formed aggregates, hiding epitopes from the detection antibody and thus giving an incorrect result.

LC-MS/MS

By using liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) peptide ions can be selected, isolated and analyzed in the presence of many other peptides. This method was used to detect the secreted Aβ42 from our different cell lines. The reason for using this method was that the levels of secreted Aβ42 were under the detection limit of the ELISA system. Another reason was that we hoped to be able to identify longer versions of the peptide (≥Aβ43), which was not possible by our Aβ40/Aβ42 ELISA. The Aβ was first concentrated by co-IP with antibody-coupled tosylactivated magnetic beads before successive cleavage by cyanogen bromide. Samples were concentrated and desalted before subjected to LC-MS/MS analysis.

MITOCHONDRIAL IMPORT ASSAY

The import was performed using rat liver mitochondria, which were incubated with Aβ40 or Aβ42 in the presence of different inhibiting agents to assess which mitochondrial uptake mechanism is used by Aβ. In addition, a positive control, S^{35} labeled $pF_1\beta$ (precursor of the ATP synthase from *N. plumbaginifolia*) was imported. The result was analyzed using either Western blot, fluorescence-activated cell sorting (FACS) or immunoelectron microscopy. The reason for using FACS analysis was that the import-competent mitochondria were contaminated by other membranes, which could potentially bind Aβ. By using fluorescent Aβ40 in the import assay and then use a mitochondrial stain (MitoFluor Red) it was possible to measure the mitochondrial uptake of Aβ with high specificity by FACS. This technique also made it possible to perform quantitative measurements of the mitochondrial Aβ. In addition, immunoelectron microscopy was used to further analyze the mitochondrial uptake of Aβ to verify the mitochondrial localization of Aβ.

RESULTS AND DISCUSSION

In this part the results from paper I-V will be summarized and discussed. Some of the figures, from the individual papers, will also be presented here to improve the understanding and to support the discussion.

γ-SECRETASE LOCALIZATION TO MITOCHONDRIA

The γ-secretase complex has been reported to be localized to the secretory pathway (ER, Golgi, plasma membrane and endosomes) (Annaert et al., 1999) and to the lysosome (Pasternak et al., 2003). *PSEN1* FAD-mutations have been shown to cause mitochondrial dysfunction and to sensitize the cell to apoptosis (Begley et al., 1999; Suh and Checler, 2002). This data made it interesting to study if PS1 could be localized to mitochondria. In 2002 Ankarcrona and Hultenby was able to show that PS1 was localized to mitochondria (Ankarcrona M et al 2002). Together these reports represent the background for studying the mitochondrial localization of γ-secretase complex in paper 1 and paper II. In **paper I** we performed an iPSORT data base search looking for targeting sequences in the four known γ-secretase components. A dual ER/mitochondrial targeting sequence in the N-terminal region of Nct was identified. The first 8-32 amino acid residues represent a ER-targeting signal domain and the sequence immediately following resembles a mitochondrial targeting peptide with positive amino acid residues at positions 38, 39, 46, 52, and 58 (Figure 8). Moreover, all γ-secretase components could be detected in mitochondrial fractions from rat brain by Western blot and immunoelectron microscopy. In addition, the four known γ-secretase components formed a 500kDa complex as analyzed by BN-PAGE and were pulled down by co-IP, showing that the individual proteins were part of a complex.

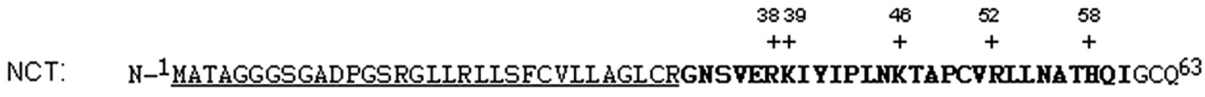

Figure 8. Mitochondrial targeting sequence in Nct.
Amino acids 1-32 represent an ER-targeting signal, whereas sequence 33-60 represent a mitochondrial-targeting signal

To investigate if the mitochondrial γ-secretase complex was active we used the cell-membrane based γ-secretase activity assay using BD8 cells or recombinant C100-flag as substrates. The mitochondrial γ-secretase complex showed activity, but it appeared lower than the γ-secretase complex in the control membrane fraction. In **paper II** we

elucidated how much of γ-secretase complex that was localized to mitochondria as compared to the whole cell. Since Nct contains a chimeric mitochondrial signal and our immunoelectron microscopy data suggests that there is more Nct staining in mitochondria compared to the other γ-secretase components (Figure 9), we also decided to measure the levels of Nct in mitochondria compared to whole cell. For these experiments Nct was detected on Western blot and a γ-secretase inhibitor (GCB)-pull down were used to pull down the active γ-secretase complex. Thus the GCB-pull down was used not only to be able to assess the amount of γ-secretase complexes, but the amount of active complexes. The results demonstrated that approximately 1.5% of the whole cell γ-secretase activity is located to mitochondria and that approximately 3% of the Nct content is mitochondrial. This data nicely fits with our previous immunoelectron microscopy data demonstrating that Nct is more abundant in mitochondria. Interestingly, it has been reported that the skeletal muscle cell express 50% more Nct than PS1 and that if 50% of the Nct expression is knocked down the γ-secretase activity is not affected, implicating a γ-secretase independent role of Nct (Ilaya et al., 2004; Brijbassi et al., 2007).

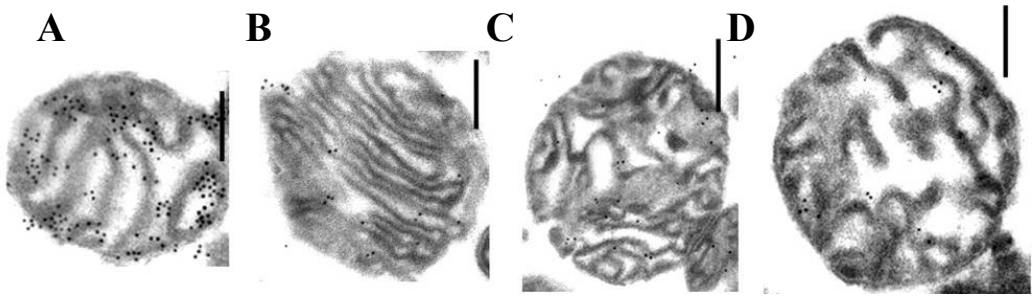

Figure 9. ?-secretase components in isolated mitochondria.
A) Nct, **B)** PS1, **C)** Aph-1 and **D)** Pen-2

It is still not known how the γ-secretase complex is imported to mitochondria. Is the complex assembled in ER and then translocated to mitochondria? Do some components act as carriers for the others to be able to be localized to mitochondria, or is each component individually imported into mitochondria and then forming a complex? One might here argue that it is less likely that the whole γ-secretase complex would be able to pass the import pore since it would be very large. We performed co-IPs and found that Nct interacts with Tom20 in the mitochondrial import machinery. In addition, different PS and Nct deficient cell lines were used to investigate if the different components could be detected independently of each other. We found that Nct is imported into mitochondria independently of PS and that Aph-1 was imported in both

PS and Nct deficient cells. In addition, full length PS1 was detected in Nct deficient cells. This suggests that some of the γ-secretase components can be imported independently of each other and that the complex thus can be formed in mitochondria after import. We also performed proteinase K treatment of mitochondria and crude mitoplasts to try to assess if the γ-secretase has the same topology as previously has been described at the ER. The Nct C-terminal was protected in mitochondria and degraded in crude mitoplast and PS C-terminal was partially protected in the crude mitoplast, indicating that these components have the same topology as been reported at the plasma membrane. Together with the results from immunoelectron microscopy, these data suggest that the γ-secretase complex is mostly localized to the inner mitochondrial membrane. The mitochondrial γ-secretase substrate candidates, which have been found in our laboratory, are also localized to the inner mitochondrial membrane.

It is possible that there are several γ-secretase complexes with different functions in the same cellular organelle. In our laboratory we have found both PS1 and PS2 in mitochondria and Behbahani *et al* showed that lack of PS2 affected the mitochondrial morphology and membrane potential. Lack of PS1 did not have these effects (Behbahani et al., 2006). This data support the notion of different types of mitochondrial γ-secretase complexes.

Aβ LOCALIZATION IN MITOCHONDRIA

Aβ has been shown to cause mitochondrial dysfunction and also to be localized in mitochondria from AD brain and APP transgenic mice (Lustbader et al., 2004; Caspersen et al., 2005). Interestingly, APP has been shown to be localized in AD brain mitochondria and to block the import pore due to an acidic stop transfer signal at residue 220 (Anandatheerthavarada et al., 2003). This incomplete import leaves the APP transmembrane domain and the Aβ region outside the mitochondria, making it a less likely substrate candidate for the mitochondrial γ-secretase. This suggests that the Aβ peptide is not generated locally but transported into mitochondria. There are several possible pathways for this to occur. Aβ is a very hydrophobic peptide and it has been shown to be able to form membrane pores (Bezprozvanny and Mattson, 2008). Mitochondria have channels, which Aβ could pass through, like the VDAC, the Tom40 pore or the pore formed by the mitochondrial permeability transition. In **paper III** a mitochondrial import assay was used to investigate these different possible pathways and we found that both Aβ40 and Aβ42 could be imported into mitochondria via the

TOM complex. In addition to our import assay, we used cortex samples obtained from patients with normal pressure hydrocephalus with or without amyloid plaque pathology. We found that patients with plaque pathology and affected mini mental scores also had Aβ42 in their mitochondria (Figure 10).

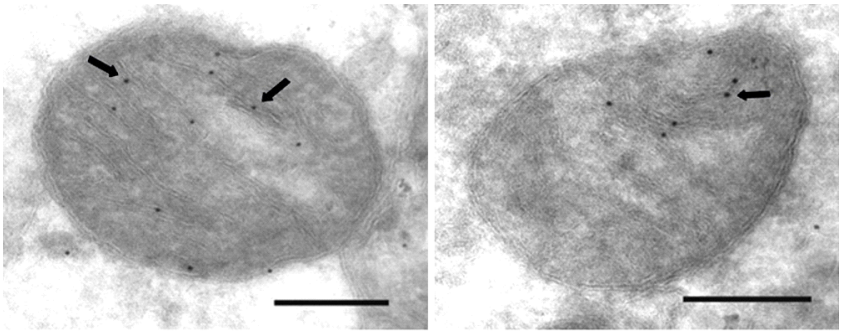

Figure 10. Immunoelectron microscopy of Aß42 in mitochondria from human brain biopsy from patient with normal-pressure hydrocephalus and amyloidosis. Arrows indicates labelling of Aß42.

It is here important to mention that it is not uncommon for patients suffering from normal pressure hydrocephalus to show affected mini mental scores. Therefore, we cannot know if there is any connection between the amyloid pathology and the decrease in the mini mental score. It would be interesting to make a follow-up study on these patients, to asses if the ones with mitochondrial Aβ as compared to the ones lacking mitochondrial Aβ, will develop AD.

To further investigate the mitochondrial localization of Aβ, subfractionation and immunoelectron microscopy analysis of the mitochondria after import, was performed. Both the imported Aβ and the Aβ detected in human cortex was predominantly located to mitochondrial cristae and not to matrix as previously reported (Caspersen et al., 2005). It is possible that the Aβ during the time that AD develops can pass through the inner membrane and accumulate in matrix, which would then explain the discrepancy between our result and the results shown in post-mortem brain (Caspersen et al., 2005). However, Aβ has been to shown to exert its neurotoxicity by affecting the electron transport chain, which is located to the inner mitochondrial membrane. Indeed we could detect a 5-10% decrease in mitochondrial membrane potential after Aβ import, suggesting that the peptide may affect the inner membrane and its functions. Furthermore, we report that Aβ applied extracellularly can be taken up by neuroblastoma cells and reach mitochondria.

γ-SECRETASE COMPLEX IS ACTIVE DURING CELL DEATH.

In the second part of this thesis project the γ-secretase complex stability, composition and activity during cell death was investigated in neuroblastoma cells (**paper IV)**. We found that the γ-secretase complex is active during staurosporine (STS)-induced apoptosis and after six hours we could see an increasing amount of caspase processed PS1-CTF. This PS1-caspCTF was pulled down with Nct, suggesting that the PS1-caspCTF was part of the γ-secretase complex. Furthermore, the γ-secretase activity was investigated in STS-induced apoptotic neuroblastoma cells using the cell-membrane based γ-secretase activity assay. In addition, the Aβ40 was measured in STS-treated HEK-APPswe cells. Here it was clear that the activity of the γ-secretase complex was preserved in neuroblastoma and HEK cells undergoing apoptosis, as shown by AICD and Aβ40 production, respectively. Still this did not answer the question whether the PS1-caspCTF containing γ-secretase complex remains active. To answer this question a construct mimicking the PS1-caspCTF was co-transfected with PS1-NTF into BD8 cells (PS deficient cells) and the γ-secretase activity was measured by the luciferase based γ-secretase activity assay. The PS1-caspCTF reconstituted the γ-secretase activity, cleaving the C99 to levels similar to the PS1-CTF.

Interestingly, Deng *et al* has shown that the deletion of hydrophilic loop in PS1 results in an increased Aβ42/Aβ40 ratio (Deng et al., 2006). This deletion is not a very likely event occurring in human brain, but caspase processed PS1-CTF may cause a similar effect. To be able to study the caspCTF containing γ-secretase complex and if it could affect the Aβ42/Aβ40 ratio, another cell model was needed. This was because the STS-treated SH-SY5Y cells produced low levels of Aβ and also contained a mix of both PS1-wtCTF and PS1-caspCTF. These problems were solved in **paper V** by producing stable cell lines expressing C99 (direct γ-secretase substrate for Aβ generation), PS1-NTF and caspCTF or CTF. γ-Secretase complex formation was investigated by GCB-pull down, the activity of the different complexes was measured by membrane based γ-secretase activity assay and the Aβ42/Aβ40 ratio were assessed by ELISA. Interestingly, cells containing caspCTF γ-secretase complexes had an increased intracellular Aβ42/Aβ40 ratio. The ability of caspCTF cells to produce Aβ42 was confirmed by LC-MS/MS. Furthermore, the PS1-caspCTF was pulled down with Nct antibody in post-mortem brain from control and AD cases, showing that these complexes are present in human brain.

CONCLUSIONS

This thesis is focused on the role of mitochondria and some of the key proteins involved in AD.

The γ-secretase complex has been shown to be localized to the secretory pathway in the cell and is involved in the regulation of many different cellular functions (e.g. cell adhesion and development).

We have identified a mitochondrial γ-secretase complex **(paper I)** and shown that notable levels (~1.5%) of the cells active γ-secretase complexes are localized to mitochondria **(paper II)**. This makes it worthwhile to investigate the substrates of the mitochondrial γ-secretase. The complexes are primarily found in the inner mitochondrial membrane and so are the substrate candidates. The import mechanism of the mitochondrial γ-secretase is not known but we have found that Nct contains a chimeric N-terminal ER/mitochondrial targeting signal and interacts with the Tom20 receptor of the mitochondrial import machinery. Furthermore Nct can be imported into mitochondria independently of PS1 and PS2.

Aβ has previously been shown to be localized to mitochondria in post-mortem brains from AD patients and brains from an APP-transgenic mouse. The pathway for this mitochondrial uptake of Aβ has not been known.

Our results suggest that both Aβ40 and Aβ42 are imported through the TOM machinery independently on the mitochondrial membrane potential in a mitochondrial import assay **(paper III)**. Furthermore, we found that Aβ42 is localized to the cristae of human brain mitochondria, in patients suffering from normal pressure hydrocephalus with amyloid plaques. The localization pattern of Aβ40 and Aβ42 after import resembles that of the human brain mitochondria suggesting that the import assay is a valid technique to study Aβ uptake into mitochondria and can be used to study the effect of Aβ on mitochondria.

It has earlier been suggested that caspase activity results in an increased secretion of Aβ. The mechanisms responsible for this increase have been hypothesized to be either through caspase cleavage of APP or γ-secretase.

We report that γ-secretase is active during cell death and that caspases can process the C-terminal loop of PS1 (PS1-caspCTF) **(paper IV)**. Furthermore, the γ-secretase complexes containing PS1-caspCTF are still active. Stably transfected BD8 expressing either PS1-caspCTF containing γ-secretase or PS1-CTF showed that cells containing PS1-caspCTF γ-secretase complexes have a significant increased Aβ42/Aβ40 ratio **(paper V)**. This could then result in vicious cycle where elevated levels of Aβ42 could cause further activation of caspases, with increased generation of Aβ42 as a result. Interestingly, we also report that the PS1-caspCTF containing γ-secretase complex is present in human post-mortem samples from AD and control brains.

FUTURE PERSPECTIVES

Much of AD research is focused on the discovery of new molecular targets for novel treatment strategies. In spite of this not many new targets have emerged since the amyloid-β hypothesis was proposed in 1992. The neurodegenerative processes observed in AD are still believed to be caused by the action of Aβ. The function of this peptide is not known and to complicate things it has been given both neurotoxic and neurotrophic properties. Aβ is cleaved out from APP by sequential proteolysis by the β-secretase and γ-secretase. Aβ has been shown to exert its toxicity through intracellular aggregation and actions causing mitochondrial and ER dysfunction and oxidative stress. Mitochondria are the main site for the formation of oxidative radicals.

We and others have been able to show that mitochondria from human brains with amyloid plaques contain Aβ. In paper III we describe how Aβ is imported through the mitochondrial import machinery, the TOM-complex, in isolated rat liver mitochondria and accumulates to the inner mitochondrial membrane. Furthermore, we have identified a mitochondrial γ-secretase complex in paper I and II. In paper IV and V we studied the effect of cell death and caspase cleaved PS1 on the γ-secretase activity and found that the Aβ42/ Aβ40 ratio was increased. These findings may not present the ideal drug targets, since they are involving several important and vital cellular processes. Caspase inhibitors have been suggested as drug target. But the prospect of inhibiting caspases for long time spans, which would be required for treating AD, is problematic since they are key actors in many apoptotic pathways. Furthermore, the mitochondrial import machinery is necessary for supporting cellular respiration and every aspect of mitochondrial function is dependent on protein import making it a difficult drug target. However, if an exact binding-epitope for Aβ was identified and the Aβ-TOM interaction could be inhibited without affecting the mitochondrial import, mitochondrial Aβ uptake could become a successful target.

The roles of the mitochondrial γ-secretase complex need further investigation and the substrate candidates are currently studied in our laboratory. Interestingly, the previous studies on PS2 and PS1 deficient cells in our laboratory have given some implications that PS2 deficiency result in lower mitochondrial membrane potential and swollen morphology (Behbahani et al., 2006). It is possible that the cell contains many different γ-secretase complexes (PS1/PS2 and Aph-1a/Aph1b), which have different functions and specificity. I think we need more knowledge about these different γ-

secretase complexes before initiating new clinical trials with γ-secretase inhibitors. To conclude, the findings presented in this thesis need to be further investigated before deciding whether any of them could become a possible drug target. Even so, these findings can give new insights in basic cellular biology and contribute to the understanding of the pathological mechanisms behind AD.

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