Processing of the APP family by the α-secretases ADAM10 and TACE

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Licentiate thesis

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ISBN 978-91-7447-000-0

Printed in Sweden by Universitetsservice US-AB, Stockholm 2010
Distributor: Department of Neurochemistry, Stockholm University

To my family

List of publications

I Holback, S., Adlerz, L., Gatsinzi, T., Jacobsen, K.T., Iverfeldt, K.
PI3-K- and PKC-dependent up-regulation of APP processing enzymes by retinoic acid.
Biochemical and Biophysical Research Communications. 2008; 365(2):298-303.

II Jacobsen, K.T., Adlerz, L., Multhaup, G., Iverfeldt, K.
IGF-1-induced processing of amyloid-β precursor protein and APP-like protein 2 is mediated by different enzymes.
Manuscript

Additional publications

Jacobsen, K.T. and Iverfeldt, K.
Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. (Review)
Cellular and Molecular Life Science. 2009; 66:2299-2318

Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disease, which is characterized by formation of amyloid plaques in the brain. The major constituent of these plaques is the hydrophobic peptide Aβ. Aβ accumulation is considered to be the main cause of the pathology seen in AD brains. Aβ is produced through sequential cleavage of the amyloid precursor protein (APP). APP can be processed by two different enzymatic pathways. Formation of Aβ requires cleavage of APP by β- and γ-secretase. However, most proteolytic processing of APP does not result in Aβ formation. Instead, APP is mainly cleaved by α-secretase, which not only precludes formation of the toxic Aβ peptide but also generates the neuroprotective sAPPα fragment. Increasing the α-secretase processing of APP is thereby a potential therapeutic strategy for AD. APP is a member of a conserved gene family, also including the APP-like proteins-1 and -2 (APLP1 and APLP2). The APP family members have essential and overlapping functions and have been reported to be processed in a similar way by the same enzymes. The processing of all APP family members is increased in response to several stimuli, including retinoic acid (RA) and insulin-like growth factor-1 (IGF-1), which also induce a shift towards α-secretase processing. The aim of this thesis was to investigate the mechanisms and signaling involved in induced α-secretase processing of the APP family. The main α-secretase candidates are ADAM10 and TACE. In this thesis we wanted to study the effects on expression levels of ADAM10 and TACE during RA treatment. We also wanted to investigate the mechanism behind IGF-1-induced processing of APP and APLP2. We found that both ADAM10 and TACE are up-regulated in response to RA, but that the signaling pathways involved differed between the two enzymes. Similarly, we showed that IGF-1-induced processing of APLP2, but not of APP, is dependent on PKC. Furthermore, we showed that ADAM10 is the main α-secretase for APP, whereas TACE cleaves APLP2 in response to IGF-1. We conclude that although APP and APL2 proteolytic processing are induced by the same stimuli, the processing is dependent on different signaling pathways and processing enzymes, which in turn are differentially regulated.

Contents

Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's Disease
ADAM	A disintegrin and metalloprotease
AICD	APP intracellular domain
ALID	APP-like intracellular domain
ANOVA	Analysis of variance
APH-1	Anterior pharynx defective-1
APLP	APP-like protein
APP	Amyloid-β precursor protein
BACE	B-site cleaving enzyme
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
C83	APP C-terminal stub of 83 amino acids
C99	APP C-terminal stub of 99 amino acids
CRABs	Cellular retinoic acid binding proteins
CS GAG	Chondroitin sulphate glycosaminoglycan
ECL	Enhanced chemiluminescence
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmatic reticulum
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IP	Immunoprecipitation
MAPK	Mitogen activated protein kinase
NFTs	Neurofibrillary tangles
NGF	Nerve growth factor
NMDA	N-methyl D-aspartate
PCR	Polymerase chain reaction
PEN2	Presenlilin enhancer 2
PI3-K	Phosphatidyl inositol-3 kinase
PKC	Protein kinase C
PLC	Phospholipase C
PS	Presenilin
RA	Retinoic acid
RAR	Retinoic acid receptor

RARE	Retinoic acid responsive element
RBP	Retinol binding protein
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
RXR	Retinoid X receptor
sAPP	Secreted APP
SDS	Sodium dodecyl sulphate
siRNA	Short interfering RNA
TACE	TNF-α converting enzyme
TGF	Transforming growth factor
TIMPs	Tissue inhibitors of metalloproteases
TMB	Tetremethylbenzidine
TNF	Tumor necrosis factor

1. Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a devastating neurodegenerative disease that 7500 people in Sweden are diagnosed with every year (www.alzheimerforeningen.se). The most affected brain area is the hippocampus, which is a part of the limbic system. As a result the disease causes cognitive defects, such as memory deficits, problems to speak and disorientation. The histopathological hallmarks of AD are (1) neurofibrillary tangles (NFTs); which are intracellular lesions consisting of the hyperphosphorylated microtubule-associated protein Tau, (2) chronic inflammation, and (3) amyloid plaques; which are extracellular aggregates composed of the neurotoxic peptide amyloid-β (Aβ) (reviewed in Duyckaerts et al. 2009). The pathology of AD was first characterized more than 100 years ago, but the cause of most cases is still unknown. However, according to the main theory "the amyloid cascade hypothesis", the accumulation of Aβ is considered to be the key event, leading to formation of NFTs, inflammation and neurodegeneration (Glenner et al. 1984). There are several facts that support this theory, the most important ones being the genetics involved in AD. Although only a small part of AD cases are caused by genetic defects, these mutations are all linked to increased production or accumulation of Aβ. However, not everyone agrees with the amyloid cascade hypothesis, mainly since the number of Aβ deposits in the brain does not correlate well with the degree of cognitive impairment (reviewed in Hardy 2009).

1.2 The APP family

Aβ is produced through sequential cleavage of the amyloid precursor protein (APP). More than 20 years have passed since the molecular cloning of the human APP gene. Soon after its discovery, APP was found to be evolutionary highly conserved. In addition, two mammalian genes encoding the homologous proteins, APP-like protein-1 and -2 (APLP1 and APLP2) were identified. The APP family proteins are intriguing with many different suggested functions. It is still not fully clear whether the proteins function as *bona fide* signaling receptors and/or adhesion molecules or whether the

physiological function is primarily mediated by a shedded soluble fragment. However, it is clear that the APP family has redundant and at least partly overlapping functions. Therefore, a comparison of the members will give a more complete picture.

1.2.1 Structure and function

All APP family members are type 1 integral membrane proteins, with a single membrane-spanning domain, a large ectoplasmic N-terminal region and a shorter cytoplasmic C-terminal region (cf., Kang et al. 1987; Dyrks et al. 1988). The APP sequence can be divided into multiple distinct domains (Fig. 1). The ectoplasmic region of APP, which constitutes the major part of the protein, can be divided into the E1 and E2 domains (reviewed in Gralle and Ferreira 2007). The E1 domain can be further divided into a number of subdomains, including a heparin-binding/growth-factor-like domain (HFBD/GFLD), a copper binding domain (CuBD) and a zinc binding domain (ZnBD). The E1 domain is followed by an acidic region rich in aspartic acid and glutamic acid (DE) and a Kunitz protease inhibitor domain (KPI; not present in APP695). The E2 region consists of another HFBD/GFLD and a random coil region (RC). The cytoplasmic region of APP contains a protein interaction motif, namely the YENPTY sequence (including the NPXY internalization signal), which is conserved in all APP homologues. The sequences of APLP1 and APLP2 can be divided into similar domain structures as APP (Fig. 1).

There are three major isoforms of mammalian APP; APP695, APP751 and APP770. Alternative splicing of APLP2 also produces multiple protein isoforms, while only one form of APLP1 has been detected. The main difference between the APP isoforms is the presence or absence of the KPI domain and a chondroitin sulfate glycosaminoglycan (CS GAG) attachment site. This is also the case for the APLP2 isoforms. APLP1 consists of 650 amino acids and lacks both the KPI domain and the CS GAG attachment site (Wasco et al. 1992; Paliga et al. 1997). APP and APLP2 are ubiquitously expressed. However, the different isoforms can be preferentially expressed in different cell types, such as the 695 amino acid long isoform of APP, which is mainly found in cells of neuronal origin. APLP1 expression has been reported to be restricted to the nervous system (Slunt et al. 1994) (Lorent et al. 1995).

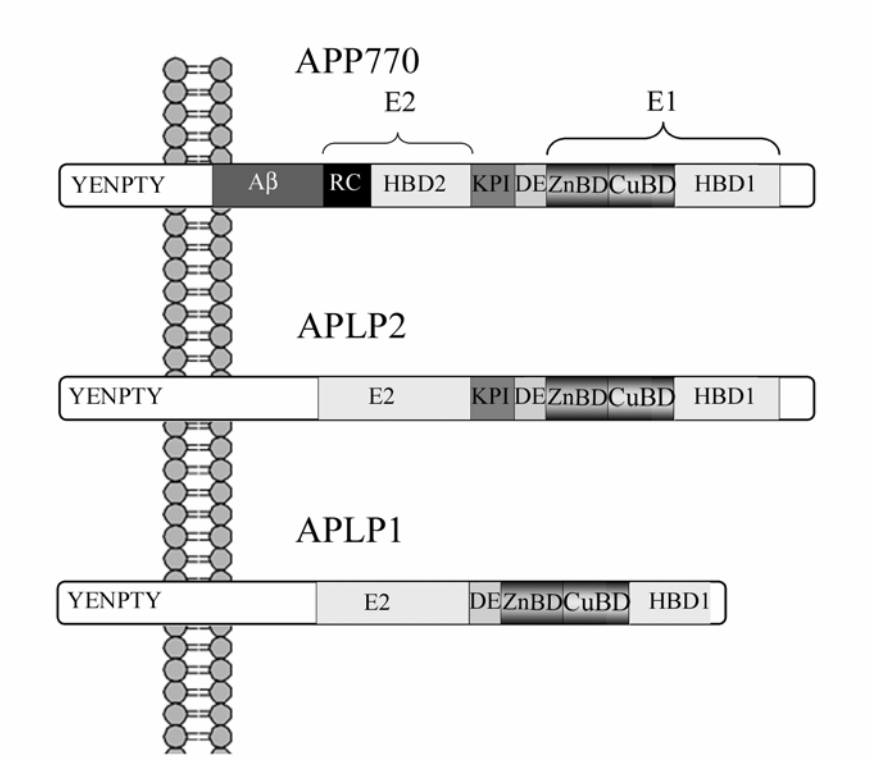

Figure 1. Schematic illustration of the domain structure of the APP family. The APP family members are integral membrane proteins with a multi-domain structure, including the ectoplasmic E1 and E2 domains. E1 and E2 can be further divided into several domains, e.g. heparin-binding/growth factor-like domains (HBD1-2), copper- and zinc-binding domains (CuBD and ZnBD), an acidic domain (DE) and a random coil region (RC). The APP family proteins all have a protein interaction motif (YENPTY) in the C-terminal. Some isoforms of APLP2 and APP, also contain a Kunitz protease inhibitor domain (KPI).

The exact biological function of APP and its homologues is still unknown. However, several *in vitro* and *in vivo* studies have yielded strong evidences for roles of APP, both in the developing and adult nervous system, in cell adhesion, neuronal survival, neurite outgrowth, synaptogenesis, vesicular transport, neuronal migration, modulation of synaptic plasticity and insulin and glucose homeostasis. Studies with knockout mice have shown that single disruptions of APP, APLP1 or APLP2 only caused minor abnormalities (Zheng et al. 1995; von Koch et al. 1997; Heber et al. 2000). In contrast, $APLP2^{-/-}/APP^{-/-}$ mice and $APLP2^{-/-}/APLP1^{-/-}$ both showed a lethal phenotype, whereas $APLP1^{-/-}/APP^{-/-}$ mice were viable (Heber et al. 2000). Tripple knockout mice showed a 100% lethal phenotype (Herms et al. 2004). These

results suggest that APLP2 has a key physiological role among the mammalian family members and that there seems to be functional redundancy among the homologues. Little is known about the function of APLP1 and APLP2. However, the functional overlap with APP has been demonstrated in several studies, providing evidence for roles of the homologues in neurite outgrowth, cell adhesion and neuronal migration.

1.2.2 Proteolytic processing of the APP family

Although APP processing has been studied extensively, there is not yet a complete picture of the processing events and the enzymes involved. All APP family members have been reported to be processed in a similar way by the same enzymes. The identity of the enzymes which process APP was initially unknown, and they were instead referred to as α-, β- and γ-secretase, after the sites where they cleaved APP.

1.2.2.1 Secretases

α-Secretase has been characterized as a zinc metalloproteases that cleaves APP at the Lys613-Leu614 bond (in the APP695 isoform) (Roberts et al. 1994). Several α-secretase candidates exist and they are all members of the ADAM (a disintegrin and metalloprotease) family. The two most likely candidates are ADAM10 and ADAM17, also known as TACE (tumor necrosis factor-α converting enzyme), but also ADAM9 has been proposed to play a role.

β-Secretase is an aspartyl protease that cleaves APP at the Met597-Asp598 bond, which constitutes the first step towards Aβ production. Two β-secretases have been identified; BACE (β-site APP-cleaving enzyme) and BACE2 (Hussain et al. 1999; Sinha et al. 1999; Vassar et al. 1999; Yan et al. 1999). BACE is more widely expressed in the brain and BACE knock-out animals do not produce any detectable levels of Aβ (Luo et al. 2001; Roberds et al. 2001). BACE is a single domain integral protein with its active site located on the ectoplasmic side of the membrane (Hussain et al. 1999; Vassar et al. 1999). The optimal pH of BACE activity is approximately 4.5, indicating that the β-site cleavage of APP takes place in more acidic cellular compartments, such as the endosomes (Vassar et al. 1999). BACE cleavage has also been shown to occur in lipid rafts (Ehehalt et al. 2003).

γ-Secretase is an aspartyl protease with low sequence specificity that cleaves the substrate within its transmembrane domain. The enzyme is a protein complex that consists of anterior pharynx defective 1 (APH-1), nicastrin, PS1 or PS2, and presenilin enhancer-2 (PEN-2) (Goutte et al. 2000; Yu et al. 2000; Francis et al. 2002; Edbauer et al. 2003). PS1 is a 9 transmembrane

domain (TM) protein and is considered to harbor the active site of the enzyme (Yu et al. 1998). Two highly conserved aspartate residues (Asp257 and Asp385 in human PS1) within TM6 and TM7 constitute the core of the catalytic site (Wolfe et al. 1999). Nicastrin has one TM and a large ectodomain, proposed to function as a gatekeeper to the PS1 active site (Shah et al. 2005). Nicastrin also functions as a substrate receptor and is important for the assembly process of the γ-secretase complex. The functions of APH1 and PEN2 are not yet fully understood. However, APH1 has been implicated in stabilization of PS1 and plays an important part during assembly of the complex, whereas PEN2 stabilizes the final complex as well as is involved in endoproteolysis of PS1 (reviewed in Verdile et al. 2006; Wolfe 2006).

1.2.2.2 Processing of APP

The proteolytic processing of APP can be divided into two different pathways (Fig. 2), the amyloidogenic pathway, which leads to generation of Aβ, and the non-amyloidogenic pathway. Both pathways include at least three cleavage events. The amyloidogenic processing of APP is initiated through cleavage by β-secretase, which leads to secretion of the large N-terminal ectodomain, sAPPβ. The remaining C-terminal stub of 99 amino acids (C99) can then be further processed by γ-secretase, generating Aβ and a soluble APP intracellular domain (AICD). AICD can translocate into the nucleus where it may function as a transcription factor (Cao and Südhof 2001). γ-Secretase cleavage between Val637 and Ile638 generates Aβ40, and cleavage after Ala639 results in production of Aβ42 (reviewed in Esler and Wolfe 2001). The remaining C-terminal fragments, after γ-secretase cleavage, were expected to be 57 or 59 amino acids long (C57 and C59). However, instead a 50 amino acids long C-terminal fragment (C50) has been identified as AICD (Sastre et al. 2001). Consequently, the final processing step has been suggested to be a result of three cleavage events. The C99 fragment is first cleaved at the ε-site at the Leu646-Val647 bond (Yu et al. 2001; Weidemann et al. 2002), followed by cleavage at the ζ-site between Val643 and Ile644, and finally the peptide is cut at the γ-site (Zhao et al. 2004).

In the non-amyloidogenic pathway, generation of Aβ is precluded since APP is initially cleaved by α-secretase within the Aβ sequence near the ectoplasmic side of the plasma membrane (Esch et al. 1990; Sisodia et al. 1990). The α-secretase cleavage of APP releases the N-terminal ectodomain, sAPPα from the cell surface leaving an 83 amino acid long C-terminal membrane-bound fragment (C83). The remaining C83 fragment can be further processed by γ-secretase in a similar way as in the amyloidogenic pathway, giving rise to the small peptide p3 and AICD.

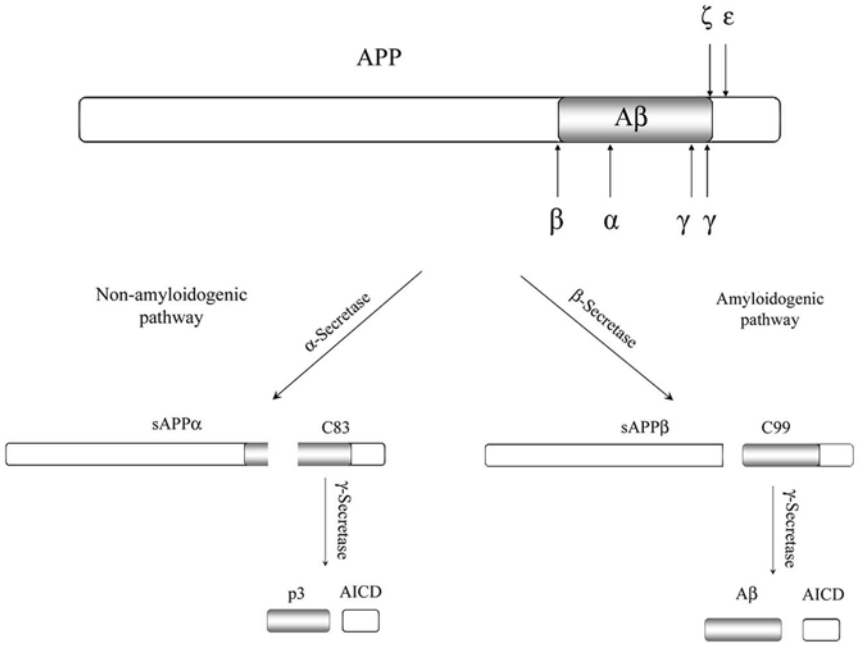

Figure 2. Schematic illustration of APP processing. In the non-amyloidogenic pathway, APP is initially cleaved at the α-site, generating the secreted sAPPα fragment. In the amyloidogenic pathway, APP is instead cleaved at the β-site, generating sAPPβ. The remaining C-terminal membrane-bound stubs, C83 or C99, are subsequently cleaved by γ-secretase, releasing AICD and p3 or Aβ.

1.2.2.3 Processing of APP homologues

Although the amino acid sequences in APP, where the secretases cleave, are very different in the two mammalian homologues, both APLP1 and APLP2 are believed to be proteolytically processed in a similar way as APP. However, the exact cleavage sites have still not been determined. The first indication that APLPs were cleaved by γ-secretase was the detection of elevated levels of an APLP1 membrane-bound C-terminal fragment in brain tissues from animals deficient in PS1 (Naruse et al. 1998). Cleavage of APLPs by secretases generates not only fragments corresponding to membrane-bound APP C-terminal fragments, but also to AICDs, denoted ALID1 and 2 (APP-like intracellular domain 1 and 2) (Gu et al. 2001). Substantial evidences show that APLP2 can be processed by α- and β-secretase, while the processing events are less certain for APLP1 (Eggert et al. 2004; Endres et al. 2005).

Like APP, APLP2 and APLP1 processing has been demonstrated to be induced by phorbol esters (Xu et al. 2001; Eggert et al. 2004). Furthermore, overexpression of either ADAM10 or TACE has been shown to increase the secretion of sAPLP2 (Endres et al. 2005). α-Secretase processing of APLP1 was demonstrated in APLP-1 transfected human neuroblastoma SH-SY5Y cells, since the production of an APLP1-derived p3-like fragment was strongly reduced by the ADAM inhibitors batimastat and TAPI-2.

1.3 ADAM10 and TACE

ADAMs are type 1 integral membrane glycoproteins with a multi-domain structure (Fig. 3), including signal peptide, pro-domain, catalytic metalloprotease domain, disintegrin/cystein-rich domain, transmembrane domain and a short cytoplasmic domain (Howard et al. 1996). The pro-domain is cleaved off by a cystein switch mechanism in order to activate the protease. Due to the presence of a disintegrin domain, which has been shown to bind integrins on cell surfaces, ADAMs have been implicated to be involved in regulation of cell-cell or cell-extracellular matrix contacts, as well as in extracellular matrix degradation. About half of the presently known ADAMs are zinc-dependent proteases that share an extended catalytic site sequence, HEXGHXXGXXHD. The three histidines bind a Zn^{2+}, and the glutamate is believed to be the catalytic residue. A major function of ADAM proteases is the ectoplasmic cleavage of cell membrane proteins, also called ectodomain shedding. Many functionally diverse proteins, such as cytokines, growth factors and their receptors, and cell-adhesion proteins are initially synthesized as membrane-anchored proteins, which are released upon proteolysis mediated by ADAMs. This includes the conversion of membrane anchored precursors of epidermal growth factor receptor (EGFR) ligands into soluble growth factors, which in turn have essential roles in development and diseases such as cancer. Both TACE and ADAM10 have been implicated as α-secretases, but it is still debated whether both enzymes are able to cleave APP constitutively and/or during regulated conditions. The general idea in this field seems to have been that ADAM10 cleaves APP constituently, whereas TACE cleaves APP during regulated conditions. This seems to be a much too simplified idea, since some stimuli activate TACE (e.g. phorbol esters) and other stimuli activate ADAM10 (e.g. calcium). Furthermore, TACE and ADAM10 both have a broad substrate specificity that somewhat overlaps. Understanding the substrate recognition and selectivity of ADAM10 and TACE has recently become an extensively studied area that is making much progress. Here, a background on TACE and ADAM10 will be presented, and their roles as α-secretases will be discussed.

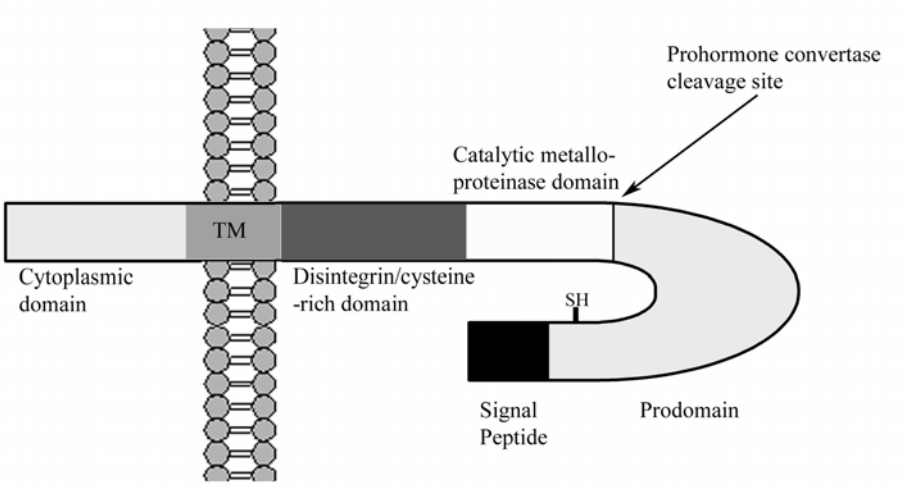

Figure 3. Schematic illustration of the domain structure of ADAMs. Most ADAMs including ADAM10 and TACE contain a cytosolic domain (sometimes containing phosphorylation sites, e.g. TACE), a transmembrane domain (TM), a disintegrin/cystein-rich domain, a catalytic metalloproteinase domain followed by a prohormone convertase cleavage site, a pro-domain, containing a cysteine (SH) switch and a signal peptide.

1.3.1 TACE

TACE was originally identified as the enzyme responsible for cleavage of the membrane-bound pro-protein of tumor necrosis factor α (TNF α) (Black et al. 1997; Moss et al. 1997). TNFα is a cytokine which can induce inflammation and apotosis. The protein exists as a membrane-bound precursor with a molecular mass of 26 kDa, which can be processed by TACE, to generate the secreted 17 kDa mature TNFα. Inactivation of TACE, by elimination of its zinc-binding domain, *in vivo* ($tace^{\Delta Zn/\Delta Zn}$ mice) demonstrated the role of TACE in shedding of ligands for the epidermal growth factor (EGF) receptor family, including transforming growth factor α (Peschon et al. 1998; Sunnarborg et al. 2002). Several other substrates have since been identified, e.g. Notch (Brou et al. 2000) and APP (Buxbaum et al. 1998).

TACE is found in most tissues and is constitutively expressed in neurons, vascular cells and leukocytes. The cellular localization of ADAMs is thought to be important for their shedding activity. Immunofluorescence and biotinylation studies have shown that the majority of mature TACE is localized to perinuclear compartments, with only a small amount being present on the cell surface (Schlondorff et al. 2000). Pro-TACE is activated by a furin-like cleavage, which removes the pro-domain. Furin as well as

several other pro-hormone convertases (PCs) has been implicated in this maturation of TACE (Srour et al. 2003). This cleavage takes place in a late Golgi compartment. The activity of most ADAMs is regulated by a group of physiological inhibitors, namely the tissue inhibitors of metalloproteases (TIMPs). *In vitro* studies with recombinant TACE have shown that the activity of TACE is inhibited by TIMP-3 (Amour et al. 1998).

Stimulation with phorbol esters has been shown to enhance the shedding activity of TACE. In a study by Soond et al, it was demonstrated that phorbol ester stimulation increased phosphorylation of pro-TACE at Thr735 in the intracellular domain (Soond et al. 2005). The phosphorylation was ERK-dependent and increased maturation of TACE as well as increased the translocation from the ER to the cell surface. TACE has further been shown to colocalize to the plasma membrane with one of its substrates, CD44, within 10 min after phorbol ester stimulation (Nagano et al. 2004). These studies suggest that phosphorylation of TACE affects the subcellular localization, leading to its activation. In a study by Cissé and colleagues it was demonstrated that mutations at Thr735 abolished the carbachol-induced increase of TACE phosphorylation as well as the induced processing of the cellular prion protein (Cissé et al. 2007). This study demonstrates a clear and direct correlation between TACE phosphorylation and activity induced by a physiological stimulus. However, in one study the cytoplasmic domain of TACE was shown not to be required for phorbol ester stimulated shedding of TNFα (Reddy et al. 2000), which have made the significance of TACE phosphorylation a debated area.

1.3.1.1 TACE as α-secretase

In situ hybridization in mice during prenatal and postnatal development and in adulthood demonstrated only a partial overlap of APP and TACE expression. However, in human AD brains TACE was observed to co-localize with neurons, senile plaques, and neurofibrillary tangles (Skovronsky et al. 2001).

TACE was identified as an α-secretase candidate since it was shown that it could cleave an α-site spanning peptide (Buxbaum et al. 1998). TACE was further shown to be involved in α-site processing of APP since phorbol ester-induced sAPPα secretion was completely blocked in embryonic fibroblast derived from TACE knock-out mice. However, the constitutive sAPPα secretion was unaffected by TACE knock-out. In another study by Merlos-Suarez et al. the mercurial compound 4-aminophenylmercuric acetate (APMA), frequently used to activate *in vitro* recombinant matrix metalloproteases, was shown to induce sAPPα secretion in a TACE-dependent manner (Merlos-Suarez et al. 2001). These results all point to that TACE is involved in regulated, and not constitutive α-secretase processing

of APP. In contrast, APP and TACE co-transfection in HEK293 cells increased basal sAPPα secretion in a dose-dependent manner in relation to TACE cDNA expression (Slack et al. 2001). In the same study, muscarine-stimulated α-secretase activity was unaffected by TACE overexpression. This was interpreted as evidence that TACE does not participate in regulated α-secretase processing. Likewise, carbacol has been shown to induce sAPPα secretion in a TACE independent manner (Parkin et al. 2002). It is today known that ADAM10 and TACE are activated by different stimluli, ADAM10 by increased calcium influx, and TACE by PKC activation (phorbol ester) (Nagano et al. 2004; Horiuchi et al. 2007). Based on this, it can be speculated that the enzyme involvement in regulated α-secretase processing of APP is dependent on the nature of the stimulus.

Phorbol ester-induced sAPPα secretion could not be detected in furin-deficient LoVo cells, but constitutive sAPPα secretion still occurred (Lopez-Perez et al. 2001). Overexpression of ADAM10, but not TACE increased the sAPPα secretion in these cells, which made the authors speculate that ADAM10 and not TACE is involved in the constitutive α-secretase processing of APP. Another interpretation could be that furin is more important for TACE maturation that for ADAM10. This would explain why phorbol esters, which activate TACE and not ADAM10 failed to increase sAPPα secretion in these cells. If TACE fails to mature in these cells, it would also explain why overexpression of TACE had no effect on sAPPα secretion. It should be noted however, that furin has been shown to affect maturation of both TACE and ADAM10, but other PCs, PC7 for ADAM10 and PC1-6 for TACE, also play a role in maturation (Anders et al. 2001; Srour et al. 2003). Indeed, overexpression of PC7 in furin-deficient LoVo cells increased sAPPα secretion (Lopez-Perez et al. 2001), probably through activation of ADAM10. Endogenous constitutive sAPPα secretion in human glioblastoma A172 cells was shown to decrease upon gene silencing using dsRNA directed against either TACE or ADAM10 (Asai et al. 2003). Together, these results indicate that TACE can cleave APP constitutively, and that TACE most likely is responsible for PKC-dependent, or at least the phorbol ester-induced APP processing. Interestingly, no study has shown that TACE cleaves APP in response to a physiological stimulus. Therefore, the physiological importance of TACE processing of APP during endogenous conditions still remains to be investigated.

1.3.2 ADAM10

ADAM10 is predominantly expressed in the Golgi and trans-Golgi network (TGN) (Gutwein et al. 2003). However, mature ADAM10 is also found at the cell surface (Lammich et al. 1999) and in the nucleus (McCulloch et al. 2004). In epithelial cells, ADAM10 has been detected at the basolateral

plasma membrane, primarly at cell-cell contacts (Wild-Bode et al. 2006). The basolateral signal sequence was identified as the proline rich sequence Pro708-Pro715 located in the cytoplasmic region. APP also contains a basolateral sorting sequence (Haass et al. 1995), and is sorted in basolateral membranes in polarized Madin-Darby canine kidney (MDCK) cells (Capell et al. 2002). Parallels have been drawn between basolateral sorting in epithelial and dendritic sorting in neurons (Dotti and Simons 1990) (Jareb and Banker 1998). A number of basolaterally sorted proteins have been shown to localize to somatodendritic regions in cultured hippocampal neurons, relying on the same sorting sequence in both cell types. Indeed, in a study by Marcello et al, immunocytochemical labeling of ADAM10 in cultured hippocamplal neurons revealed a diffuse somatodendritic staining pattern (Marcello et al. 2007). In the same study, ADAM10 was found to co-localize with synapse-associated protein-97 (SAP-97), which traffics proteins to the excitatory synapse, being enriched in isolated post-synaptic densities. SAP-97 interacts with ADAM10 through its SH3 domain. NMDA receptor activation increased the interaction between SAP-97 and ADAM10, leading to ADAM10 trafficking from dendritic shaft to spine-like structures.

ADAM10 shares many substrates with TACE, which will be further discussed later, including pro-TNFα (Rosendahl et al. 1997), APP (Lammich et al. 1999) and Notch (Hartmann et al. 2002). However, some substrates are selectively cleaved by one of the enzymes. ADAM10 has for example been shown to be the main regulator of epithelial cell-cell adhesion and cell migration, through cleavage of E-cadherin (Maretzky et al. 2005). ADAM10 activity is not affected by phorbol esters, but is instead increased upon treatments that promote cellular calcium influx, such as calcium ionophores (Nagano et al. 2004; Horiuchi et al. 2007). *In vitro* the activity of ADAM10 has been demonstrated to be inhibited by the physiological inhibitors TIMP-1 and TIMP-3 (Amour et al. 2000). Furin and PC7 have been shown to induce ADAM10 maturation (Anders et al. 2001).

Recently, Tousseyn et al made the remarkable finding, that ADAM10 not only sheds other proteins, but is also itself shedded (Tousseyn et al. 2009). ADAM9 and ADAM15 were identified as ADAM10 shedding proteases, since the accumulation of ADAM10 C-terminal fragments was reduced *in vivo* in brains of ADAM9/ADAM15-deficient mice. ADAM10 proteolysis generates a soluble ADAM10 protease which is proteolytically active, as well as an ADAM10 C-terminal fragment, which is subsequently cleaved by γ-secretase. This generates a soluble ADAM10 intracellular domain, which translocates to a nuclear speckle-like compartment. Speckles are interchromatin bodies that concentrate proteins involved in mRNA production, splicing and maturation.

1.3.2.1 ADAM10 as α-secretase

ADAM10 was established as an α-secretase candidate, since it was able to cleave a synthetic peptide spanning the α-secretase cleavage site (Lammich et al. 1999). In addition, overexpression of ADAM10 in HEK293 cells significantly increased the levels of sAPPα. As discussed earlier, ADAM10 has been considered as the enzyme responsible for the constitutive α-secretase processing of APP. This was based on studies that demonstrated that TACE overexpression/silencing had no or little effect on basal sAPPα release (Merlos-Suárez et al. 1998; Blacker et al. 2002) whereas ADAM10 overexpression in furin-deficient LoVo cells significantly increased the constitutive sAPPα secretion (Lopez-Perez et al. 2001).

ADAM10 immunostaining was shown to be reduced in the brains of AD patients (Bernstein et al. 2003). Neuronal overexpression of ADAM10 in transgenic mice carrying a human APP mutation (V717I), increased the secretion of sAPPα, reduced the production of Aβ and prevented its deposition in plaques (Postina et al. 2004). However, sAPPα generation was not affected in fibroblasts isolated from ADAM10-deficient mice (Hartmann et al. 2002), indicating that ADAM10 is not important for constitutive sAPPα production.

In vivo experiments demonstrated that disruption of the interaction between ADAM10 and the synapse trafficking protein SAP-97, impaired the ADAM10 localization in postsynaptic membranes, leading to decreased sAPPα secretion (Marcello et al. 2007). Furthermore, TIMP-1, which inhibits ADAM10 but not TACE (Amour et al. 2000), was shown to totally abolish NMDA-induced sAPPα secretion in hippocampal neurons.

1.3.3 Substrate selectivity of TACE and ADAM10

TACE and ADAM10 have a higher sequence homology to each other, than to any other mammalian ADAM (Black et al. 1997). Like all members of the ADAM family, they both have broad substrate specificity that seems to somewhat overlap. However, some substrates are considered to be preferentially cleaved selectively by ADAM10 or TACE, e.g. TGFα is cleaved by TACE (Peschon et al. 1998) and E-cadherin by ADAM10 (Maretzky et al. 2005). This partial overlap makes it difficult to understand the factors important for substrate selectivity. The sequence of the cleavage site is usually not an important factor for substrate recognition (Tsakadze et al. 2006; Deuss et al. 2008). Instead, it seems like the membrane proximity and the length of the stalk (i.e. the susceptible membrane-proximal region between the ectoplasmic globular domain and the transmembrane domain) are more important. The substrate recognition of TACE and ADAM10 was

further studied by Caescu and colleagues (Caescu et al. 2009). They demonstrated by peptide library screening and analysis of individual substrates that TACE and ADAM10 have distinct amino acid preferences at multiple positions surrounding the substrate cleavage site. Furthermore, they performed mutation studies of the active sites of the enzymes and found that certain amino acids in the active site could partially explain why some substrates are selective for either ADAM10 or TACE.

Interestingly, even though certain substrates are thought to be selective for either ADAM10 or TACE, Le Gall and colleagues demonstrated that their substrate selectivity can be manipulated (Le Gall et al. 2009). The group had previously demonstrated that TACE was required for constitutive and phorbol ester-induced TGFα shedding (Peschon et al. 1998), and were surprised to find shedding activity in $TACE^{-/-}$ cells upon ionomycin stimulation. Acute treatment of wild type cells with a selective TACE inhibitor showed that TACE was the main sheddase for TNFα and TGFα. However, upon chronic inhibition of TACE, ADAM10 rescued the shedding activity of these substrates. This study indicates that many studies in which APP and/or ADAM10/TACE have been overexpressed or knocked-out may not be of physiological significance since ADAM10 and TACE obviously can be pushed to cleave substrates that they under physiological conditions do not cleave. In agreement with LeGall et al, Hikita and colleagues found that even though TACE was the major sheddase for TNFα in most cell types investigated (Hikita et al. 2009), ADAM10 was shown to be the major sheddase in some cell types. The significance of ADAM10 and TACE as sheddases for TNFα could be correlated to the expression levels, but also to the abundance of TIMPs.

In some cases, the overlapping substrate specificity may reflect a selective use of ADAM10 and TACE as sheddases depending on the stimulus. Both TACE and ADAM10 have been shown to cleave Notch1. However, in a study by Bozulak et al. the role of the two proteases was further investigated. It was found that ADAM10 was required for Notch1 signaling induced by ligand binding, whereas signaling independent of ligand required TACE. TACE was not able to rescue ADAM10 processing of Notch1 in $ADAM10^{-/-}$ mouse embryonic fibroblasts. Together, these results illustrate why the α-secretase processing of APP is so difficult to completely understand.

1.4 Factors regulating α-secretase activity

sAPPα is constitutively secreted into the medium of most cell cultures, and several stimuli have been shown to induce a shift in the processing of APP towards the α-secretase pathway. Direct activation of PKC by phorbol ester

stimulation was one of the earliest α-secretase activating stimuli identified (Caporaso et al. 1992; Buxbaum et al. 1993). Activation of muscarinic and growth factor receptors, such as epidermal growth factor, insulin/insulin-like growth factor receptors and tyrosine receptor kinase B (TrkB) has also been shown to enhance the secretion of sAPPα, through activation of PKC, MAPKs and PI3-K (Allinson et al. 2003; Holback et al. 2005; Adlerz et al. 2007). Interestingly, synaptic NMDA receptor activation was recently shown to induce α-secretase processing of APP in primary cultured neurons (Hoey et al. 2009). In our studies we have used the neurotrophic factors retinoic acid, brain-derived neurotrophic factor and insulin-like growth factor-1 to induce α-secretase processing.

1.4.1 Retinoic acid

Retinoic acid (RA) is a vitamin A derivate and a physiological signaling molecule that is involved in neuronal differentiation, axon outgrowth and neuronal plasticity (reviewed in Maden 2007). Retinol is taken up through the diet and is transported into the cells by retinol binding proteins (RBPs), which interact with membrane receptors. In the cytoplasm, retinol is metabolized to RA, which can leave the cell and signal in an autocrine or paracrine fashion. RA exerts its effect by translocating to the nucleus, assisted by cellular RA-binding proteins (CRABs). RA then binds to the RA receptor (RAR) or the retinoic X receptor (RXR), which both are ligand activated nuclear receptors. RAR and RXR function as heterodimers, and bind to RA response elements (RAREs) in the target genes. Binding of RA to the receptor complex induces gene transcription of the target genes, e.g. various transcription factors, cell signaling molecules, structural proteins etc. RA in complex with RAR can also signal through rapid non-genomic pathways, through direct interaction and subsequent activation of protein kinase C (Hoyos et al. 2000), PI3-K and MAPK (Masia et al. 2007).

Interestingly, a connection between RA signaling and AD has been observed, since vitamin A deprieved mice displayed Aβ accumulation and RARα downregulation (Corcoran et al. 2004). Furthermore, RA has been shown to up-regulate the expression of APP, ADAM10, TACE and BACE, as well as to induce a shift in the APP processing toward the α-secretase pathway (Adlerz et al. 2003; Holback et al. 2005; Holback et al. 2007).

1.4.2 Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a member of the neutrophin family, and is involved in cell survival, cell growth and differentiation. BDNF signals mainly through the TrkB receptor, but can also like all other neurotrophins activate the p75NTR receptor (reviewed in Reichardt 2006).

Upon BDNF binding, the TrkB receptor dimerizes leading to autophosphorylation of the cytoplasmic tyrosine residues. This in turn leads to recruitment of adaptor protein such as Shc, Grb2, and phospholipase C (PLC), which then can activate different signaling pathways. These include the MAP kinase pathway, the PI3-K/Akt pathway as well as the inositol triphosphate dependent Ca^{2+} release and diacylglycerol depended PKC activation. BDNF levels have been shown to be significantly reduced in the brains of Alzheimer's patients (Connor et al. 1997) and an increased APP synthesis and sAPPα secretion has been observed in response to BDNF (Holback et al. 2005).

1.4.3 Insulin-like growth factor-1

Insulin-like growth factor-1 (IGF-1) is a hormone that regulates cell survival and differentiation. IGF-1 mainly signals through the IGF-1 receptor (IGF-1R), which is part of the receptor tyrosine kinase family. However, since IGF-1 is structurally related to insulin, it can also bind to the insulin receptor; however with 100-fold lower affinity than to IGF-1R. Similar to BDNF activation of TrkB, IGF-1 binding to IGF-1R leads to activation of MAPK, PI3-K and PLC signaling pathways. In contrast to the TrkB receptor, IGF-1R does not undergo dimerization in response to ligand binding, but instead undergoes a conformational change leading to autophosphorylation.

Like RA and BDNF, IGF-1 has been shown to induce a shift in the processing of APP, increasing the levels of sAPPα (Adlerz et al. 2007). In addition, impaired insulin and IGF-1 signaling has been suggested to be a part of AD pathology, since AD patients have been shown to have lower insulin and IGF-1 levels in the cerebrospinal fluid (CSF) and higher plasma insulin levels than non-AD individuals (Steen et al. 2005). Furthermore, increased serum IGF-1 levels have even been demonstrated to decrease Aβ levels in the brain of aging rats (Carro et al. 2002), and in AD transgenic mice (Carro et al. 2006).

2. Methodological considerations

2.1 Cell lines

In both papers, the human neuroblastoma SH-SY5Y cell line was used as a model system to study APP family processing and ADAM10 and TACE levels. SH-SY5Y is a third generation clone from SK-N-SH, which was isolated from a metastatic tumor in the bone marrow of a four year old girl in 1971 (Biedler et al. 1978). SH-SY5Y cells can be differentiated into a more neuron-like phenotype by treatment with RA or IGF-1(Pahlman et al. 1984; Påhlman et al. 1991). During RA-differentiation, SH-SY5Y cells express TrkB receptors (Encinas et al. 1999), which then can be activated by BDNF. SH-SY5Y cells have been shown to endogenoulsy express all members of the APP protein family (Beckman and Iverfeldt 1997; Adlerz et al. 2003).

2.2 Cell treatments

Throughout these studies, SH-SY5Y cells were treated with 10 or 1 μM RA in serum containing or serum free medium, respectively for 6 days. In addition to RA treatment, the cells are also treated with RA concomitantly with 50 ng/ml BDNF or with 10 nM IGF-1 alone for 6 days. In both papers, the effect of various kinases was investigated by co-treating the cells with pharmacological inhibitors for the last 18 h of the treatment. To inhibit PKC we used Bisindolylmaleimide XI (BIM11), which like staurosporine is cell-permeable and blocks PKC activity by competitively inhibiting the binding of ATP by the kinase. BIM11 is more selective for PKC over other kinases than stauropsorine. BIM11 inhibits all PKC isoforms, but with varying potency with a 3- to 10-fold preference for the PKCα isoform. LY29002 (LY) is a reversible, cell-permeable inhibitor of PI3-K that acts through competitive binding to the ATP-binding site of the catalytic subunit of the kinase. At low concentrations it does not affect the activity of MAPK, PKC or PI4-K. PD098059 (PD) was used to inhibit MEK1. It interacts with the dephopshorylated form of MEK1, thereby preventing it from activation. PD also works as a weak inhibitor of MEK2.

2.3 siRNA gene silencing

In paper II we wanted to investigate the effect of TACE on APP and APLP2 processing, and since no specific pharmacological inhibitor was available on the market at the time, we chose to knock-down the expression of TACE with short interfering RNA (siRNA). siRNA, like the name suggests, are small RNA sequences which interfere with the expression of a specific gene. siRNA incorporates into the RNA-induced silencing complex (RISC), which cleaves the target mRNA strand complementary to the bound siRNA, leading to post-transcriptional gene silencing. Two strategies were applied to knock-down the expression of TACE. SH-SY5Y cells were transfected using Lipofectamine or Fugene with either 100 nM of a mix of 4 siRNA sequences directed against TACE, or with 5-50 nM of one single sequence (two different sequences were used). The use of different sequences makes it possible to determine the specificity of the knock-down, and to exclude off-target effects. In addition, a negative control composed of non-targeting siRNA was used.

2.4 BCA assay

The protein content in the cell lysate and cell medium was determined using bicinchoninic acid (BCA) assay. The principle of the BCA assay is similar to the Lowry procedure. However, in the BCA assay the mechanism can be divided into two reactions. First, cysteines, tryptophans, tyrosines, and the peptide bond will reduce Cu^{2+} from the cupric sulfate present in the BCA stock solution, to Cu^{1+}. Then, Cu^{1+} will form a purple-blue complex, by chelating with two BCA molecules. This complex has a strong absorbance at 562 nm. The amount of protein present in a solution can be quantified by measuring the absorption spectra and comparing with protein solutions, e.g. bovine serum albumin (BSA) with known concentrations. The BCA assay is more sensitive than Bradford and Lowry, and is also less susceptible to detergents.

2.5 ^{32}P-labeling and immunoprecipitation

In paper II we wanted to study if TACE was phosphorylated in response to IGF-1. A common method to study phosphorylation of proteins is ^{32}P-labeling. Cells are incubated with ^{32}P-orthophosphate and during this incubation the ^{32}P will be taken up by the cells and incorporated into ATP. In this study, we labeled phosphoproteins in cells in the presence or absence of a protein kinase C inhibitor. After the labeling, the cells were treated with IGF-1 before harvesting. To isolate TACE from the cell lysate we performed

immunoprecipitation (IP) with an antibody directed against the C-terminal of TACE. IP is based on specific binding of an antibody against the target protein and immobilization on sepharose or agarose beads. The complex consisting of sepharose/agarose beads, antibody and target protein can then be precipitated by centrifugation. The immunoprecipited TACE sample was resolved by SDS-PAGE and the levels of phosphorylated TACE was the visualized by exposure to a phosphoimager screen.

2.6 Western Blot

Western blot is a frequently used method to analyze the expression levels of a specific protein. The protein sample is first boiled together with sodium dodecyl sulphate (SDS), which denaturates the proteins and gives them a negative net charge in proportion to their size. This difference in charge can then be used to separate proteins according to size on a polyacrylamide gel that is run in an electric field. The separated proteins in the gel are then transferred to a membrane with the help of an electric current. The protein of interest can now be probed for by using a specific antibody directed against the target protein. A secondary antibody, coupled to horseradish peroxidase (HRP) that binds to the primary antibody is added in order to visualize the protein by enhanced chemiluminescence (ECL). When adding a solution containing hydrogen peroxide (H_2O_2) and luminol, HRP will catalyze the oxidation of luminol by H_2O_2, generating acridium ester intermediates, which in turn will react with peroxide and generate an excited state that emits fluorescent light as it decays to a lower energy level. This signal is captured on a film. The relative abundance of the protein is quantified by densiometric analysis.

2.7 ELISA

Enzyme-linked immunosorbant assay (ELISA) was carried out in paper II to analyze the levels of secreted $A\beta_{40}$, since this is a more sensitive method compared to Western blot. The method, like Western blot is based on the binding of an antigen (in this case $A\beta_{40}$) to a specific antibody. We used sandwich ELISA, in which a capture antibody directed against the N-terminal of $A\beta_{40}$ is immobilized on the bottom surface of the wells in a 96 well plate. The cell medium sample was then added to the wells, resulting in $A\beta_{40}$ binding to the antibody. Without the first layer of capture antibody, any proteins in the sample may competitively adsorb to the plate surface. A detection antibody directed against $A\beta_{40}$ and a secondary, HRP-linked antibody was used before colorimetric detection. For colorimetric detection, the antibody-antigen-antibody-antibody/HRP complex (or sandwich) is

incubated with a chromogen, in this case tetramethylbenzidine (TMB). TMB is oxidized during the enzymatic degradation of H_2O_2 by HRP. The oxidized product of TMB has a deep blue color, but after addition of an acidic stop solution, a clear yellow color is formed. The optical density of the yellow color is measured at 450 nm, and the intensity reflects the amount of $A\beta_{40}$. In order to translate intensity to concentration, standard samples containing known concentrations of $A\beta_{40}$ are run alongside the cell medium samples.

3. Aim of the study

AD is an irreversible, progressive neurodegenerative disease. A potential therapeutic strategy is to early on in the disease increase the α-secretase processing of APP, thereby decreasing Aβ generation and subsequent plaque formation. Since the APP family members have essential and overlapping functions, such therapy should aim to only affect APP processing. The aim of this thesis was to investigate the components and mechanism involved in α-secretase processing of the APP family.

The specific aims of the thesis were:

- To investigate the mechanism behind RA-, and IGF-1-induced α-secretase processing of the APP family (Paper I-II).
- To study the effects on expression levels of ADAM10 and TACE during RA-induced differentiation (Paper I).
- To investigate the difference in mechanism behind IGF-1-induced processing of APP and APLP2 (Paper II).

4. Results and discussion

4.1 RA-induced expression of ADAM10 and TACE (Paper I)

Retinoic acid induces differentiation of human neuroblastoma SH-SY5Y cells (Pahlman et al. 1984). Previous studies in this lab have demonstrated that all members of the APP family are up-regulated in SH-SY5Y cells during RA-induced differentiation (Beckman and Iverfeldt 1997; Adlerz et al. 2003). In addition, the processing of all APP members was increased, and a shift towards the α-secretase pathway was observed (Holback et al. 2005). Here, we wanted to study if the RA-induced effect on APP family processing was a result of increased expression of the main putative α-secretases, ADAM10 and TACE, or a decreased expression of the β-secretase, BACE. We found that the protein levels of all three enzymes were up-regulated in response to RA. However, the increase of BACE levels, although significant, was minor compared to the increase of ADAM10 and TACE levels. BDNF has previously been shown to further increase the α-secretase processing of the APP and APLP2 in RA-differentiated SH-SY5Y cells (Holback et al. 2005). Based on this, we expected to see a similar effect on ADAM10 and TACE levels in response to BDNF as to RA. Surprisingly, BDNF was shown to counteract the effect of RA on ADAM10 and TACE. The effect of BDNF on APP family processing clearly involves other mechanism than enzyme up-regulation, such as post-translational modifications of ADAM10/TACE or the APP family members.

4.2 RA-induced effects on ADAM10 levels are dependent on PI3-K, whereas TACE levels are dependent on PKC (Paper I)

The mechanisms behind RA-induced differentiation still remain rather unclear. Classically, RA activates RA receptors which translocate to the nucleus and activate gene transcription. However, different signaling pathways have been shown to be involved in RA-signaling (Lee and Kim 2004) (Pan et al. 2005). To investigate the involvement of different signaling pathways in RA-induced up-regulation of ADAM10 and TACE, we used

various kinase inhibitors. We found that the RA-induced expression of the three enzymes is regulated by different signaling pathways. The RA-induced increase of ADAM10 levels is dependent on PI3-K activation, whereas RA-induced increase of TACE levels is independent of this signaling pathway. On the contrary, RA-induce increase of TACE levels was shown to be dependent of PKC, whereas ADAM10 levels were unaffected by PKC inhibition. Interestingly, RA-induced up-regulation of ADAM10 was further increased upon inhibition of MAPK. We speculate that activation of MAPK counteracts the effect of RA on ADAM10. RT-PCR analysis showed that RA also up-regulates the ADAM10 mRNA levels. However, no effect of the PI3-K inhibitor on the mRNA levels could be detected. This indicates that the effect of PI3-K on RA-induced ADAM10 levels are posttranscriptional.

Previous studies have shown that the ADAM10 gene contains two potential RAREs in its promoter region (Prinzen et al. 2005), and the ADAM10 promoter activity has been shown to increase in response to RA in a RAR-dependent manner (Tippmann et al. 2009). RA-induced promoter activity of TACE has not been studied. It is also possible that RA-induced up-regulation of ADAM10 and TACE is a secondary effect of RA-induced APP family processing. The mechanism behind this would include non-genomic protein kinase activation by RA, leading to post-translational modifications of either ADAM10/TACE or the APP proteins, resulting in increased processing. The RA-induced generation of AICD/ALIDs could then be responsible for the up-regulation of ADAM10/TACE by inducing gene transcription. This kind of feed-forward mechanism is of course purely speculative.

4.3 RA- and IGF-1-induced secretion of sAPLP2 is dependent on PKC (Paper II)

Since the RA-induced effect on ADAM10 and TACE was shown to be dependent on different kinases, we wanted so investigate if this was true also for the RA-induced processing of APP and APLP2. Indeed, it was found that PI3-K activation is required for RA-induced APP processing. However, RA-induced processing of APLP2 was unaffected by PI3-K inhibition. On the contrary, RA-induced processing of APLP2 was found to be dependent on PKC, whereas APP processing was not. In addition to RA, IGF-1 has also been shown to induce a shift in the processing of all APP family members, but without affecting their expression (Adlerz et al. 2007). It was shown that the IGF-1-induced processing of the different members of the APP family, were dependent on different signaling pathways. IGF-1-induced secretion of sAPPα was completely dependent on PI3-K activation and APLP1 shedding

involved both PI3-K and MAPK. IGF-1-induced processing of APLP2 was independent of these signaling pathways. Here, we demonstrate that IGF-1-induced secretion of sAPLP2 is completely dependent on PKC. IGF-1-induced processing of APP and APLP1 was also affected by PKC inhibition. The results from the present study together with our previous studies (Adlerz et al. 2007) suggest that IGF-1 stimulation activates a PKC-dependent pathway that is independent of both PI3-K and MAPK, possibly through receptor tyrosine kinase activation of PLCγ (cf., Hong et al. 2001). We also observed that the IGF-1-induced processing of APP was dependent on PKC, but not to the same extent as for APLP2. Since PKC inhibition only partially blocked the IGF-1-induced secretion of sAPPα, one plausible explanation could be that another PKC isoform, that acts downstream of PI3-K is involved.

4.4 ADAM10 activity is required for IGF-1-induced secretion of sAPPα (Paper II)

Our results show that APLP2 processing is dependent on PKC, whereas APP processing mainly is dependent on PI3-K. Combined with the results from paper I, which demonstrated that ADAM10 levels are regulated by a PI3-K-dependent mechanism and TACE levels by PKC, we speculated that APLP2 is cleaved by TACE, and APP by ADAM10 during induced conditions. This theory was supported by the use of a specific ADAM10 inhibitor, which abolished the IGF-1-induced processing of APP. On the contrary, the ADAM10 inhibitor did not have any significant effect on the IGF-1-induced secretion of sAPLP2. To further investigate the effect of ADAM10 inhibiton on APP processing, the $A\beta_{40}$ levels were analyzed. ELISA measurements of conditioned cell medium demonstrated that IGF-1 treatment induced an approx. 40% decrease of secreted $A\beta_{40}$. This effect was completely reversed by co-treatment with the ADAM10 inhibitor.

4.5 TACE activity is required for IGF-1-induced secretion of sAPLP2 (Paper II)

In paper II we used siRNA directed against TACE to silence the expression of the enzyme. The siRNA used succesfully down-regulated the expression levels of TACE by approx. 60%. Even though the down-regulation was not complete, TACE down-regulation completely blocked the IGF-1-induced secretion of sAPLP2. The reduced levels of TACE had no significant effect on the IGF-1-induced processing of APP.

Since both ADAM10 and TACE obvioulsy are activated during these conditions, and they have both been shown to be able to cleave both APP (Buxbaum et al. 1998; Lammich et al. 1999) and APLP2 (Endres et al. 2005), it is reasonable to ask the question "why are APP and APLP2 cleaved by ADAM10 and TACE, selectively in response to IGF-1?". One can speculate that the answer lies within the subcellular localization and that upon IGF-1 treatment TACE colocalizes with APLP2 and ADAM10 with APP. This is supported by a study that demonstrated that TACE colocalized to the plasma membrane with one of its other substrates, CD44, within 10 min after phorbol ester stimulation (Nagano et al. 2004). The localization of ADAM10 was unfortunately not investigated in this study. If localization is important for substrate selectivity, the previous mentioned studies on APP/APLP2 processing by ADAM10/TACE may not be of physiological importance, since overexpression may cause these proteins to localize to compartments in which they normally are not found. Furthermore, as discussed in the introduction (section 1.3), the substrate selectivity of ADAM10 and TACE seems to be altered in response to chronic silencing or overexpression (Le Gall et al. 2009). In our study, we have used a physiological stimulus and analyzed the effects on processing during endogenous conditions. We propose that although APP can be cleaved by TACE, ADAM10 is the main secretase cleaving APP in response to a stimulus like IGF-1 which activates both ADAM10 and TACE.

4.6 TACE phosphorylation is increased in response to IGF-1 (Paper II)

To further investigate the mechanism behind the IGF-1-induced activation of TACE, we studied its phosphorylation status. Previous studies have shown that TACE is phosphorylated in response to several physiological stimuli, including NGF, EGF (Diaz-Rodriguez et al. 2002), high glucose (Reddy et al. 2009), and carbachol (Cissé et al. 2007), as well as in response to phorbol ester (Diaz-Rodriguez et al. 2002). We demonstrate through ^{32}P-labelling and immunoprecipitation that IGF-1 induces phosphorylation of TACE in a PKC-dependent way. Cissé and colleagues demonstrated that mutation of the Thr735 site in the cytoplasmic domain of TACE abolished the carbachol-induced processing of the cellular prion protein (Cissé et al. 2007), suggesting a direct correlation between phosphorylation and activation of TACE. The question "how does phosphorylation of TACE induce its processing activity?" still remains to be answered. Based on a previous study by Soond, which demonstrated that phosphorylation of TACE induced translocation to the cell surface, it can be speculated that IGF-1-induced phosphorylation of TACE leads to co-localization with APLP2. It can be

further speculated that the phosphorylation of TACE affects its interactions with other proteins. This could for example be interaction with the small GTPase Rac, which has been shown to be responsible for phorbol ester-induced translocation of TACE to the cell-surface (Nagano et al. 2004), or the interaction with the inhibitor TIMP-3, leading to reduced TACE inhibition. This of course is purely speculative and more research is needed to investigate this mechanism. However, our study clearly demonstrate that phosphorylation of TACE is an important regulatory mechanism involved in the induced processing of APLP2.

5. Conclusions

This thesis has provided insight to the regulation of APP family processing by the α-secretases ADAM10 and TACE. Based on the results in this thesis we conclude that although APP and APLP2 proteolytic processing seems to be induced by the same stimuli, the processing relies on different signaling pathways and processing enzymes, which in turn are differently regulated.

The major findings of this thesis are listed below:

- The α-secretase pathway stimulator RA induces up-regulation of ADAM10 and TACE levels.
- RA-induced up-regulation of ADAM10 is PI3-K dependent, whereas the RA effects on TACE levels are dependent on PKC.
- The BDNF-induced α-secretase processing of APP and APLP2 are not a result of increased ADAM10 or TACE levels.
- IGF-1-induced processing of APLP2, but not of APP, is dependent on PKC.
- TACE phosphorylation is increased in a PKC-dependent manner in response to IGF-1.
- ADAM10 is the main α-secretase for APP, whereas TACE cleaves APLP2 upon IGF-1 stimulation.

6. Acknowledgements

Känns som det inte alls var länge sen jag började doktorera här på Neurokemi, men redan har nästan halva tiden förflutit! Om jag skulle försöka summera min doktorandtid hittils, så skulle den nog bäst beskrivas som en berg-och-dalbana. När jag först började rullade allt på och jag tänkte att det nog inte fanns något bättre än att få forska. Sen stötte man ju på problem efter problem, och helt plötsligt kändes det lite kämpigare. Trots att det ibland känns jobbigt så är jag i grunden väldigt glad att jag har chansen att få jobba så pass fritt med projekt som jag såklart tycker är otroligt intressanta. Därför vill jag först och främst tacka Kerstin, min handledare och stora stöd både när saker går bra och dåligt på lab. Tack för att du alltid finns där, för alla våra diskussioner och för att du delar med dig din kunskap, nyfikenhet och glädje för forskningen!

Sen vill jag tacka alla i gruppen. Jag är otroligt glad att jag hamnat i en sån rolig grupp där alla bjuder på sig själva och alltid ställer upp med en hjälpande hand om det behövs. Veronica, det har varit jätte kul att ha rekombinant kursen tillsammans med dig, du är en jätte rolig tjej som alltid tänker ett steg längre än alla andra. Tom, jag är så glad att du är den du är; glad, frågvis, sprallig och nyfiken, skulle bli tråkigt utan dig! Linda, tack för allt ditt stöd på sistonde det har betytt jätte mycket för mig, snart är det din tur och det kommer gå hur bra som helst. Veronica, det nyaste tillskottet i gruppen, det var väldigt imponerande att du tog hand om lab 2 på Rek kursen bara nån vecka efter du börjat. Jag vill även tacka Sofia, som ju tyvärr inte längre är kvar i gruppen, men som betytt väldigt mycket för mig. Tack för alla samtal på kontoret och hjälp på labbet, och lycka till med allt i framtiden. En annan före detta gruppmedlem som jag vill tacka är Linda Adlerz, som handledde mig på mitt examensarbete.

Tack även till alla doktorander på institutionen, utan er hade allt känts mycket jobbigare och tråkigare. Ett speciellt tack till Nina, som nästan är i vår grupp. Du är en inspirationskälla, ge aldrig upp, du är så himla duktig. Marie, jag är jätte glad att vi delar kontor, du en rolig, glad tjej som lyckas hålla sig lugn genom det mesta. Tack till alla lärare på institutionen, Anders, Anna, Anna-Lena och Ülo, er kunskap och drivkraft är inspirerande! Stort tack till Siv, Ulla, Marie-Louise och Birgitta som håller koll och tar hand om

oss alla! Tack även till mina examensarbetare, Jenny (tack även för din vänskap) och Jelena (hoppas du kommer tillbaka till oss).

Tack även till Monique, Linda, Katti, Jenny, Märta och Camilla. Er vänskap är otroligt viktig för mig och jag är så himla glad att jag har er! Tack alla klätterkompisarna, speciellt Anders, Anton, Lollo och Erik. Sist, men absolut inte minst, stort tack till min familj, ni betyder allt för mig! Tack för allt stöd genom alla år.

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