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Identification of the physiologically relevant α-secretase of the Alzheimer's disease-related amyloid precursor protein and its relationship with β-secretase

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Huanhuan Wang

aus Hangzhou, China

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Betreuer: Priv. Doz. Dr. rer. nat. Stefan Lichtenthaler

Zweitgutachterin bzw. Zweitgutachter: Prof. Dr. rer. nat. Jörg Tatzelt

Dekan: Prof. Dr. med. Dr. h. c. Maximilian Reiser, FACR, FRCR

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Declaration

I hereby declare that the thesis is my original work and I have not received outside assistance. All the work and results presented in the thesis were performed independently. Anything from the literature was cited and listed in the reference. Part of the results have been published in the paper Kuhn P. H.*, Wang H.*, Dislich B., Colombo A., Zeitschel U., Ellwart J. W., Kremmer E., Roßner S., Lichtenthaler S. F. (2010) ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. EMBO J. 29(17): 3020-32 (* Contributed equally to the paper). No unauthorized data was included.

All the data presented in the thesis will not be used in any other thesis for scientific degree application.

The work for the thesis began from Oct. 2008 with the supervision from PD Dr. Stefan Lichtenthaler in Deutsches Zentrum für Neurodegenerative Erkrankungen (DZNE), Munich and the Adolf-Butenandt Institute, Ludwig-Maximilians University Munich, Germany.

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(Huanhuan Wang)

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Abstract

Alzheimer's disease (AD) represents the most prevalent form of dementia and is characterized by the cerebral deposition of the neurotoxic amyloid beta (Aβ) peptide. Aβ is produced upon sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretase. In addition, APP is constitutively shed by another protease activity referred to α-secretase. This is considered to be an important mechanism preventing the generation of Aβ, as cleavage of APP by α-secretase occurs within the Aβ domain. The protease activities of α- and β-secretase are thought to be inversely coupled, as they are assumed to compete for the same substrate APP. The molecular identity of α-secretase is unknown, but appears to be a member of a disintegrin and metalloprotease (ADAM) family of proteases. The aim of this thesis was to study the processing of APP by α-secretase in more detail. Therefore, a novel α-secretase-cleavage site-specific antibody was developed. RNAi-mediated knockdown of putative proteases with α-secretase activity showed that only the loss of function of ADAM10, but not of ADAM9, 17 or the matrix metalloproteinase 14 (MMP14), completely suppressed APP α-secretase cleavage in immortalized cell lines and in neuronal like cells. ADAM10 was essential for the constitutive α-cleavage of APP, whereas other proteases were unable to compensate for the loss of α-cleavage activity. Surprisingly, upon the genetically or pharmacologically induced loss of function of either ADAM10 or the β-secretase BACE1, almost no compensatory relationship between these two protease activities towards APP cleavage was observed. Instead, α- and β-secretase contributed to the proteolytic processing of APP in a stable ratio. However, ADAM10 could partially compete with γ-secretase for the cleavage of a C-terminal APP fragment generated by β-secretase. In conclusion, it was shown that ADAM10 is the only physiologically relevant, constitutive α-secretase of APP.

Zusammenfassung

Die Alzheimer Krankheit, die häufigste Form von Demenz, wird durch eine zerebrale Ablagerung von beta Amyloid (Aβ) Peptiden gekennzeichnet. Aβ entsteht bei der Spaltung des Amyloid-Vorläufer-Proteins (Amyloid precursor protein, APP) durch die beiden Proteasen β- und γ-Sekretase. In einem anderen Proteolyseprozess wird APP durch die α-Secretase konstitutiv gespalten. Dieser Prozess ist ein wichtiger Mechanismus, der die Bildung der Aβ-Peptide verhindert, indem die α-Secretase innerhalb der Aβ-Domäne spaltet; es wird angenommen, dass in der Regel arbeiten α-and β-Secretase umgekehrt gekoppelt arbeite. Die α-Sekretase scheint eine Metalloprotease der ADAM (A disintegrin and metalloproteinase)-Familie zu sein, wobei diese Identität noch etabliert werden muss. In dieser Arbeit wurde ein spezifischer Antikörper zur Untersuchung der α-Sekretase-Spaltung entwickelt. Das RNAi-basierte Ausschalten verschiedener Proteasen in Zellen zeigte, dass nur der Funktionsverlust von ADAM10, aber nicht von ADAM9, 17 oder MMP14 (Matrix metalloprotease 14) die α-Sekretase Spaltung von APP in immortalisierten Zell-Linien komplett unterdrückte. ADAM10 war für die konstitutive α-Spaltung von APP essentiell, während andere Proteasen den Verlust an α-Spaltung nicht kompensieren konnten. Überraschenderweise zeigte der Funktionsverlust von ADAM10 und β-Sekretase BACE1 untereinander fast gar keine kompensierende Beziehungen bezüglich der APP α-Spaltung, weder genetisch noch pharmakologisch in immortalisierten Zellen; α- und β-Sekretase trugen in einem stabilen Verhältnis zum APP proteolytischen Prozess bei. Dagegen konnte ADAM10 teilweise mit der γ-Sekretase um die Spaltung eines C-terminalen APP Fragments konkurrieren, das durch die β-Sekretase erzeugt wurde. Abschließend lässt sich sagen, dass ADAM10 die einzige physiologisch gesehen relevante konstitutive α-Sekretase von APP ist.

1 Introduction

1.1 Alzheimer's disease (AD)

The research on Alzheimer's disease (AD), which is named after the famous German psychiatrist and neuropathologist Alois Alzheimer, has been carried out since 1906 (Alzheimer, 1907; Stelzmann *et al.*, 1995). In the following 100 years, interdisciplinary studies have been unlocking the secrets of biochemical aspects the disease. AD-related amyloid beta (Aβ) peptide was purified (Glenner & Wong, 1984); the amyloid precursor protein (APP) was decoded (Kang *et al.*, 1987); and the disease-related genetic mutations were revealed (Goate *et al.*, 1991; Sherrington *et al.*, 1995). The following table lists the most important milestones in the AD research (Table 1). The fascinating achievements provide possible direction of AD and related molecular biochemical research.

Table 1 A historical perspective of AD research.

Years	Events	References
1906	Clinical phenotype definition	Alzheimer, 1907; Stelzmann *et al.*, 1995
1984	Aβ purified from amyloid plaques and sequenced	Glenner & Wong, 1984
1986	Phosphorylated tau protein found in neurofibrillary tangles	Grundke-Iqbal *et al.*, 1986
1986	APP sequence decoded	Kang *et al.*, 1987
1991	The first APP mutation related to familial AD decoded	Goate *et al.*, 1991
1995	PS mutations related to familial AD decoded	Sherrington *et al.*, 1995
1999	BACE1 identified as β-secretase of APP	Vassar *et al.*, 1999

Due to the dramatic rise in life expectancy during the 20th century, neurodegenerative disorders have become more threatening to the elder population. AD is becoming the most prevalent adult-onset dementia form nowadays (reviewed by Selkoe, 2001; reviewed by Karran *et al.*, 2011). Although there are two types of AD described - the familial AD and the non-familial sporadic AD - they are phenotypically highly similar (reviewed by Selkoe, 2001). The phenotypic similarity makes it reasonable to assume that they also share similar molecular pathology and mechanism. The greatest risk factor for sporadic AD is age. With the predicted demographic increase of elderly populations, it is estimated that well over 100 million patients will develop AD by 2050 (reviewed by Karran *et al.*, 2011).

1.1.1 Clinical symptoms of AD

AD patients undergo progressive decline in various aspects of behavior, including memory impairment, disordered cognitive function, altered psychological performance, loss of social appropriateness and decline in language ability (reviewed by Selkoe, 2001; reviewed by Sisodia & St George-Hyslop, 2002). The clinical symptoms develop in various stages according to the severity of dementia (Table 2). In the first stage, the mild or early dementia stage, patients only show short-term memory impairment. They do not remember a conversation or activity and have difficulties to keep new information. After the first couple of years, most of them develop additional problems with cognitive functions, including spatial orientation and language disability. Patients may start to have growing problems to easily and correctly execute complex tasks. The personalities and emotions of patients would also receive a remarkable change, like losing interest to their surroundings and being often aggressive. Over several years or even a decade or more, patients are completely mentally disoriented with full disorientation, profound memory impairment and global cognitive deficits (reviewed by Selkoe & Schenk, 2003).

Table 2 Clinical symptoms of AD development.

Step 1	Step 2	Step 3
Amnestic stage	Progressive memory and motor decline	Marked dementia
• mild or early dementia • short-term memory impairment • difficulties to keep new information	• problems with orientation and language • incorrect execution of complex tasks • changes in personality and emotion	• completely mentally disoriented

1.1.2 Pathological features of AD

The molecular pathological description of AD began with the identification of a marked decrease in choline acetyltransferase (ChAT) in the cerebral cortex (reviewed by Selkoe & Schenck, 2003) and later characterized by abnormal protein deposition. Starting from 1980s and during the decade following, the two brain lesions were recognized as diagnostic criteria of AD: the amyloid (senile) plaques and the neurofibrillary tangles (Figure 1) (reviewed by Selkoe, 1998; reviewed by Sisodia & St George-Hyslop, 2002; reviewed by Serrano-Pozo *et al.*, 2011).

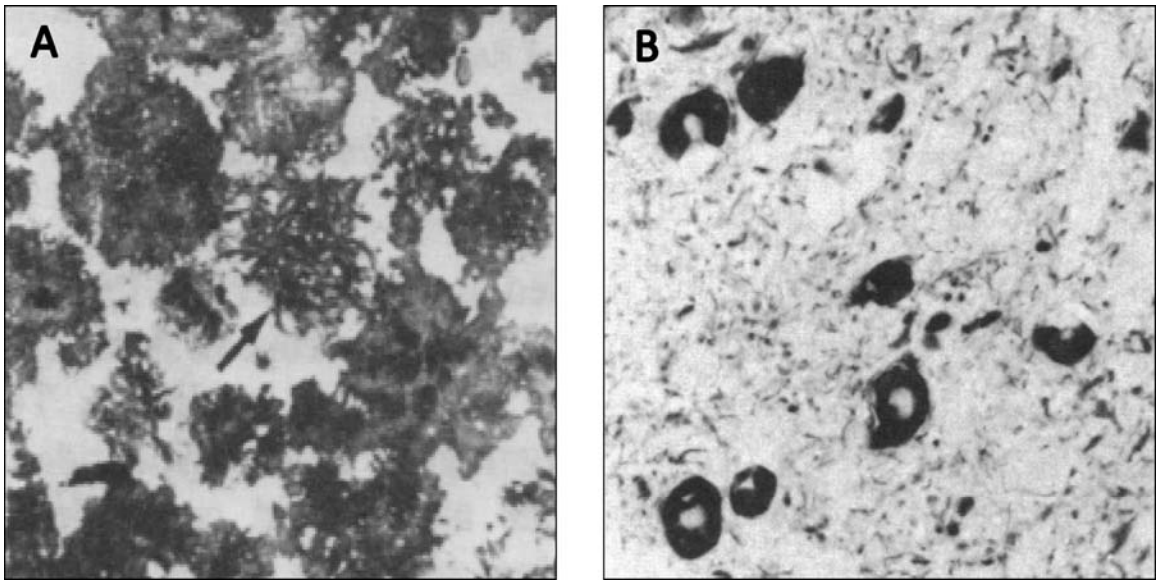

Figure 1 Morphological hallmarks of AD pathology.
A) Amyloid deposits: plastic-embedded sections showing a mixture of spherical cores and an amorphous deposit (figure from Masters *et al.*, 1985).
B) Neurofibrillary tangles: immunochemical staining section of AD hippocampus with protein tau antibody (figure from Grundke-Iqbal *et al.*, 1986).

Amyloid plaques are deposits of aggregated amyloid beta (Aβ) peptides (Glenner & Wong, 1984; Masters *et al.*, 1985), which are found almost exclusively in the extracellular space of the brain and the cerebral vascular system (reviewed by Selkoe, 1999). In amyloid plaques, fibrillar Aβ peptides are found mainly as a 42-amino acid-long form (Aβ42) (Iwatsubo *et al.*, 1994). This is the more hydrophobic form that aggregates particularly easily and quickly (Jarrett *et al.*, 1993; reviewed by Harper & Lansbury, 1997; Walsh *et al.*, 1997). Together with Aβ42, a low proportion of a 40-amino acid-long form (Aβ40) is found in the plaque, although it is normally more abundantly produced by cells (Asami-Odaka *et al.*, 1995; reviewed by Selkoe, 1999). Amyloid plaques have a dense amyloid core, infiltrated by the dystrophic axons and dendrites and colocalized with activated microglia and reactive astrocytes (Pike *et al.*, 1994). It indicates a potential inflammatory component in the process. This form of plaques is specific for AD. An alternative form of amyloid plaques is called diffuse plaques or pre-amyloid deposits. The diffuse plaques contain only Aβ42 (Iwatsubo *et al.*, 1994; Lemere *et al.*, 1996; Lambert *et al.*, 1998) and are not surrounded by activated glial cells and neurons (Itagaki *et al.*, 1989). These plaques are considered the immature form

of amyloid plaques. The diffuse plaques in the brain, however, can also be found in non-demented elderly people (Tagliavini *et al.*, 1988; Dickson *et al.*, 1995).

Neurofibrillary tangles are the second neuropathological hallmark of AD. They are intracellular lesions and consist mainly of a microtubule-associated abnormally phosphorylated form of the tau protein (Grundke-Iqbal *et al.*, 1986), whose physiological function is to stabilize the axonal microtubule-associated cytoskeleton (reviewed by Mandelkow & Mandelkow, 1998; reviewed by Friedhoff *et al.*, 2000). Under pathological conditions the protein is hyperphosphorylated, leading to a destabilization of microtubules and consequently to reduced mass transport and impaired impulse in neurons, which may be caused by an imbalance of various kinases and phosphatases (Illenberger *et al.*, 1998; reviewed by Mandelkow & Mandelkow, 1998; reviewed by Selkoe, 2001).

However, the two hallmarks of AD can be observed only in postmortem brain and considered to indicate the late stage of the disease. Thus, they are unlikely to provide crucial information for early diagnosis or epidemiological research. But still they shed light on the molecular biochemical research on AD pathology and help to better understand the etiology and molecular mechanisms behind.

1.1.3 Amyloid cascade

After the observation of two neuropathological hallmarks of AD, the idea that they are the result of disease-associated biochemical steps became accepted. Some more research was carried out to reveal the complete biochemical metabolism process. The identification of the Aβ peptide as a component of amyloid plaques is a milestone in the study of AD (Glenner & Wong, 1984; Masters *et al.*, 1985). Later Kang *et al.* decoded the full length gene of precursor protein APP (Kang *et al.*, 1987), whose proteolytic processing leads to the Aβ peptide generation. At the beginning of 1990s, there was an enthusiasm towards AD biochemical and genetic studies. The search for genetic linkages provided clues that missense mutations in APP caused autosomal dominant, early-onset AD. These mutations occurred in or around the Aβ region or γ-secretase cleavage site of

APP (Chartier-Harlin *et al.*, 1991; Goate *et al.*, 1991; Murrell *et al.*, 1991) and increased the production of Aβ (Citron *et al.*, 1992; Cai *et al.*, 1993; Haass *et al.*, 1994; Suzuki *et al.*, 1994). Some more mutations were found in the genes encoding presenilins (PSs), the subunits of γ-secretase (Levy-Lahad *et al.*, 1995; Rogaev *et al.*, 1995; Sherrington *et al.*, 1995), an enzyme involved in Aβ production. These mutations increased the production of Aβ42 (Borchelt *et al.*, 1996; Duff *et al.*, 1996; Scheuner et *al.*, 1996; Citron *et al.*, 1997; reviewed by Hardy, 1997; Tomita *et al.*, 1997; Xia *et al.*, 1997; Holcomb *et al.*, 1998). Another strong genetic risk factor for late-onset AD is the ε4 allele of apolipoprotein E (APOE) gene (Strittmatter *et al.*, 1993). These findings, together with observations that Aβ readily forms neurotoxic, threadlike structures called fibrils *in vitro* or *in vivo* (Hilbich *et al.*, 1991; Pike *et al.*, 1993; Lorenzo & Yankner, 1994; LaFerla *et al.*, 1995), bolstered the view that the accumulation of Aβ is a common initiating event that ultimately leads to neuronal dysfunction and eventually clinical manifestation of the disease in both familial and sporadic AD. The so called "amyloid cascade hypothesis" (Figure 2), positing that the deposition of the Aβ in the brain is a central event in AD pathology, has dominated research for the past twenty years (reviewed by Karran *et al.*, 2011).

Although the direct relationship between the occurrence of amyloid plaques and neuronal degeneration in the central nervous system is so far not clear, the formation of plaques is thought to be the crucial primary insult in AD pathogenesis. However, evidence also assumed that the plaques were insufficient to cause neuronal death *in vivo*. The soluble Aβ oligomers may be responsible for neuronal dysfunction, while the plaques only form a reservoir for these species (Hartley *et al.*, 1999; reviewed by Hardy and Selkoe, 2002; reviewed by Kawasumi *et al.*, 2002; Walsh *et al.*, 2002; reviewed by Glabe, 2006; reviewed by Haass & Selkoe, 2007; review Walsh & Selkoe, 2007). At the beginning of the cascade, changes happen to Aβ metabolism, such as an increased Aβ formation, a reduced degradation of Aβ, or a shift in the Aβ balance in favor of Aβ42 and the formation of diffuse plaques, which can induce the toxic effect to neurons directly or indirectly. The next step is to convert diffuse Aβ plaques to Aβ deposit fibrillars. The conversion activates microglia and astrocytes, triggering a local inflammatory reaction

and thereby damaging the surrounding neurons. The aggregation of Aβ peptide leads directly to an increased damage of neurons and changes in the intracellular milieu of cells, resulting in changes in kinase and phosphatase activities. Neurofibrillary tangles occur under such condition, which enhance neuronal dysfunction and death, together with amyloid plaques. The wide-spread neuronal dysfunction is regarded as the immediate cause of the disease (reviewed by Hardy & Selkoe, 2002; reviewed by Sisodia & St George-Hyslop, 2002).

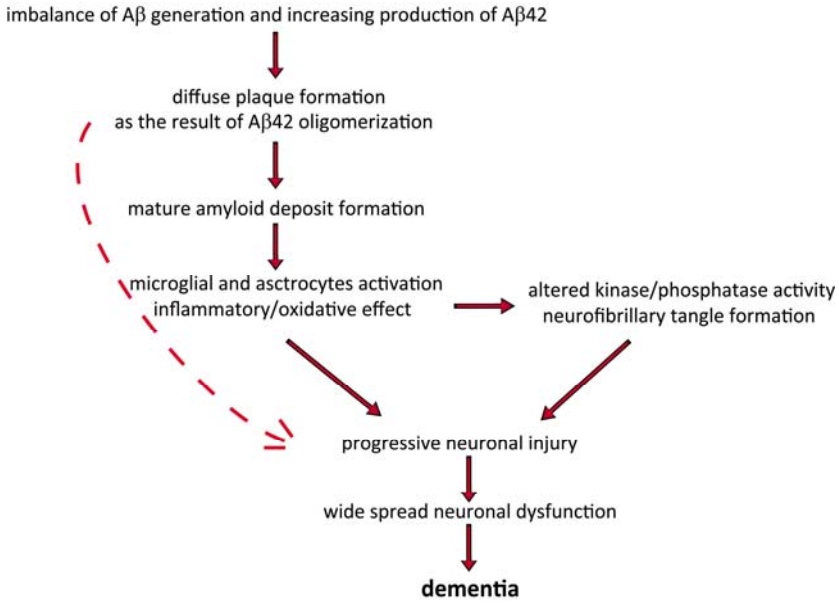

Figure 2 Amyloid cascade hypothesis.
A shift in the Aβ balance in favor of Aβ42 leads to the formation of diffuse amyloid plaques, which may have the indirect toxic effect on neurons. Diffuse Aβ deposition then develops to mature amyloid plaques, which activates microglia and astrocytes, triggering a local inflammatory reaction from which damages the surrounding neurons. It also leads to changes in kinase and phosphatase activities resulting in the formation of neurofibrillary tangles. The tangles together with the Aβ deposition progressively give stress to neurons and therefore lead to wide spread neuronal dysfunction, which is regarded as the immediate cause of the disease. (modified picture from Hardy & Selkoe, 2002)

1.1.4 Therapeutic approaches of AD

Current therapeutic approaches of AD towards symptomatic aspects include the acetylcholinesterase (AChE) inhibitors (galantamine and rivastigmine), psychotropic drugs modifying behavioral symptoms and potential neurotrophic strategies (reviewed by Selkoe & Schenck, 2003; reviewed by Karran *et al.*, 2011). These drugs improve some of the disease symptoms but do not treat the underlying mechanism, so the effects are limited.

Because Aβ is at the beginning of the amyloid cascade, considerable attention has been focused on attempts to develop therapies for AD towards metabolic pathways that involve Aβ (reviewed by Sisodia & St George-Hyslop, 2002). Such therapeutic approaches include: inhibition of Aβ monomers developing into toxic oligomers, or enhancement of clearance and disaggregation of fibrillar aggregates from cerebral cortex (Kisilevsky *et al.*, 1995; Soto *et al.*, 1998; Fraser *et al.*, 2001); modulation of the fate and toxicity of Aβ by using antibodies against Aβ (Schenk *et al.*, 1999; Bard *et al.*, 2000; Janus *et al.*, 2000; Morgan *et al.*, 2000) or anti-inflammatory strategy; inhibition of the production of Aβ, by inhibiting either β- or γ-secretase that generates Aβ from APP. β- and γ-secretase are two proteases that participate in APP cleavage resulting to Aβ generation (see details in 1.2.3 and 1.2.4). Although there have been already some highly potent inhibitors identified to block γ-secretase activity, they still have trouble in moving further on the clinical trial. That is because γ-secretase also plays an important role in shedding of many other proteins, like Notch receptor (reviewed by Annaert & De Strooper, 1999); the inhibition of γ-secretase may induce some unrespectable side effect. Thus, β-secretase inhibitor is considered a better therapeutic target, because the phenotype of BACE1 knockout mice was much milder than γ-secretase-deficit mice (Cai *et al.*, 2001; Luo *et al.*, 2001b).

1.2 Amyloid precursor protein (APP)

Aβ proteolytically derives from its precursor protein APP, which is ubiquitously present in all tissues (Tanzi *et al.*, 1987). The three major APP isoforms are APP695, APP751, and APP770. APP695 is the only isoform lacking a 57-residue domain homologous to the family of kunitz serine protease inhibitors (KPI) and is the isoform most highly expressed in neurons (Goedert, 1987; LeBlanc *et al.*, 1991).

1.2.1 Structure of APP

APP is a type I transmembrane protein with an amino-terminal signal peptide, a large N-terminal ectodomain, a transmembrane domain and a short cytoplasmic domain (Figure 3) (Kang *et al.*, 1987; Dyrks *et al.*, 1988). There are two APP homologues, namely amyloid-

precursor like protein 1 (APLP1) and amyloid-precursor like protein 2 (APLP2). They share the conserved structure and several identical motifs. The intracellular domain exhibits the highest sequence identity between APP, APLP1 and APLP2. However, Aβ sequence is unique to APP.

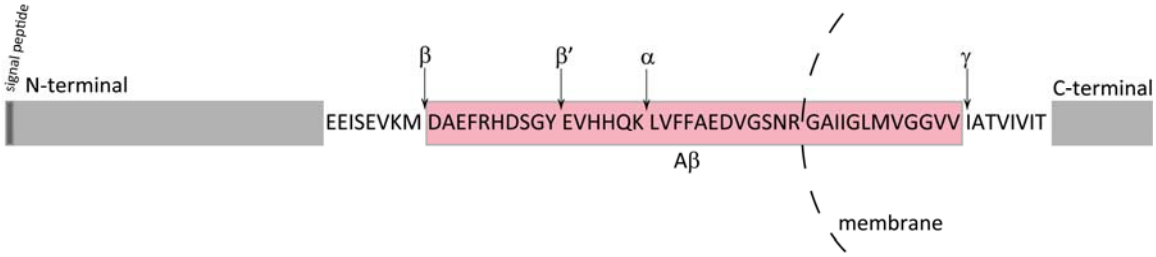

Figure 3 APP structure and Aβ sequence.
APP structure and the cleavage sites of α-, β- and γ-secretases. β- and γ-secretase cleavages generate the Aβ peptide, while α-secretase and an alternative β-cleavage site (β') occur inside the Aβ domain precluding Aβ production.

1.2.2 Physiological functions of APP

While the role of APP processing in AD is becoming clearer, physiological functions of APP are not completely elucidated (reviewed by De Strooper & Annaert, 2000). Because of the domain structure and the striking similarity of proteolytic processing of APP to the Notch receptor, a receptor function for APP has been postulated (Kang *et al.*, 1987; reviewed by Annaert & De Strooper, 1999; reviewed by Selkoe & Kopan, 2003). APP has also been suggested to have a role in synaptic plasticity, adhesion and cell migration via dimerization (Qiu *et al.*, 1995; Perez *et al.*, 1997; Soba *et al.*, 2005; Weyer *et al.*, 2011). Some other research by gene knockout animals suggested an essential function of APP and its homologues during early development, postnatal survival, compromised neuronal or muscular function (Zheng *et al.*, 1995; Heber *et al.*, 2000; Herms *et al.*, 2004; Wang *et al.*, 2005); and the secreted APP α-cleavage fragment (sAPPα) has been even reported to rescue APP knockout phenotype (Ring *et al.*, 2007; Weyer *et al.*, 2011), which raises its possible therapeutic importance. More interestingly, the ectodomain fragments of APP, especially sAPPα, were supposed to have the neuroprotective and synaptotrophic functions (Furukawa *et al.*, 1996; Meziane *et al.*, 1998; Stein *et al.*, 2004; Thornton *et al.*, 2006; Gralle *et al.*, 2009); while the secreted APP β-cleavage fragment (sAPPβ) was supposed to bind the death receptor 6 (DR6) to induce cell death (Nikolaev

et al., 2009); and the intracellular domain of APP was considered to be involved in phosphorylation and protein-protein interaction (reviewed by Zheng & Koo, 2011).

1.2.3 Proteolytic processing of APP

APP proteolysis has recently been recognized as a special case of regulated intramembrane proteolysis (RIP), a process that generates intra- and/or extracellular fragments from transmembrane proteins involved in signal transduction (reviewed by Edwards *et al.*, 2008; reviewed by Lichtenthaler *et al.*, 2011). APP can be processed by two different protease activities, called α- and β-secretase (Figure 4). β-secretase is the aspartyl protease BACE1 (β-site APP cleaving enzyme 1). It cleaves APP at N-terminus of the Aβ domain to release sAPPβ and the remaining C-terminal fragment (CTF) C99 which can be further cleaved by γ-secretase resulting in the formation of the Aβ peptide (Vassar *et al.*, 1999) (Figure 3, 4). This process is called the APP amyloidogenic proteolytic pathway. In the alternative non-amyloidogenic pathway, APP is cleaved by α-secretase within the Aβ domain, between the 16^{th} (Lysine, Lys) and 17^{th} (Leucine, Leu) amino acid of Aβ domain (Esch *et al.*, 1990; Anderson *et al.*, 1991; Wang *et al.*, 1991) (Figure 3, 4). This cleavage releases the large, soluble ectodomain sAPPα into the medium and allows the resultant 83-residue, membrane-retained CTF C83 to be cleaved by γ-secretase, generating the small p3 peptide (Haass *et al.*, 1992b). Another alternative cleavage performed by β-secretase takes place at the β'-cleavage site which is between α- and β-cleavage site (Figure 3).

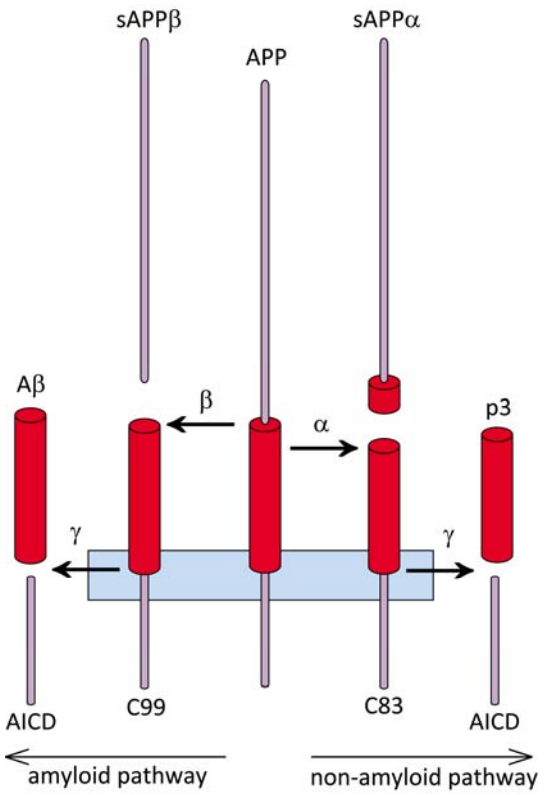

Figure 4 APP proteolytic processing.
APP can be cleaved by α-secretase releasing the sAPPα. The remaining fragment C83 can be further cleaved by γ-secretase to generate the small peptide p3. It can also be cleaved by β-secretase releasing sAPPβ. The remaining fragment C99 can be further cleaved by γ-secretase to generate the Aβ peptide.

1.2.4 Secretion and transportation of APP

The vast majority of secretory proteins share a common biosynthetic origin in the rough endoplasmic reticulum (ER), from where they are transported to the Golgi complex. The protein destined for secretion is then further processed through the Golgi apparatus and finally ends up in vesicles that fuse with the plasma membrane (reviewed by Halban & Irminger, 1994). APP also complies by such trafficking route. APP matures post-translationally while being transported through the secretory pathway, modified by *N*- and *O*-glycosylation and tyrosine-sulfation while moving through the trans-Golgi network (TGN) to the cell surface (Weidemann *et al.*, 1989; Oltersdorf *et al.*, 1990). Immature APP (being *N*-glycosylated only) can be proteolytically processed in the ER, in the TGN, at cell surface or in the endocytic vesicles. The mature APP is degraded rapidly during the way where it is either transported to the cell surface via a biosynthetic pathway or from the cell surface via an endocytic pathway.

Evidence is accumulating that α-, β-, and γ-secretases localize differentially within the cell. α-secretase cleavage of APP appears to occur either in the late compartment of constitutive secretory pathway (TGN or post-Golgi compartment) or mostly at the plasma membrane (Sambamurti *et al.*, 1992; De strooper *et al.*, 1993; Kuentzel *et al.*, 1993). Early research also suggested that membrane association was required for APP cleavage (Sisodia, 1992) as well as its internalization via the endocytic pathway (Carey *et al.*, 2005). On the other hand, the β-cleavage of APP seems to happen after APP endocytosis (Golde *et al.*, 1992; Koo & Squazzo, 1994; Grbovie *et al.*, 2003; reviewed by Tang, 2009). Aβ production by cleavage of both β- and γ-secretase occurs in the endosomal compartment (Shoji *et al.*, 1992; Siman *et al.*, 1993; Schrader-Fischer & Paganetti, 1996; Yamazaki *et al.*, 1996; Peraus *et al.*, 1997; Perez *et al.*, 1999). γ-secretase is suggested to work at plasma membrane (Chyung & Selkoe, 2003; Chyung *et al.*, 2005; reviewed by Kaether *et al.*, 2006a) or in early endosome (Kaether *et al.*, 2006b), in parallel or even earlier than β-secretase. However, proteolytic metabolism of sAPPβ and Aβ was also reported to happen in trans-Golgi compartment in absence of vesicle formation under disease condition or in the presence of certain APP mutations (Xu *et al.*, 1997). *In vivo* evidence proved that endocytosis is required for Aβ generation (Cirrito *et al.*, 2008). The exact cleavage compartment by β-secretase is still not revealed completely. The exosomal organelle is considered necessary for the production and transportation of a fraction of the secreted APP fragments (sAPP), including sAPPα, sAPPβ or Aβ (Yu *et al.*, 2004; Rajendran *et al.*, 2006; Vingtdeux *et al.*, 2007).

Piling all the evidences together, APP can be processed in different pathways (Figure 5): in the constitutive secretory pathway, APP is transported towards the cell surface where it meets α-secretase and releases sAPPα (Figure 5 secretory pathway); the full length APP which is not cleaved by α-secretase can be rapidly internalized to enter the endosomal-lysosomal pathways and can be cleaved by β- and γ-secretase (Figure 5 endocytic pathway) (Hare, 2010); a part of APP recycles back to the cell surface (Figure 5 recycling pathway) (Marquez-Sterling *et al.*, 1997; reviewed by Small & Gandy, 2006; reviewed by Sannerud & Annaert, 2009), or it is degraded directly in the lysosome. Given the short

half-life of APP, even subtle changes in APP transportation through these various secretase-containing compartments may have large effects on the net steady-state levels of sAPP and Aβ.

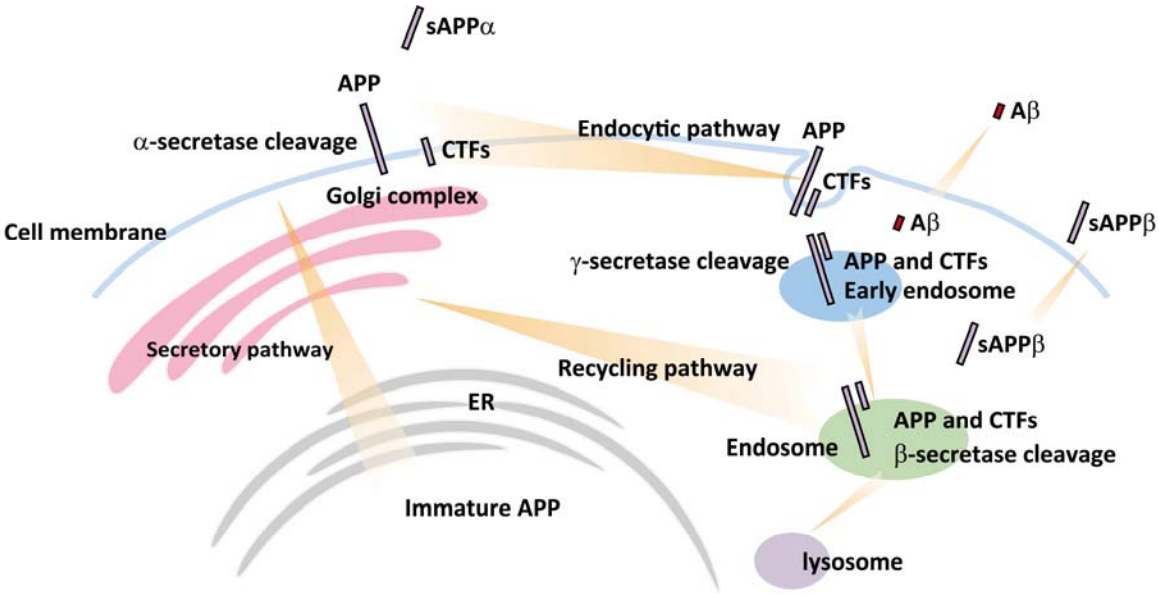

Figure 5 APP trafficking and ectodomain shedding.
In the constitutive secretory pathway, APP is transported through ER and TGN towards the cell surface where it comes across α-secretase and releases sAPPα; the full length APP or CTFs could then rapidly enter the endosomal-lysosomal pathways and be cleaved by γ- and β-secretases; The rest of APP and CTFs enter the recycling pathway reaching the cell membrane again or is degrade directly in the lysosome.

1.2.5 Secretases responsible for APP cleavage

Several key players involved in APP proteolytic processing, such as β- and γ-secretase, have been precisely identified or at least had a rough idea. In contrast, the identity of α-secretase remains unclear but it is assumed to belong to a disintegrin and metalloproteinase (ADAM) family (see details in 1.3).

β-secretase leads to the cleavage of APP at the N-terminus of the Aβ domain in the amyloidogenic route. In 1999, Vassar *et al.* identified the aspartyl protease BACE1 to be β-secretase of APP. Overexpression of BACE1 increased the amount of β-secretase cleavage products, and antisense inhibition of BACE1 messenger RNA decreased the amount of sAPPβ (Vassar *et al.*, 1999). BACE1 is a 501 amino acid long type I transmembrane, glycosylated aspartyl protease with an N-terminal signal peptide, a prodomain, a large catalytic domain, a transmembrane domain and a small cytoplasmic

domain (Hussain *et al.*, 1999). This finding was subsequently confirmed by several research groups using different experimental approaches (Sinha *et al.*, 1999; Yan *et al.*, 1999; Lin *et al.*, 2000). BACE1 is expressed highly in brain tissue (Vassar *et al.*, 1999), consistent with its role in amyloidogenic pathway. BACE1 was shown to be the unique protease for APP β-proteolytic processing (Cai *et al.*, 2001; Luo *et al.*, 2001b). Nevertheless, APP is not the unique substrate for BACE1, although the identification of other BACE1 substrate is poorly understood (reviewed by Haass, 2004). A BACE1 homologue, BACE2, has also been characterized (Acquati *et al.*, 2000; Solans *et al.*, 2000). Interestingly, although lowly expressed in the brain (Bennett *et al.*, 2000), BACE2 cleaves APP predominately inside the Aβ domain closer to the transmembrane domain than α-cleavage site. For that reason BACE2 would be rather classified as an alternative α-secretase.

After α- or β-secretase cleavage, the remaining membrane bound CTFs of APP are cut within the transmembrane region by γ-secretase. The cut can be carried out at different positions in C99 to generate different lengths of Aβ peptides. It arises predominantly Aβ40, while the much more pathogenic form Aβ42 makes up only 5-10% percentage (Selkoe, 1999). The γ-secretase is a high molecular weight complex comprising the four subunits: presenilin1 (PS1) or the homologous presenilin2 (PS2), APH-1 (anterior pharynx defective 1), nicastrin and Pen-2 (presenilin enhancer protein 2). These four subunits are necessary for the activity of the complex (Francis *et al.*, 2002) and also sufficient (Edbauer *et al.*, 2003). PSs are the active catalytic subunits of γ-secretase and belong to the family of GxGD aspartyl proteases (De Strooper *et al.*, 1998; Wolfe *et al.*, 1999; Haass & Steiner, 2002; reviewed by Wolfe, 2006). Missense mutations in the PS genes are the major cause of familial AD (Sherrington *et al.*, 1995), which is biochemically characterized by a shift of the C-terminal cleavage site of γ-secretase, leading to more Aβ42 generation.

1.3 Proteases involved in α-secretase cleavage of APP

Despite that β- and γ-secretase have been identified since a decade ago, the molecular identity of α-secretase is still unclear, controversial and remains to be fully established.

Some proteases of ADAM and matrix metalloprotease (MMP) family were supposed to be involved in APP α-secretase cleavage. Furthermore, because α-secretase cleavage has the potential to prevent Aβ generation and sAPPα has even been shown to have neuroprotective effects (see details in 1.2.2); it is considered to be a crucial target in the therapeutic approach for AD treatment.

1.3.1 ADAM proteases family

The α-secretase of APP was considered to be a metalloprotease due to the fact that APP α-secretase cleavage could be blocked by certain metalloprotease inhibitors. TAPI-1, -2 and 10-phenanthroline inhibits metalloproteases by removal and chelation of metal ions required for their catalytic activity, leaving an inactive apoenzyme (Arribas *et al.*, 1996). Some further researches concerning to metalloproteases inhibition revealed that metalloproteases were involved in APP α-cleavage process (Buxbaum *et al.*, 1998; Koike *et al.*, 1999; Lammich *et al.*, 1999; Skovronsky *et al.*, 2000; Lopez-Perez *et al.*, 2001; Slack *et al.*, 2001; Kim *et al.*, 2008). The most frequently named ones are three members of the ADAM family: ADAM9, 10 and 17 (see details in Table 3).

1.3.1.1 Structure of ADAM proteases

ADAMs are type I transmembrane proteins belonging to the zinc-dependent protease superfamily. They compromise a signal sequence, a prodomain, a large luminal domain containing the catalytically active metalloprotease domain and cysteine-rich domain, an EGF (epidermal growth factor)-like domain, a transmembrane domain and a short cytoplasmic domain (Figure 6) (reviewed by Seals & Courtneidge, 2003). The prodomain contains a conserved cysteine residue and the catalytic domain has to be coordinated with the zinc ions. In the trans-Golgi compartment, ADAM proteases can be activated by the removal of prodomain by a furin-like proprotein convertase (Lammich *et al.*, 1999; Anders *et al.*, 2001; Endres *et al.*, 2003). In ADAM10, proprotein convertase recognition sequence (RKKR) is essential for activation of the zymogen, and both furin and PC7 can act as proprotein convertases (Anders *et al.*, 2001). The cytoplasmic domain varies the most among the different ADAM proteases. It contains the motifs for protein-protein interactions and serves for signal transduction and cellular transport of ADAM proteases.

ADAM9, 10 and 17 have PXXP motifs and serine, threonine and tyrosine residues, which can be phosphorylated. The modifications of PXXP motifs and phosphorylation provide binding sites for SH3 and SH2 domain-containing proteins (Howard *et al.*, 1999).

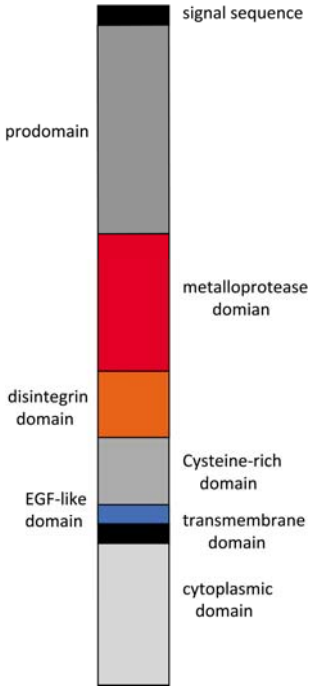

Figure 6 ADAM protease structure.
ADAM proteases are type I transmembrane protein with a signal sequence, a prodomain, a large luminal domain containing the catalytically active metalloprotease domain and cysteine-rich domain, a transmembrane domain and a short cytoplasmic domain.

1.3.1.2 Physiological functions of ADAM proteases

The ADAM proteases catalyze the cleavage of different type of type I transmembrane proteins, including cytokines and cytokine receptors, growth factors and their receptors, extracellular matrix proteins and adhesion proteins. Of the 21 human ADAMs identified, only 13 are proteolytically active. ADAMs shown to exhibit protease activity include ADAM9, 10, 12, 15, 17, 19, 28 and 33 (reviewed by Duffy *et al.*, 2011). Because of the diversity and variety of substrates, ADAM proteases are supposed to regulate many different functions in the various types of cells, like membrane fusion, cell migration, and also early development, fertilization and cell fate determination (reviewed by Seals & Courtneidge, 2003).

1.3.1.3 ADAM9, 10 and 17

ADAM9, 10 and 17 are the best described candidates providing APP α-secretase activity. Research about involvements of these three ADAM proteases in APP α-proteolytic process has been performed by various research group using different techniques in either *in vitro* cell lines or *in vivo* animal experiments. The following table summarizes the research about APP α-secretase candidates (Table 3).

Table 3 Research summary of APP α-secretase candidates (ADAM9, 10 and 17).

	Cell lines and ADAMs manipulations	**References**
in vitro		
Cell free assay	ADAM9	Roghani *et al.*, 1999
	ADAM10	Lammich *et al.*, 1999
Cell based assay		
Overexpression of individual protease	APP-COS ADAM9 ↑	Koike *et al.*, 1999
	HEK ADAM9 ↑	Taylor et al., 2009
	HEK/APP-HEK ADAM10 ↑	Lammich *et al.*, 1999
	LoVo ADAM10 ↑	Lopez-Perez *et al.*, 2001
	HEK ADAM10 ↑	Taylor et al., 2009
	APP-HEK ADAM17 ↑	Slack *et al.*, 2001
	LoVo ADAM17 ↑	Lopez-Perez *et al.*, 2001
gene knockdown or knockout of individual protease	ADAM9 KO-MEF	Weskamp *et al.*, 2002
	COS ADAM9 ↓	Asai *et al.*, 2003
	HEK ADAM9 ↓	Taylor et al., 2009
	ADAM10 KO-MEF	Hartmann *et al.*, 2002
	COS ADAM10 ↓	Asai *et al.*, 2003
	SH-SY5Y ADAM10 ↓	Allinson *et al.*, 2004
	1321N1 ADAM10 ↓	Camden et al. 2005
	U373 ADAM10 ↓	Freese et al., 2009
	HEK ADAM10 ↓	Taylor et al., 2009
	ADAM10 KO-neuron	Jorissen *et al.*, 2010
	COS ADAM17 ↓	Asai *et al.*, 2003
	SH-SY5Y ADAM17 ↓	Allinson *et al.*, 2004
	1321N1 ADAM17 ↓	Camden et al. 2005
in vivo		
overexpression	ADAM10-mouse brain	Postina *et al.*, 2004

"↑" represents gene overexpression; "↓" represents gene knockdown or knockout.

Some research was performed by *in vitro* enzyme activity assay. The corresponding α-cleavage product together with the exact α-cleavage site was verified between the 16th and 17th amino acid of Aβ domain (Lammich *et al.*, 1999; Roghani *et al.*, 1999). Other studies tried to elucidate the identity of α-secretase by candidate overexpression. ADAM9, 10 and 17 overexpressions in different cell lines resulted to an increase of APP

α-cleavage product (Koike *et al.*, 1999; Lammich *et al.*, 1999; Lopez-Perez *et al.*, 2001; Slack *et al.*, 2001; Hotoda *et al.*, 2002; Postina *et al.*, 2004). Catalytic inactive mutations of ADAM9 and 10 overexpressions could either inhibit the APP α-cleavage or at lease keep sAPPα generation at normal level (Koike *et al.*, 1999; Lammich *et al.*, 1999).

However, since the overexpression of a protease could only up-regulate APP α-secretase cleavage artificially or indirectly, the physiological relevance of a candidate protease needs to be shown using the corresponding protease knockdown or knockout cells. In fact, different kinds of cells derived from ADAM9-, 10- or 17-deficient mice showed either no or a variable degree of reduction of APP shedding (Buxbaum *et al.*, 1998; Hartmann *et al.*, 2002; Weskamp *et al.*, 2002; Jorissen *et al.*, 2010). Besides, cell based gene disruption experiments showed that ADAM9, 10 and 17 were all involved in the APP α-shedding. RNAi-mediated knockdown of the individual proteases in cultured cells led to the reduction of APP shedding to different extents (Asai *et al.*, 2003; Allison *et al.*, 2004; Camden *et al.*, 2005; Freese *et al.*, 2009; Taylor *et al.*, 2009).

The finding that APP shedding was never fully suppressed has led to the conclusion that ADAM9, 10 and 17 may contribute to α-secretase activity all together and they may exhibit considerable functional redundancy. However, this is in clear contrast to the fact that many other ADAM protease substrates are predominantly cleaved by a single ADAM protease, such as transforming growth factor α (TGFα), EGF, the low-affinity immunoglobulin E receptor CD23 and N-cadherin (Sahin *et al.*, 2004; Reiss *et al.*, 2005; Weskamp *et al.*, 2006; Le Gall *et al.*, 2009; reviewed by Pruessmeyer & Ludwig, 2009). This raises the doubt that whether all three candidates of APP α-secretase provide the physiologically relevant α-secretase activity.

Moreover, some MMPs and other ADAMs may also have to be involved in the α-secretase activity. At least, upon overexpression of MMP2, 9, 14, 16 and ADAM8, 19, APP α-cleavage increased (Ahmad *et al.*, 2006; Naus *et al.*, 2006; Talamagas *et al.*, 2007; Tanabe *et al.*, 2007; Vaisar *et al.*, 2009). Whether they are relevant as α-secretase at

endogenous level remains unclear. They were also supposed to cleave APP at other site different from α-cleavage site.

1.3.2 Problems of APP α-secretase identification research

Investigation of APP α-secretase has always been complicated by two major factors. One aspect is the fact that APP is cleaved by distinct proteases at different sites in close proximity (Figure 3). For example, there is a secondary β-secretase cleavage site (termed β'-site) within the Aβ sequence in close proximity to the α-secretase-cleavage site. Antibodies used in previous studies, like WO2 or 6E10, have not specifically detected sAPPα, but also the alternative β-secretase-cleavage product sAPPβ' (Miles *et al.*, 2008), which may have confounded the study of α-secretase cleavage. The other difficulty to study APP shedding is that the endogenous APP is expressed at very low levels, typically at the limit of assay sensitivity, especially in non-neuronal cells. Indeed the major part of research about APP shedding was performed using APP overexpressing cell lines, which simplified the analysis of APP shedding behavior but in an artificial environment. The heterogeneous results may also be caused by the off-target effects raised up by the overexpression of APP. Furthermore, many studies were carried out using activators of APP ectodomain shedding, like protein kinase C (PKC) signaling activator phorbol ester, which would imply the identity of α-secretase under regulated condition (see details in 1.4.1) but not constitutive condition.

1.4 Regulation of APP α-secretase

APP is processed in two different proteolytic pathways by α- and β-secretase (Figure 4). APP is a common substrate for both proteases. So theoretically upregulation of one's activity would lead to the reduction of the ectodomain shedding by the other secretase. This is the reason why in general the regulation of sAPPα and Aβ production is thought to occur in an inversely coupled manner. An increase of APP α-secretase activity is considered a possible therapeutic approach for AD, as it could be preventive in terms of Aβ generation. In contrast, a reduction of the α-cleavage is assumed to increase the risk of AD by leading to enhanced β-secretase cleavage and Aβ levels. Understanding the regulatory mechanisms of both secretases and their reciprocal coupling is of main

importance since the modulation of their activities could be promising therapeutic approach for AD.

1.4.1 Regulated α-secretase of APP

α-secretase activity has two components: a constitutive part and a regulated part. Both pathways contribute to APP α-proteolytic process. The constitutive pathway plays its role under normal physiological conditions; additionally, α-secretase activity can be increased above the constitutive level by some stimulation, which is called the regulated pathway. Due to its therapeutic potential, the activation of the regulated α pathways has roused the interest of AD community in the last decade. Different kinds of stimuli have been suggested to increase the secretion of sAPPα under certain conditions. The first evidence about the regulated α-secretase was shown in 1992. In that report, the release of sAPP was stimulated by muscarinic acetylcholine receptor (mAChR) agonist (Nitsch *et al*., 1992). Later, some other activators were also successfully tested, such as activation of G protein-coupled receptors (GPCRs) and receptor tyrosine kinases, like PKC, mitogen-activated protein kinase (MAPK), phosphoinositid-3-kinase (PI3K), cAMP and calcium (reviewed by Postina, 2011). The following table summarizes some physiological stimuli involved in the modulation of APP α-secretase activity (Table 4). Another most frequently used sAPPα stimuli is phorbol-12-myristate-13-acetate (PMA) (see details in 1.4.2).

Table 4 Research summary of physiological APP secretion stimuli.

Sorts	Stimuli	References
Cytokines	Interleukin-1 α (IL-1α)	Bandyopadhyay *et al.*, 2006
	Interleukin-1 β (IL-1β)	Dash & Moore, 1995; Chong & Lee, 1999; Gitter *et al.*, 2000; Ma *et al.*, 2005; Tachida *et al.*, 2008; Kong *et al.*, 2009
Growth factors	EGF	Slack *et al.*, 1997; Santiago-Josefat *et al.*, 2007
	Insulin-like growth factor 1 (IGF-1)	Adlerz *et al.*, 2007; Jacobsen *et al.*, 2010
Hormones	Estrogen	Manthey *et al.*, 2001; Zhang *et al.*, 2005; Amtul *et al.*, 2010
	Insulin	Solano *et al.*, 2000; Chen *et al.*, 2007
Neurotransmitters	Glutamate receptor (GluR) agonist	Lee *et al.*, 1995; Nitsch *et al.*, 1997; Jolly-Tornetta *et al.*, 1998; Marcello *et al.*, 2008; Hoey *et al.*, 2009
	mAChR agonist	Buxbaum *et al.*, 1992; Nitsch *et al.*, 1992; Nitsch *et al.*, 1993; Slack *et al.*, 1995; Wolf *et al.*, 1995; Caccamo *et al.*, 2006; Cho *et al.*, 2006; Shirey *et al.*, 2009; Davis *et al.*, 2010

However, for most of them it remains to be shown whether they activate α-secretase cleavage through ADAM9, 10, 17 or yet other metalloproteases. The identification of such a regulated α-secretase of APP would be helpful for AD therapy development.

1.4.2 Relationship between α- and β-secretase on APP cleavage

Evidence from early reports demonstrated that Aβ release could be modulated physiologically or pharmacologically by a number of agonists which increase APP α-shedding through phospholipase C and/or PKC activation (Table 5). PKC activation by PMA or phorbol 12,13-dibutyrate (PDBu), or activation of neurotransmitter receptors linked to phospholipase C, like mAChR, increased the α-cleavage of APP in cell lines with endogenous or overexpressed APP or Swedish APP (K670N, M671L) (Citron *et al.*, 1992). As a result, sAPPα and p3 production increased apparently, while the toxic product Aβ decreased correspondingly (Hung *et al.*, 1993; Jacobsen *et al.*, 1994; Wolf *et al.*, 1995; Citron *et al.*, 1996; Buxbaum *et al.*, 1998; Kioke *et al.*, 1999; Skovronsky *et al.*, 2000; Zhu *et al.*, 2001; Allinson *et al.*, 2004; Kim *et al.*, 2008; Fu *et al.*, 2009). PMA

treatment in neurons from Tg2576 mouse (transgenic mice overexpressing APP Swedish mutation) (Hsiao *et al.*, 1996) also produced a significant increase in the levels of sAPPα, which was associated with a significant decrease in the levels of soluble Aβ (Qiu *et al.*, 2001). Postina *et al.* reported that neuronal overexpression of ADAM10 in human APP_{V717I} overexpressed mice increased the secretion of the neurotrophic sAPPα, reduced the formation of Aβ peptides and prevented their deposition in plaques (Postina *et al.*, 2004). Other research related to AD diagnosis or therapy implied the compensative or competitive position of α- and β-secretase on APP cleavage (Colciaghi *et al.*, 2002; Yao *et al.*, 2011). However, in all these research, sAPPβ level was hardly measured. Thus, the activated α-secretase seems to compete with β-secretase on APP cleavage and upregulation of α-secretase activity is considered a potential therapeutic application in AD.

Conversely, PKC activation in human primary neurons was shown to not affect Aβ production or even increase it (LeBlanc *et al.*, 1998; Blacker *et al.*, 2002). Another report showed that Aβ and sAPPβ levels were significantly reduced after intracortical injection of PMA in human APP overexpressing mouse, whereas sAPPα level was unchanged (Savage *et al.*, 1998). On the other hand, pharmacological down-regulation of α-secretase cleavage of APP led to variant results about β-cleavage changes (Skovronsky *et al.*, 2000; Gandhi *et al.*, 2004; Kim *et al.*, 2008). Using an ADAM10 conditional knockout mice, Saftig and colleagues found an obvious diminished appearance of CTFα and sAPPα together with a paradoxical reduction in CTFβ, sAPPβ, and total Aβ peptide production including the amount of Aβ40 and Aβ42 (Jorissen *et al.*, 2010). Due to these heterogeneous findings, the scenario about the relationship between α- and β-secretase on APP cleavage is still unclear. The following table summarizes the regulation of α-secretase and the corresponding compensative and competitive effect from β-secretase on APP cleavage (Table 5).

Table 5 Research summary of relationship of α- and β-secretase on APP cleavage.

			APP shedding behaviors	References
Compensation α-secretase activity down-regulation	*in vivo*	ADAM10-/- mouse	sAPPα ↓ CTFα ↓, sAPPβ ↓ CTFβ ↓ Aβ ↓	Jorissen *et al.*, 2010
	in vitro	TAPI (metalloprotease inhibitor)	sAPPα ↓ C83 ↓, sAPPβ -- C89 ↑ C99 ↑	Skovronsky *et al.*, 2000
		TAPI	sAPPα ↓ CTFα ↓, sAPPβ – CTFβ – Aβ --	Kim *et al.*, 2008; Gandhi *et al.*, 2004
Competition α-secretase activity up-regulation	*in vivo*	ADAM10 mouse	sAPPα ↑ CTFα ↑, sAPPβ ↓ CTFβ – Aβ ↓	Postina *et al.*, 2004
	in vitro	PMA	sAPPα ↑ p3 ↑ Aβ --	Dyrks, 1994; Blacker *et al.*, 2002; Gandhi *et al.*, 2004
		PMA/PDBu	sAPP ↑ p3 ↑ CTFα ↑, sAPPβ ↓ CTFβ ↓ Aβ ↓	Hung *et al.*, 1993; Jacobsen *et al.*, 1994; Citron *et al.*, 1996; Buxbaum *et al.*, 1998; Koike *et al.*, 1999; Skovronsky *et al.*, 2000; Qiu *et al.*, 2001; Zhu *et al.*, 2001; Allinson et al., 2004; Kim, 2008; Fu *et al.*, 2009
		PMA/PDBu	sAPP ↑, Aβ ↑	LeBlanc *et al.*, 1998
		PMA	sAPPα --, sAPPβ ↓ Aβ ↓	Savage *et al.*, 1998

"↓" represents reduction; "↑" represents increase; "--" represents no change.

Because of the discrepancies from the literature, the complex relationship between α- and β-secretase is not yet totally clear. It seems to be affected by factors from various aspects, like cell lines, *in vivo*/*in vitro* environments, the way of regulation achieved and APP trafficking involvement. Therefore, this topic has to be further investigated, in order to draw a final conclusion. In particular, most of the previous reports were performed using cell lines overexpressing APP or AD related APP mutation because of the difficulty of endogenous APP fragments detection. It would be extremely fruitful to investigate the relationship of α- and β-secretase cleavage at endogenous APP expression level. On the other hand, the majority of the research describing the relationship of these two proteases was performed under the condition of activity up-regulation. Little evidence has been shown their compensatory relationship under the condition of proteases activity down-

regulation. So it would provide additional evidence for the relationship between these two proteases under the condition of protease inactivation.

2 Aim of the work

Alzheimer's disease (AD), the most prevalent form of dementia, is characterized by the deposition of amyloid beta (Aβ) peptides in the brain. Aβ is a proteolytic product of the larger amyloid precursor protein (APP). There are two different proteolytic activities, referred to as α- and β-secretase, responsible for the cleavage of the APP ectodomain. Ectodomain shedding of APP by β-secretase and the subsequent cleavage of the remaining C-terminal fragment by γ-secretase contribute to Aβ generation; whereas cleavage by α-secretase occurs within the Aβ domain, releasing secreted APP α-fragment (sAPPα) and therefore precludes Aβ generation. Unlike β- and γ- secretase, the molecular identity of α-secretase is still ambiguous. Proteases belonging to a disintegrin and metalloproteinase (ADAM) family, especially ADAM9, 10 and 17 are the most named α-secretase candidates. The exact identification of APP α-secretase would help to complete the understanding of APP processing and facilitate pharmacological intervention; and the potential compensatory relationship between α- and β-secretase offers therapeutic potential. Despite the large body of evidence showing the relationship between these two proteases under activity upregulation condition, few studies based on inhibition or knockdown have been performed. Therefore, two major questions should be addressed in this thesis:

• Which protease represents the physiologically relevant, constitutive α-secretase of APP? In order to facilitate the specific detection of APP α-cleavage, new cleavage-site specific sAPPα antibodies should be generated. Afterwards, the APP α-secretase candidates ADAM9, 10, 17 and metalloprotease 14 (MMP14) should be knocked down in order to identify the proteases that contribute to APP α-cleavage under endogenous APP expression conditions. Different cell lines should be analyzed to account for the potential cell type-specific expression levels of the studied proteases. To analyze the proteases under short- and long term loss of function conditions, transient knockdowns by siRNA transfection as well as stable knockdowns upon viral transduction of shRNAs should be performed. The concentration and sequences of applied siRNAs/shRNAs should be optimized in order to minimize off-target effects. Different APP ectodomain fragments

should be analyzed to reveal the shedding contribution of α- and β-secretase. In addition, the identification of regulated APP α-secretase will be carried out upon PMA stimulation.

• What is the relationship between α- and β-secretase in terms of APP cleavage?

In order to address the compensative relationship between α- and β-secretase under physiological conditions, different cell lines, from peripheral tumor cell lines to neuron-like cells, should be studied. The loss of function of α- or β-secretase should be achieved by different methods, both pharmacologically and genetically. Pharmacological inhibition by protease inhibitors as well as siRNA/shRNA mediated knockdowns of α- and β-secretase should be used to monitor compensatory effects of one protease in the absence of the other.

3 Materials and methods

3.1 General materials

3.1.1 Equipments and consumables

Equipments/consumables	Manufacturers
Analytical balance (200 - 0.0001 g)	Ohaus
Autoclave (Tuttnauer 3850 EL)	Systec
Balance (2000 - 0.01 g)	Ohaus
Falcon tube	Sarstedt
Fridge (Santo 4 °C)	AEG
Freezer (-20 °C)	Liebherr
Freezer (-80 °C)	Heraeus
Micro tubes (1.5 ml, 2 ml)	Sarstedt
Milli Q plus filtration system	Millipore
Multi-channel pipette (300 µl)	Eppendorf
pH electrode	Schott
pH meter (Inolab pH Level 1)	WTW
Pipettes (1 ml, 200 µl, 20 µl)	Gilson
Pipettes (10 µl)	Eppendorf
Pipettes (Accu-Jet)	Brand
Pipette tips (1 ml, 200 µl, 20 µl, 10 µl)	Sarstedt
Pipette tips (25 ml, 10 ml, 5 ml)	Sarstedt
Thermomixer compact	Eppendorf
Vortex VF2	IKA labortechnik

3.1.2 Reagents

Reagents	Manufacturers
Calcium chloride ($CaCl_2$)	J.T. Baker
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma
Dimethyl sulfoxide (DMSO)	Merck
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Ethanol	Merck
Glycerol	Sigma
Hydrogen chloride (HCl)	Merck
Magnesium chloride ($MgCl_2$)	Merck
Potassium chloride (KCl)	Merck
Potassium dihydrogen phosphate (KH_2PO_4)	J.T. Baker
Sodium chloride (NaCl)	Roth
Tris hydroxymethyl aminomethane (Tris)	Biomol

3.1.3 Buffers

All solutions were dissolved with Mili-Q plant deionized H_2O (double-distilled water, ddH_2O) if not specifically described, whose electric resistance was purified to › 18.2 MΩ at 25 °C.

Buffers	Recipes
10* PBS	80 g NaCl 2 g KCl 14.4 g Na_2HPO_4 2 g KH_2PO_4 add ddH_2O to 1 L

3.2 Molecular biological techniques

3.2.1 Equipments and Consumables

Equipments/consumables	Manufacturers
Cooling centrifuge (Avanti J-20XP)	Beckman Coulter
Centrifuge biofuge	Heraeus
Electrophoresis	Owl separation system
Microwave	Alaska
Multifuge 3	Heraeus, Thermo Fischer
NucleoBond Xtra Midi kit	Macherey-Nagel
NucleoSpin extract II kit	Macherey-Nagel
NucleoSpin plasmid kit	Macherey-Nagel
Thermal cycler	Bio-Rad
Thin-walled PCR tubes	Sarstedt
UV lamp	Intas

3.2.2 Reagents

Reagents	Manufacturers
10* complete PCR buffer	Peqlab
10* T4 DNA ligase buffer	Fermentas
1 kb DNA ladder	Gibco
Acetic acid	Merck
Agar	BD
Agarose NA	Invitrogen
Ampicillin	Sigma
Ethidium bromide (EB)	Roth

Reagents	Manufacturers
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Biomol
Orange G	Sigma
PCR Nucleotide Mix (dNTP) (10 mM)	Roche
Pwo DNA polymerase (1 U/µl)	Peqlab
T4 DNA ligase (5 U/µl)	Fermentas
Tryptone	BD
Yeast extract	BD

3.2.3 Buffers

Buffers	Recipes
4* DNA sample buffer	15 ml Glycerol 1 ml 0.5 M EDTA 25 mg Orange G add ddH_2O to 50 ml
50* TAE	24.2 g Tris 57.1 ml Acetic Acid 100 ml 0.5 M EDTA (pH 8.0) add ddH_2O to 1 L
1000* Ampicillin solution	100 mg/ml ampicillin in 70% ethanol
LB agar gel	15 g/L agar solution is autoclaved 1.2 kPa for 20 min. The solution was poured into the plate and cooled down below 50 $^{\circ}$C.
LB medium	1% tryptone 0.5% yeast extract 0.5% NaCl ddH_2O solution pH was adjusted to pH 7.0. The solution was autoclaved at 1.2 kPa for 20 min.
SOB medium	0.2% tryptone 0.05% yeast extract 0.5% NaCl ddH_2O solution pH was adjusted to 7.0.
TB buffer	10 mM HEPES buffer 15 mM $CaCl_2$ solution 250 mM KCl solution 55 mM $MgCl_2$ solution ddH_2O solution pH was adjusted to 6.7.

3.2.4 Plasmids and oligonucleotides

The following plasmids were used in the thesis (Table 6). The table lists the names of the plasmids, the cDNA or shRNA, the source and the restriction endonucleases (RE) sites. The restriction enzymes and their specific buffers were from Fermentas or New England Biolabs.

Table 6 Plasmids used in the thesis.

Plasmids	Vectors	cDNA/shRNA	RE sites	sources
P12-APP695	Peak12	APP695	HindIII/NotI	Dr. S. Lichtenthaler
P12-sAPPα	Peak12	sAPPα	HindIII/NotI	Cloned from P12-APP695
P12-sAPP15	Peak12	sAPP15	HindIII/NotI	Cloned from P12-APP695
P12-sAPP14	Peak12	sAPP14	HindIII/NotI	Cloned from P12-APP695
P12-sAPPβ'	Peak12	sAPPβ'	HindIII/NotI	Cloned from P12-APP695
P12-sAPPβ	Peak12	sAPPβ	HindIII/NotI	Cloned from P12-APP695
P12-Hygro-hAPP	Peak12	hAPP695	MfeI	Dr. P. Kuhn
PLVTH-control	PLVTH	Control shRNA	XhoI/NotI	Dr. P. Kuhn
PLVTH-ADAM10-sh6	PLVTH	ADAM10-sh6	XhoI/NotI	Dr. P. Kuhn
PLVTH-ADAM10-sh9	PLVTH	ADAM10-sh9	XhoI/NotI	Dr. P. Kuhn
PLKO-control	PLKO	Control shRNA	XhoI/NotI	Dr. P. Kuhn
PLKO-ADAM10-sh7	PLKO	ADAM10-sh7	XhoI/NotI	Dr. P. Kuhn
PLKO-ADAM10-sh9	PLKO	ADAM10-sh9	XhoI/NotI	Dr. P. Kuhn
PLKO-BACE1-sh1	PLKO	BACE1-sh1	XhoI/NotI	Dr. P. Kuhn
PLKO-BACE1-sh2	PLKO	BACE1-sh2	XhoI/NotI	Dr. P. Kuhn
sPAX2	sPAX2		XhoI/NotI	Dr. P. Kuhn
pcDNA3.1-Delta.Zeo (-)-VSV-G	pcDNA 3.1	VSV-G	XhoI/NotI	Dr. P. Kuhn

Table 7 lists the template plasmids, the primers used in the PCR reaction, the primers used in plasmid sequencing and the final plasmids. Primers were purchased from ThermoFisher or Sigma.

Table 7 Primers used in the thesis.

Template plasmids	Primer sequences	Primer sequences (sequencing)	Final plasmids
P12-APP695	P12-APP695-PmlI-for P12-sAPPα-NotI-rev	P12-APP695-PmlI-for	P12-sAPPα
P12-APP695	P12-APP695-PmlI-for P12-sAPP15-NotI-rev	P12-APP695-PmlI-for	P12-sAPP15
P12-APP695	P12-APP695-PmlI-for P12-sAPP14-NotI-rev	P12-APP695-PmlI-for	P12-sAPP14
P12-APP695	P12-APP695-PmlI-for P12-sAPPβ'-NotI-rev	P12-APP695-PmlI-for	P12-sAPPβ'
P12-APP695	P12-APP695-PmlI-for P12-sAPPβ-NotI-rev	P12-APP695-PmlI-for	P12-sAPPβ

Table 8 lists the sequences of primers. The oligonucleotides used were dissolved in ddH_2O by vortexing. The stock solution (100 mM) was stocked at -20 °C, and a 10 mM solution served as working solution.

Table 8 Sequences of primer used in the thesis.

primers	Sequences
P12-APP695-PmlI-for	5'-GATCGGCCTCGTCACGTGTTCAATATGC-3'
P12-sAPPα-NotI-rev	5'-GATCGCGGCCGCCTATTTTTGATGATG-3'
P12-sAPP15-NotI-rev	5'-GATCGCGGCCGCCTATTGATGATGAACTTCATATC-3'
P12-sAPP14-NotI-rev	5'-GATCGCGGCCGCCTAATGATGAACTTCATATC-3'
P12-sAPPβ'-NotI-rev	5'-GATCGCGGCCGCCTAATATCCTGAGTCATGTCG-3'
P12-sAPPβ-NotI-rev	5'-GATCGCGGCCGCCTACATCTTCACTTCAGAGATC-3'

3.2.5 Methods

3.2.5.1 Polymerase chain reaction (PCR)

The PCR reaction solution was prepared according to the following recipe:

Pwo polymerase (1 U/μl)	1 μl
10* complete reaction buffer	5 μl
dNTP (10 mM)	1 μl
Forward primer (10 mM)	4 μl
Reverse primer (10 mM)	4 μl
Template	100 ng
add ddH_2O to	50 μl

ddH_2O was used instead of template as the negative control.

The reaction solution was prepared in thin-walled PCR tubes. Subsequently, the amplification of the target sequence was carried out in a PCR machine. The amplification was carried out for 30 cycles, each cycle consists of the following individual steps:

1. Denature: disconnection of the deoxyribonucleic acid (DNA) duplexes for 30 s at 95 °C;

2. Primer hybridization: annealing of primers to the DNA single strands for 30 s (the annealing temperature depends on the GC temperature of primer pairs);

3 Elongation: synthesis of the complementary strand at 72 °C (the elongation time depends on the base pair number of the target duplex, approximately 750 kb/min).

Before the first cycle, there was a 5 min melting procedure at 95 °C. And after the completion of the total 30 cycles, the reaction was kept at 72 °C for 10 min for stabilization. Then it was kept at 4 °C.

3.2.5.2 Agarose gel electrophoresis

DNA fragments were separated by 1-1.5% agarose gel electrophoresis. The gels were prepared by agarose solution in TAE buffer and added 0.2 mg/ml EB before use. The comb was put into the gel until the gel was coagulated. The DNA samples were mixed with 4* DNA loading buffer and loaded on the gel. The 1 kb standard DNA ladder was used as the marker. Electrophoresis was performed at 120 V in TAE buffer for 20-40 min.

3.2.5.3 DNA extraction from agarose gel

The desired DNA fragments were carefully excised under UV light with a scalpel from the gel. Subsequently, the DNA was purified by the NucleoSpin extract II Kit following manufacturer's instructions. The gel was first lysed with 200 µl binding buffer by incubating the tube at 50 °C until the gel was melted. The gel lysis was then transferred into the column. The column was centrifuged at 13,000 rpm/1 min to make DNA combining with the silica membrane. The silica membrane was washed with 600 µl wash buffer (add ethanol each bottle before use) and centrifuged at 13,000 rpm/1 min to wash down the impurity except DNA. The silica membrane was dried with centrifugation at 13,000 rpm/2 min. DNA was eluted from the silica membrane by 15-50 µl elution buffer after combination at room temperature (RT) for 1 min and centrifugation at 13,000 rpm/1 min.

3.2.5.4 Subcloning of target DNA into expression vector

To subclone the target cDNAs into the expression vectors, the PCR fragments and the respective vectors were digested. Two different REs were used to ensure correct orientation of the cDNA into the vectors. The reaction buffers were selected according to http://fermentas.com/en/tools/doubledigest/?country_code=DE. The digestion reaction buffer was mixed at RT and then incubated at 37 °C in a heating block for 1-3 h.

The digestion reaction solutions were prepared according to the following recipe:

PCR fragments	80 ng
or vector	800 ng
10* RE reaction buffer	3 µl
REs	0.8 µl for each
Add ddH_2O to	30 µl

The target DNA or vector fragments were separated and purified by agarose gel electrophoresis (see 3.5.3.2) and NucleoSpin extract Kit (see 3.5.3.3). Subsequently, the ligation of vector and insert was performed at RT for 2 h or 16 °C overnight.

The ligation reaction solutions were prepared according to the following recipe:

vector	1 µl
insert	6 µl
T4 ligase	1 µl
10* T4 ligase buffer	2 µl
add ddH_2O to	20 µl

ddH_2O was used instead of insert as the negative control.

3.2.5.5 Preparation of competent DH5α *E. coli* bacterial strain

DH5α *Escherichia coli* (*E. coli*) colonies were picked up from an agar plate under sterile conditions and these colonies were inoculated in 100 ml SOB medium at 37 °C/200 rpm for 16 h. Subsequently, these bacterials were diluted in approximately 250 ml SOB medium to an optical density (OD605) of 0.1 and incubated at 18 °C /200 rpm. After 24 h incubation, the bacterial culture solution reached an OD605 of 0.6-0.8 in 50 ml Falcon tubes and was incubated on ice for 10 min. The bacterial sediment was centrifuged at 4 °C/3500 rpm/20 min. They were resuspended in 80 ml of cold TB buffer and incubated on ice for 10 min. The bacterial sediments were centrifuged down and resuspended in 20 ml TB buffer with 7% DMSO at 4 °C. The bacterial suspension was aliquoted and flash frozen in liquid nitrogen. The bacterial stock solution was kept at -80 °C.

3.2.5.6 DNA transformation

For DNA transformation, competent *E. coli* DH5α cells were thawed on ice. 10 µl DNA and 60 µl *E. coli* DH5α cells were mixed and incubated on ice for 30 min. Then they

were incubated at 42 °C for 2 min without shaking, followed by incubation on ice for another 2 min. 800 µl LB-medium was added into the mixture and incubated at 37 °C/200 rpm/30 min. For retransformation, 100 ml LB-medium (with selection antibiotics, 1:1000 dilution) and *E. coli* DH5α cells were mixed in flasks. For transformation, *E. coli* DH5α cells were seeded onto the LB-agar plate (with selection antibiotics, 1:1000 dilution) and incubated at 37 °C overnight.

3.2.5.7 Plasmid DNA purification

For DNA preparation in mini format, bacterial colonies were picked up and cultivated in 5 ml LB-medium (with antibiotics, 1:1000 dilution) at 37 °C for at least 6 h or overnight. The plasmid DNA was purified by using Nucleospin Plasmid Kit following manufacturer's instruction. Bacterial cells were harvested into a tube and spinned at 8000 rpm/4 °C/1 min. The supernatant was discarded and cells were resuspended by 250 µl Buffer A1. 250 µl Buffer A2 was added and the mixture was incubated for 3 min at RT. Then proteins were precipitated by adding 300 µl Buffer A3. The lysate was clarified by centrifugation at 8000 rpm/5 min. The supernatant was loaded on the column and spinned at 8000 rpm/1 min to combine the DNA. The silica membrane was washed by adding 600 µl Buffer A4 and centrifugation at 8000 rpm/1 min. DNA was eluted from the silica membrane by 50 µl elution buffer after combination at RT for 1 min and centrifugation at 8000 rpm/2 min.

For DNA preparation in midi format, plasmid DNA was purified by using NucleoBond AX500 Kit following manufacturer's instruction. The *E.coli* DH5α cells were poured into a Falcon tube and centrifuged at 4 °C/6000 rpm with JA-10 rotor for 20 min after overnight incubation. The supernatant was discarded and cells were resuspended with 8 ml RES Buffer (+ RNase). 8 ml LYS Buffer was added and the mixture was incubated at RT for 3 min. Then 8 ml NEU Buffer was added to precipitate the proteins. 12 ml EQU buffer was used to pre-equilibrate the column. Then cell lysates were poured into the columns. Another 5 ml EQU buffer was added to wash off the rest DNA. 8 ml wash buffer was added to wash off the rest protein. Then 5 ml ELU buffer was used to wash down the DNA. DNA solution was harvested in a Falcon tube and 3.5 ml isopropanol

was added to precipitate the DNA. The mixture was centrifuged at 4 °C/4600 rpm with 6641 rotor for 30 min. The precipitation was washed with 1 ml 70% ethanol with centrifugation at 13,000 rpm/1 min. DNA was dissolved with 300 µl ddH_2O. DNA concentrations were measured by nanometer.

3.2.5.8 DNA constructs sequencing

The sequencing of the DNA constructs was carried out by GATC Biotech AG http://www.gatc-biotech.com/en/home.html (Constance). The correctness of the sequences was checked by VectorNTI.

3.3 Cell culture

3.3.1 Equipments and consumables

Equipments/consumables	Manufacturers
Biological safety cabinet	Thermo Scientific
Bunsen burner	Heraeus
Cell culture dish (6 cm, 10cm)	Nunc
Cell culture flask (75 cm^2)	Nunc
Cell culture plate (24-well, 12-well, 6-well)	Nunc
CO_2 incubator	Thermo Scientific
Centrifuge Megafuge 40	Heraeus
Fridge	Libherr
Hemocytometer (Neubauer)	Optik Labor
Microscope (Wiloverts 10* 4/10/20)	Hund
N2-Tank (Chronos)	Messer Griesheim
Pipette tips, steril (2 ml, 5 ml, 10 ml, 25 ml)	Sarstedt
Syringe fliter (0.45 µM)	VWR International
Water bath	GFL

3.3.2 Reagents

Reagents	Manufacturers
5* siRNA buffer	Dharmacon
β-secretase inhibitor IV (C3)	Calbiochem
γ-secretase inhibitor IX (DAPT)	Calbiochem
Dulbecco's modified eagle medium (DMEM) high glucose	Gibco
DMEM/F12 (1:1) medium	Lonza
DMEM high glucose with pyruvat	Invitrogen

Reagents	Manufacturers
Fetal bovine serum (FCS)	Gibco
Hygromycin	Invitrogen
Lipofectamine 2000	Invitrogen
Lipofectamine RNAiMAX	Invitrogen
Non essential amino acids (NEAA)	Invitrogen
Opti-MEM I reduced serum medium with GlutaMAX I	Invitrogen
Penicillin/streptomycin (P/S)	Gibco
PMA	Calbiochem
Poly-L-lysine hydrobromide	Sigma
TAPI-1	Peptides International
Trypsin-EDTA (0.05%)	Gibco

3.3.3 Cell lines

The following cell lines were used in the thesis (Table 9). The table lists the names of the cell lines, the medium used to cell culture and the source of the cell lines.

Table 9 Cell lines used in the thesis.

Cells lines	Medium	Source
Human embryonic kidney 293 cells (HEK293)	DMEM + 10% FCS + P/S	ATCC
HEK293T low passage	DMEM + 10% FCS + P/S	ATCC
Human neuroblastoma cells (SH-SY5Y)	DMEM/F12 + 15% FCS + NEAA + P/S	Dr. S. Lichtenthaler
APP-HEK293	DMEM + 10% FCS + P/S + hygromycin	Dr. S. Neumann
APP-SH-SY5Y	DMEM/F12 + 15% FCS + NEAA + P/S + hygromycin	Generated from wide type SH-SY5Y cells

3.3.4 Methods

3.3.4.1 Cultivation of cells

The cell lines were cultured in 10-cm dishes or 75-cm^2 flasks in an incubator at 37 $^{\circ}C$ with 5% CO_2. Upon reaching 70-80% confluence, the cells were washed with 3 ml sterile PBS and replaced with 2 ml sterile PBS-EDTA solution or typsin-EDTA solution to

detach the cells. The cells were resuspended with 3 ml fresh medium. Appropriate amount of cells were splitted to a new dish or flask with 8 ml fresh medium.

3.3.4.2 Coating of cell culture dishes with poly-*L*-lysine

To improve the adhesion of some cell lines (HEK293 cells especially), the cell culture dishes have to be coated with poly-*L*-lysine. The cell culture dishes were completely covered with sterile poly-*L*-lysine solution (100 µg/ml in PBS) and incubated for at least 30 min at RT. After removing the poly-*L*-lysine solution, the dishes were washed twice with PBS and then dried.

3.3.4.3 Transient gene overexpression by DNA transfection

Transient gene overexpression was achieved by DNA transfection. The following procedure was used to transfect DNA into mammalian cells in a 24-well format. One day before transfection, 2-3*10^5 cells were seeded in 500 µl of growth medium without antibiotics so that cells would be 90-95% confluent at the time of transfection. For each transfection sample, transfection complex was prepared according to the following recipe:

a. 200 µg DNA was diluted in 50 µl of Opti-MEM and mixed gently.

b. Appropriate amount lipofectamine 2000 was diluted in 50 µl of Opti-MEM, RT/5min.

c. After the 5 min incubation, the two solutions mentioned above were mixed gently and incubated for 20 min at RT.

100 µl of complex was added to each well containing cells and medium. The cell culture medium was mixed gently by rocking the plate back and forth.

Cells were incubated at 37 °C in a CO_2 incubator for 18-48 hours prior to testing for transgene expression. Medium may be changed after 4-6 hours.

For other formats, see scaling up or down transfection mixture preparation (Table 10).

Table 10 DNA transfection mix preparation.

Culture vessel	Relative surface area	Volume of plating medium	Dilution medium	cDNA	Lipofectamine 2000
96-well	0.2	100 µl	2 x 25 µl	0.2 µg	0.5 µl
24-well	1	500 µl	2 x 50 µl	0.8 µg	2.0 µl
6-well	5	2 ml	2 x 250 µl	4.0 µg	10 µl
60 mm	10	5 ml	2 x 0.5 µl	8.0 µg	20 µl

3.3.4.4 Gene transient knockdown by siRNA transfection

ADAM9, 10, 17 and MMP14 knockdowns in SH-SY5Y or HEK293 cells were achieved by variant amount of siGENOME or on-target plus pool siRNA transfection according to the protocol described below. Table 11 lists the sequences, the concentrations and the sources of siRNA used in the gene knockdown experiments.

Table 11 siRNA used in the thesis.

Target genes	siRNA products	Cell lines	Concentration	Sources
ADAM9	siGENOME SMARTpool	HEK293	10 nM	Dharmacon
		SH-SY5Y	0.1 nM	Dharmacon
ADAM10	siGENOME SMARTpool	HEK293	10 nM	Dharmacon
		SH-SY5Y	0.1 nM	Dharmacon
		Differentiated SH-SY5Y	10 nM	Dharmacon
ADAM17	siGENOME SMARTpool	HEK293	10 nM	Dharmacon
	siGENOME on-target plus	SH-SY5Y	0.5 nM	Dharmacon
MMP14	siGENOME SMARTpool	SH-SY5Y	10 nM	Dharmacon
BACE1	Qiagen pool	Differentiated SH-SY5Y	10 nM	Qiagen

Gene transient knockdown was achieved by siRNA interference. For each well to be transfected, RNAi duplex-lipofectamine RNAiMAX complexes were prepared according to the following recipe (Table 12): siRNA was diluted in Opti-MEM Medium without serum and mixed gently. Lipofectamine RNAiMAX was added into diluted siRNA solutions and mixed gently. The mixture was then added into the wells and incubated for 10-20 min at RT. In siRNA interference, the complexes are prepared before cells and medium were added.

Table 12 siRNA interference transfection mix preparation.

Culture vessel	Relative surface area	Volume of plating medium	Dilution medium	siRNA (nM)	Liofectamine RNAiMAX
96-well	0.2	100 µl	20 µl	0.1-50	0.1-0.3 µl
48-well	0.4	200 µl	40 µl	0.1-50	0.2-0.6 µl
24-well	1	500 µl	100 µl	0.1-50	0.5-1.5 µl
6-well	5	2.5 ml	500 µl	0.1-50	2.5-7.5 µl
60 mm	10	5 ml	1 ml	0.1-50	5-15 µl
100 mm	30	10 ml	2 ml	0.1-50	15-35 µl

Appropriate number of cells was diluted in medium without antibiotics which gave 30-50% confluence 24 h after plating. The cell solution was added into each well and mixed gently by rocking the plate back and forth. Cells were incubated for 24-72 h at 37 °C in a CO_2 incubator until they were ready to assay for gene knockdown.

3.3.4.5 SH-SY5Y cells neuronal differentiation

To neuronally differentiate SH-SY5Y cells, naive cells were seeded in 75 cm^2-flask with 40% cell density in DMEM/F12 + 15% FCS + NEAA + P/S medium. 24 h later, medium was change with 25 μM ATRA in DMEM/F12 + 3% FCS + P/S. 72 h later, cells differentiated into neuronal like cells. Cells were digested up with trypsin-EDTA and seeded out for experiments.

3.3.4.6 Generation of hAPP overexpressing cell lines

APP overexpressing SH-SY5Y and HEK293 cell lines were generated by DNA transfection. Before transfection, P12-Hygro-hAPP cDNA plasmid was linearized by single restriction enzyme digestion; linearized cDNA was transfected into cells by lipofectamine 2000. APP Overexpressing cells were selected by cell culture medium with specific antibiotics (hygromycin). Then cells were kept in culture with antibiotics, and very low density of cell solution was seeded into 96-well plate. There were only one or two cells in each well. Single cell clones were picked up and cells were spreaded in larger culture format. Cell lysate from each single cell clone was collected and APP expression level was detected to finally select the hAPP highly expressed cell lines.

3.3.4.7 Generation of gene stable knockdown cell lines

ADAM10 stable knockdown SH-SY5Y and HEK293 cells and BACE1 stable knockdown HEK293 cells were generated by shRNA viral transduction. The following shRNAs were used in the thesis (Table13). The table lists the sequences and sources of the shRNA. They were cloned into different vectors for viral transduction (see details in Table 6).

Table 13 shRNA used in the thesis.

Name	shRNA sequence
Con.	5'-CCCCcaacaagatgaagagcaccaaTTCAAGAGAttggtgctcttcatcttgttgTTTTTGGAAA-3'
ADAM10-sh6	5'-CCCCaagttgcctcctcctaaaccactTTCAAGAGAgtggtttaggaggaggcaacttTTTTTGGAAA-3'
ADAM10-sh7	5'-CCCCgacatttcaacctacgaatttTTCAAGAGAaaattcgtaggttgaaatgtcTTTTTGGAAA-3'
ADAM10-sh9	5'-CCCCggacaaacttaacaacaatTTCAAGAGAattgttgttaagtttgtccTTTTTGGAAA-3'
BACE1-sh1	5'-CCCCggtacaaagactgcgtcttgattcaagagatcaagacgcagtctttgtaccTTTTTGGAAA-3'
BACE1-sh2	5'-CCCCgcgtgacagaacagagaaatcttcaagagagatttctctgttctgtcacgcTTTTTGGAAA-3'

For virus production, transfection reaction mixture was prepared according to the following recipe:

For 6-well plate format (for each well):

250 µl Opti-MEM + 6.3 µl lipofectamine 2000, RT/5 min;

250 µl Opti-MEM + 1.3 µg transfer vector (PLVTHM, PLKO) + 0.75 µg sPAX2 + 0.45 µg pcDNA3.1-Delta.Zeo (-)-VSV-G.

The combination of both parts was mixed. 500 µl mixture was added into each well, RT/20 min.

1.5 ml, $8*10^5$/ml HEK293T/low passage cells were seeded in each well in Opti-MEM + 10% FCS.

24 h later, medium was changed by 2 ml packaging medium: DMEM High Glucose + Pyruvat + NEAA + 10% FCS.

24 h later, the viral transduction was performed. Viral supernatant was filtered through 0.45 µm filter from VWR directly into a 6-well plate with about $0.5\text{-}1*10^6$ targeting cells. 24 h after the viral transduction, 2 ml fresh normal medium was changed each well. Cells were kept into culture. Stable knockdown cells then seeded in 6-cm dishes and conditioned medium was collected for analysis.

3.3.4.8 Cell lines freezing

To freeze the cell lines, cells were washed after reaching 70-80% confluence with 2 ml sterile PBS and detached by 2 ml of PBS-EDTA (PBS with 25 M EDTA) or trypsin-EDTA solution. Cells were resuspended with 3 ml fresh medium and centrifuged down at 1000 rpm/5 min. Subsequently, cell pellets were resuspended in 1 ml freezing medium (FCS with 10% DMSO) and added into the freezing tubes. The cells were frozen at -80 $^{\circ}$C and transferred for long-term storage in liquid nitrogen.

3.3.4.9 Cells treatments with chemical compounds

For pharmacological activation or inhibition of either α-secretase or β-secretase cleavage of APP, cells were treated with TAPI, PMA or C3 respectively. For HEK293 cells, cells were seeded in 6-well plates with $2*10^5$/ml density, 1.5 ml/well in DMEM + 10% + P/S; for differentiated SHSY5Y cells, cells were seeded in 6-cm dishes with $4.5*10^5$/ml density, 3 ml/dish in DMEM/F12 + 15% FCS + NEAA + P/S, followed by compounds treatment 24 h later. TAPI was added to the fresh cell culture medium at 50 μM concentration, 2 ml/dish with DMSO as the control; samples were collected 24 h later. PMA was added to the fresh cell culture medium at 1 μM concentration, 2 ml/dish with ethanol as the control; samples were collected 4 h later. C3 was added to the fresh cell culture medium at 2 μM concentration, 1 ml/dish with DMSO as the control for 2 h; medium was then changed by 2 ml fresh medium with 1 μM C3 each well; samples were collected 24 h later. For γ-secretase inhibition and APP CTFs detection, cells were treated with DAPT. Control or ADAM10 shRNA tranduced SH-SY5Y cells were seeded in 6-cm dishes with $3*10^5$/ml density, 3 ml/well in DMEM/F12 + 15% FCS + P/S. Cells were treated with DAPT (1 μM) for 24 h, then supernatant and cell lysate were collected.

3.4 Protein biochemical experiments

3.4.1 Equipments and consumables

Equipments/consumables	Manufacturers
Aβ peptides 3-plex ELISA plates	MSD
Blotter criterion	Bio-Rad
Cell Lifter	Corning
Cooling centrifuge 5417R	Eppendorf
Film processor 2000 IR	CAWO
Heating block MR Hei-Tec	Heidolph instrument
Gel Transfer filter paper	Schleicher & Schuell
Glass plates	Bio-Rad
Immobilon-P membrane, PVDF, 0.45 μM	Millipore
Microplate (96-well, transparent)	Nunc
Mini-PROTEAN 3 Electrophoresis cell	Bio-Rad
Mini-PROTEAN Tetra cell	Bio-Rad
Mini Trans-Blot Transfer	Cell Bio-Rad

Equipments/consumables	Manufacturers
Nitrocellulose membrane (Protran)	Schleicher & Schuell
Powerpac basic power supply	Bio-Rad
Power pac HC	Bio-Rad
PowerWave microplate spectrophotometer	BioTek
sAPPα+sAPPβ ELISA plate	MSD
Scanner scanjet 5590P	HP
Sector imager	Meso Scale Discovery
Shaker 3015	GFL
Shaker KM-2	Edmund Buehler
X-ray films (SuperRX)	Fujifilm

3.4.2 Reagents

Reagents	Manufacturers
Acrylamid (Tris-Tricine gels)	Biomol
Acrylamid solution (37.5:1 / 40 % (w/v))	Serva
Ammonium persulfate (APS)	Roche
BCA Assay protein quantitation Kit	Uptima, Interchim
Bovine serum albumin (BSA)	Uptima, Interchim
β-Mercaptoethanol (β-ME)	Merck
ECL Western blotting detection system	Amersham Biosciences
ECL Plus Western blotting detection system	Amersham Biosciences
E. O. S. Developer	AGFA
E. O. S. Fixer	AGFA
Glycine	Biomol
I-block	Tropix
Isopropanol	Merck
MSD read buffer T	Meso Scale Discovery (MSD)
N-N'-Methylene-bisacrylamide (bisacrylamide)	Serva
Nonidet P-40 (NP-40)	Sigma
Protein A sepharose (PAS)	Sigma
Protease inhibitor (PI)	Sigma
Sodium dodecyl sulfate (SDS)	Calbiochem
SeeBlue plus 2 pre-stained standard	Invitrogen
Skimmed milk powder (instant)	Frema
TEMED (N,N,N',N'-Tetramethylethylendiamine)	Merck
Tricine	Biomol
Tween-20	Merck

3.4.3 Buffers

Buffers	Recipes
10 * anode buffer (Tris-tricine gel)	1 M Tris-HCl (242.2 g) pH 8.9 add ddH_2O to 1 L
10 * cathode buffer (Tris-tricine gel)	0.1 M Tris-HCl (121.1 g) 0.1 M tricine (179.2 g) add ddH_2O to 1 L 10% SDS was added in working solution
4* SDS-probe buffer	4 ml 20% glycerin 4 ml 20% SDS 1 ml β-ME 1.25 ml 1 M Tris pH 6.8 10 µl 10% Bromphenol blue
10* TBS	24.23 g Tris 80 g NaCl add H_2O to 1 L, pH was adjusted to 7.4
Acrylamid solution (49.5% total, 3% crosslinker) (Tris-Tricine gel)	48 g acrylamide 1.5 g bisacrylamide add ddH_2O to 100 ml, 4 $^{\circ}$C store
Blocking buffer (ELISA)	1% BSA in TBS-T
Blocking solution (Western blotting)	5% skimmed milk powder or 0.8 g I-block in PBS-T (PBS with 0.5% Tween20)
Gel buffer (Tris-tricine gel)	3 M Tris-HCl (182 g) dissolved in 300 ml ddH_2O and adjust pH to 8.45 0.3% SDS (1.5 g) add ddH_2O to 500 ml, 4 $^{\circ}$C store
Hypotonic buffer:	10 nM Tris pH 7.4 1 mM EDTA 1 mML EGTA
Low Tris buffer	0.4% SDS in ddH_2O

Buffers	Recipes
Primary antibody solution (western blotting)	Antibodies with appropriate concentration (see details in Table 16) 0.25% BSA 0.05% sodium azide in PBS-T
SDS-PAGE electrophoresis buffer	25 mM Tris 0.2 M glycine 0.1% SDS
Secondary antibody solution (western blotting)	Antibodies with appropriate concentration (see details in Table 17) 0.25% BSA in PBS-T
Solubilization buffer	$50*10^{-3}$ M Tris (pH 7.5) $150*10^{-3}$ M NaCl 1% NP-40
STEN buffer	0.05 M Tris-HCl pH 7.6 0.15 M NaCl 2 mM EDTA 0.2% NP-40
STEN-NaCl	STEN-buffer with 175 mM NaCl
STEN-SDS	STEN-buffer with 0.1% SDS
Transfer buffer	25 mM Tris 0.2 M glycine
Upper Tris buffer	0.5 M Tris-HCl pH 6.8 0.4% SDS in ddH_2O

3.4.4 Methods

3.4.4.1 Cell lysate preparation

Cells in the plate were taken out from the incubator and put on the ice. Cells were washed with PBS in the plate. And then PBS was carefully removed. Lysis buffer (Solubilization buffer:PI 1:500 mixture) was added directly into the well and the plate was incubated on ice for 10 min. Cell lysates were piptteted up and down several times. Then cell

suspension was transferred to a tube and incubated for another 10 min on ice. The lysis solution was centrifuged at 13,000 rpm/4 °C/5 min. Cell lyates were stocked at -20 °C.

3.4.4.2 Membrane preparation

For 6-well plate format, supernatants were collected and cells were washed with 1 ml/well PBS. PBS was carefully removed and discarded. Cells were scraped up in 750 µl PBS to tubes (2 wells for 1 sample). 1/3 of cell suspension was taken out to make the cell lysate and protein concentration measurement. The rest of cell suspension was spinned down and the supernatant was discarded. Cells were resuspended by 800 µl hypotonic buffer with 1:500 PI and incubated on ice for 10 min. The lysis was pipetted up and down for 15 times with 1 ml syringe and 0.6 mm syringe tip. The lysis was spinned down at 3200 rpm/4 °C/15 min to pellet nuclei and cytoskeleton. 750 µl supernatant was transferred to a new tube and membranes were pelleted at 13,000 rpm/4 °C/30 min. Supernatants were discarded and the pellets were resuspended with sample buffer and boiled at 900 rpm/95 °C/5 min.

3.4.4.3 Protein concentration measurement

Protein lysates were measured in duplicate in a 96-well plates with 10 µl per well. BCA reagent B:A were 1:50 diluted and add 200 µl of the mixture into every well. The mixture was incubated it at 37 °C/30 min. The determination of extinction was taken place photometrically at 562 nm after extrapolation of the curve.

3.4.4.4 Immunoprecipitation (IP)

Aim proteins were concentrated by immunoprecipitation (IP). For IP, 25 µl PAS solution was incubated with specific antibodies in 500 µl PBS at 4 °C for 30 min. The antibodies used in the thesis for IP, together with their epitopes, species, dilutions and sources were listed in the following table (Table 14).

Table 14 Antibodies used for immunoprecipitation (IP) in thesis.

Antibodies	Epitopes	Species	Dilutions	Sources
5313	APP ectodomain	mouse	1:150	Eurogentec
6687	APP C-terminal	rabbit	1:100	Eurogentec
BAWT 8C10	sAPP	mouse	1:10	Dr. E. Kremmer
3552	APP ectodomain	mouse	1:400	Eurogentec

The mixture was centrifuged at 4 °C/8000 rpm/1 min. The PAS pellet was then washed with 1 ml PBS-T (PBS with 0.5% Tween20). 500 µl sample supernatant was added into each tube and incubated on the shaker at 4 °C/2 h. The mixture was centrifuged at 4 °C/8000 rpm/1 min. The PAS pellet was then washed with 1 ml STEN-NaCl, STEN-SDS and STEN respectively. Sample buffer (30 µl) was then added onto the precipitation and boiled at 95 °C/5 min.

3.4.4.5 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins according to their molecular size were separated by a one-dimensional electrophoresis separation under denatured conditions. The mini-gel system of Bio-Rad was used For the SDS-PAGE, which has a collection gel of about 1 cm and a separation gel of about 7 cm. There were only gels with 15 samples with a pocket comb thickness of 1.5 mm. Before using, the glass was cleaned by 70% ethanol and then added the resolving gel to a height of about 2 cm below the upper edge. Isopropanol was casted above. After the polymerization (about 15 min), the isopropanol was discarded and the separator was dried with absorbent paper, then the stacking gel was located above the area with a comb inserted. After the completely polymerization of electrophoresis gels (approximately 15-20 min), the comb was carefully removed and the gels can be moved to the electrophoresis chamber. The detail information about the gel preparation is listed in the following table (Table 15).

Table 15 Resolving and stacking gels preparation (for two gels).

	Resolving					stacking
	4%	**8%**	**10%**	**12%**	**15%**	
H_2O	9.8 ml	7.8 ml	6.66 ml	6.0 ml	4.0 ml	3.25 ml
30% Acrylamid	2.2 ml	4.2 ml	5.33 ml	6.0 ml	8.0 ml	0.65 ml
Low Tris	4.0 ml	4.0 ml	4.0 ml	4.0 ml	4.0 ml	1.25 ml (Upper Tris)
TEMED	30 µl	30 µl	30 µl	30 µl	30 µl	30 µl
10% APS	60 µl	60 µl	60 µl	60 µl	60 µl	60 µl

The running buffer was filled, so the pockets of the gels were rinsed several times. After comparing the protein concentrations, protein extracts were mixed with loading buffer, cooked 5 min at 95 °C and applied on the gels. 7 µl "Blue Lake plus 2" was loaded as the molecular weight standard. The stacking was carried out by 90 V initially and after the entry of proteins into the separator, the voltage was increased to 140 V.

For Tris-tricine gels, gels were prepared according to the recipe shown in Table 16. Gels were charged with separate cathode/anode buffer at 80 V first, and at 120 V later.

Table 16 Tris-tricine gels preparation (for two gels).

	16.5% separating gel	**10% spacer gel**	**4% stacking gel**
Acrylamid (49.5%)	3.5 ml	1.5 ml	0.5 ml
Gelbuffer	3.5 ml	2.5 ml	1.55 ml
Water		3.5 ml	4.2 ml
32% glycerol	3.5 ml		
10% APS	32.5 µl	35 µl	25 µl
TEMED	3.25 µl	4 µl	5 µl
For one gel	5 ml	2.5 ml	2 ml

3.4.4.6 Western-blot

Transfer:

After separation, proteins were transferred to a PVDF membrane or nitrocellulose membranes in a transfer chamber. Before the transfer, the membranes are activated by isopropanol incubation for 10 s. After activation, the membrane was briefly washed with deionized water and rinsed in transfer buffer to be equilibrated. For the transfer the Bio-rad mini-gel-transfer system was used. The transfer took place at a constant flow of 400 mA for 65 min at RT.

Transfer structure:

• cathode plate (black)

• porous sponge in transfer buffer

• two layers of gel blotting paper in transfer buffer

• SDS-polyacrylamide gel

• PVDF membrane with isopropanol and activated in transfer buffer

• two layers of gel blotting paper in transfer buffer

• porous sponge with transfer buffer

• anode plate (transparent)

Blotting

After the transfer, nonspecific protein binding sites on the PVDF membrane were saturated 30 min at RT in blocking solution. The membrane was then washed and 2 * 5 min with PBS-T (PBS with 0.5% Tween20) on the shaker. Subsequently, the membrane was incubated with the primary antibodies for 1 h at RT or overnight at 4 °C on the shaker. The primary antibodies used in the thesis, together with their epitopes, species, dilutions and sources are listed in the following table (Table 17).

Table 17 Primary antibodies used for western blot in the thesis.

Antibodies	Epitopes	Species	Dilutions	Sources
ADAM9	Human ADAM9 C-terminus	Rabbit	1:1000	Cell Signaling
ADAM10	Human ADAM10 C-terminus	rabbit	1:10000	Cell signaling
ADAM17	Human ADAM17 C-terminus	rabbit	1:1000	Oncogene
MMP14	Human MMP14 C-terminus	rabbit	1:1000	Abcam
BACE1	Human BACE1	mouse	1:2000	Dr. B. Vassar
2C11	APP C-terminus	mouse	1:10	Dr. E. Kremmer
calnexin	Calnexin	rabbit	1:2000	Sigma
β-actin	β -actin	mouse	1:5000	Sigma
22C11	APP N-terminus	mouse	1:200	Dr. K. Beyreuther
WO2	Human sAPPα+sAPPβ'	mouse	1:1000	Dr. K. Beyreuther
4B4	Human sAPPα	Rat	1:10	Dr. E. Kremmer
14D6	Human sAPPα	Rat	1:10	Dr. E. Kremmer
192wt	sAPPβ	Rabbit	1 μg/ml	Dr. D. Schenk
2D8	Aβ	Rat	1 μg/ml	Dr. E. Kremmer
6687	APP C-terminus	rabbit	1 μg/ml	Dr. C. Haass

It was followed by a washing of the membrane for 3 * 10 min with PBS-T. The corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were then incubated for 45 min at RT on the PVDF membrane. The secondary antibodies used in the thesis, together with their epitopes, species, dilutions and sources are listed in the following table (Table 18).

Table 18 Secondary antibodies used for western blot in the thesis.

antibodies	epitopes	Species	dilutions	Source
mouse-HRP	mouse IgG	goat	1:10000	Promega
rabbit-HRP	rabbit IgG	goat	1:10000	Promega
rat-HRP	rat IgG	goat	1:5000	Santa Cruz

Finally, the membranes were again four times per 5 min with PBS-T buffer washed. The detection of antibodies coupled with the proteins were carried out and developed by using X-ray films. For detection of weak signals, ECL plus western blotting detection system was used.

3.4.4.7 Enzyme-linked immunosorbent assay (ELISA)

Aβ or sAPPβ levels from cell supernatant of some experiments were measured by Aβ or sAPPα+β ELISA. For Aβ detection, Aβ triplicate plate was incubated with blocking buffer at 4 °C overnight on a horizontal shaker. The plate was washed with TBS-T (TBS with 0.5% Tween20) for three times. And then 25 µl samples were added into each well. On top of that, another 25 µl human Aβ 6E10 antibody (1:50 dilution in blocking buffer) was added. The plate was incubated at RT on a horizontal shaker for 2 h. The plate was washed with TBS-T for three times. 150 µl reading buffer T (1:2 dilution in H_2O) was added into each well, being very careful to avoid bubbles and incubated for 10 min at RT. The signals were measured on Sector Imager.

For sAPPβ detection, sAPPα+β triplicate plate was incubated with blocking buffer at 4 °C overnight on a horizontal shaker. The plate was washed with TBS-T (TBS with 0.5% Tween20) for three times. And then 25 µl samples were added into each well. The plate was incubated at RT on a horizontal shaker for 1 h. The plate was washed with TBS-T and then 25 µl sAPPα+β antibody (1:50 dilution in blocking buffer) was added each well.

The plate was incubated at RT on a horizontal shaker for 1 h. The plate was washed with TBS-T for three times. 150 µl reading buffer T (1:2 dilution in H_2O) was added each well, being very careful to avoid bubbles and incubated for 10 min at RT. The signals were measured on Sector Imager.

4 Results

4.1 Antibody characterization

Antibodies for sAPPα detection in previous work were typically WO2 and 6E10 which recognize the epitope between the β- and β'- cleavage site. That means those antibodies did not specially detect sAPPα, but also sAPPβ'. To specifically detect the APP α-secretase cleavage product sAPPα, two new monoclonal antibodies 4B4 and 14D6 were generated and used in this thesis, which was against a peptide comprising amino acids 11-16 of the Aβ sequence.

To examine the detection specificity of the antibody, cDNAs of full-length APP, sAPPβ, sAPPβ', sAPPα ending after amino acid 16 of Aβ, together with truncated sAPPα that ends at the 14^{th}, 15^{th} amino acid of Aβ domain were cloned. cDNAs of different APP fragments were transfected to human embryonic kidney 293 (HEK293) cells. Samples were collected and detected by different antibodies. Western blot results showed that 4B4 antibody detected sAPPα and the sAPPα truncated by one amino acid (sAPP15) (Figure 7 B). And indeed, it did not detect full-length APP in the cell lysate. Conditioned medium blotting also showed that 22C11 detected all species since it binds to the N-terminus of APP, WO2 detected sAPPα and β' since the epitope was between β- and β'-site. 4B4 antibody detected only sAPPα, and even, only the sAPPα fragments that ended longer than the 15^{th} amino acid which implied that Gln (glutamine) is necessary for the antibody epitope recognition and it does not detect sAPPβ'.

To further test antibody 4B4, whether under the conditions, which increase or decrease sAPPα generation, would lead to a corresponding change in the 4B4 signal was examed. HEK293 cells were treated with the metalloprotease inhibitor TAPI-1 to reduce APP shedding or with PMA to increase APP shedding (Figure 7 C). TAPI-1 inhibited almost completely sAPPα generation by 4B4 detectionn. In contrast, total sAPP shedding was not as strongly reduced by 22C11 detection, consistent with the fact that this antibody detects all sAPP species. PMA strongly up-regulsted total APP shedding, but the extent

of the increase of sAPPα by 4B4 detection was much more pronounced. Antibody W02, which detects the combined signal of sAPPα and sAPPβ', detected intermediate changes between 22C11 and 4B4. The other antibody 14D6 was also validated specific as sAPPα antibody (data not shown). Taken together, these experiments show that antibody 4B4 and 14D6 specifically detect sAPPα, not any other soluble APP fragments, in contrast to other commonly used APP antibodies. In the following experiments, sAPPα detections were all accomplished by these two antibodies ensuring the specificity of sAPPα signals.

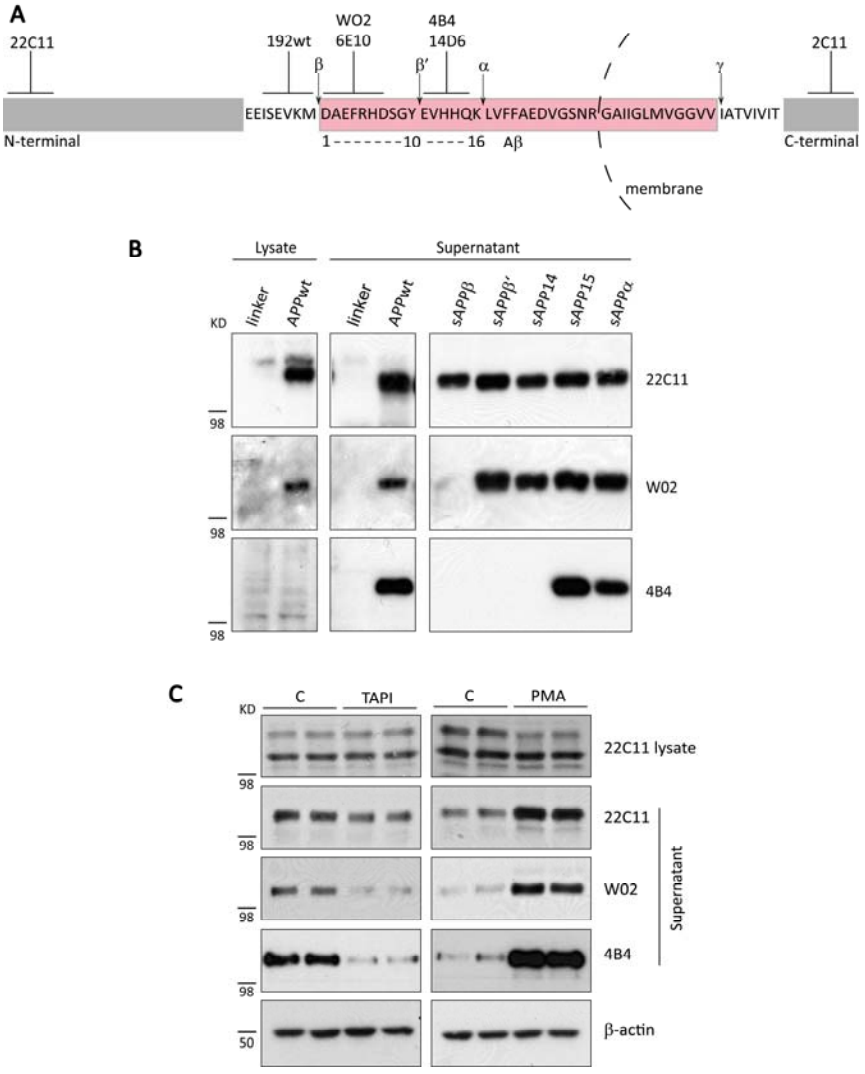

Figure 7 Characterization of newly generated sAPPα-specific antibody 4B4.

A) The exact APP cleavage site of α-, β- and γ-secretases. β- and γ-secretase cleavage generate the Aβ peptide, while α-secretase α cleaving site and β-secretase β' cleaving are located inside the Aβ domain. 22C11 and 2C11 recognize the N- and C-terminal of APP respectively. WO2 and 6E10 recognize the epitope between α- and β'-cleavage site, which means they detect both sAPPα and sAPPβ'. 4B4 and 14D6 recognize 11-15th amino acid of Aβ domain which detect specifically sAPPα.

B) Full-length APP, sAPPα, sAPP15, sAPP14, sAPPβ' and sAPPβ were transfected into HEK293 cells. Conditioned medium was collected to test different antibodies. Antibody 22C11 detects all secreted APP species, antibody W02 detects sAPPα+β', whereas antibody 4B4 specifically detect sAPPα and sAPP15.

(C) HEK293 cells were treated with TAPI-1 (50 µM, 24 h) or PMA (1 µM, 4 h). Immunoblots of conditioned media and cell lysates were probed with antibody 22C11 (total sAPP), W02 (sAPPα+β') and the sAPPα-specific antibody 4B4. Cellular APP is present in a lower molecular weight immature form and a higher molecular weight mature form and was detected with 22C11. The β-actin blot serves as a loading control. The reduction by TAPI-1 and the increase in shedding by PMA are more pronounced when analysed with the α-cleavage-specific antibody 4B4, compared with the other antibodies.

4.2 Transient knockdown of ADAM10 in wild type cells suppressed sAPPα generation

4.2.1 ADAM9, 10, 17 and MMP14 knockdowns in SHSY5Y cells

To identify the physiologically relevant α-secretase, the candidate α-secretase ADAM9, 10, 17 and MMP14 were knocked down by siRNA transfection in neuroblastoma SH-SY5Y cells. Cell toxicity was observed obvious initially when 50 nM concentration of siRNA was used, especially upon ADAM17 siRNA transfection. However, the toxic effect was abolished when lower concentration of siRNA was used. At the low concentration of siRNA pools, a good knockdown efficiency (~75% - 90%) was achieved for ADAM9, 10, 17 and MMP14 (Figure 8 A, C). Levels of cellular APP as well as of the control membrane protein calnexin were not affected. Among these knockdown experiments, only when ADAM10 was knocked down, a reduction of sAPPα generation was observed (to ~10%), which was consistent with the remaining expression level of ADAM10. The other APP cleavage fragments were also analyzed. The total sAPP detected by 22C11 decreased to 40% upon ADAM10 knockdown, while the combination of sAPPα+β' detected by WO2 decreased to 20%. When ADAM10 was knocked down, the expression levels of other ADAM proteases did not have a major change, implying that they did not compensate for the loss of ADAM10 (Figure 8 B). ADAM9, 17 and MMP14 knockdowns led to only mild changes in all APP cleavage fragments. There might be some reduction of sAPPα generation upon ADAM9, 17 and MMP14 siRNA transfection, but it was only a trend and not statistically different compared to control siRNA. The results indicated that the ADAM10 was the only protease that was required for APP α-secretase cleavage under physiological condition in SH-SY5Y cells.

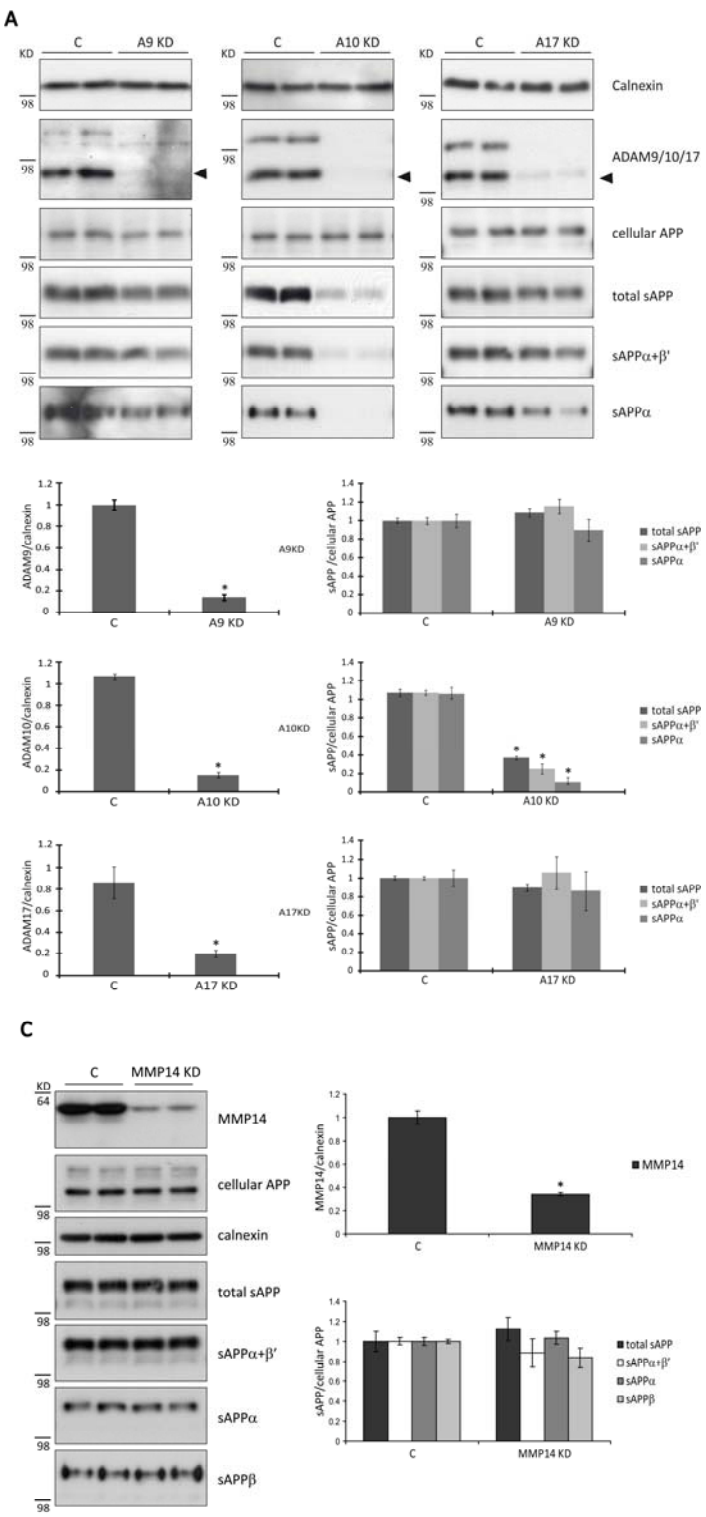

Figure 8 Transient knockdown of ADAM10 suppresses α-secretase cleavage of endogenous APP in SH-SY5Y cells.

A) SH-SY5Y cells were transfected with siGENOME siRNA pools directed against the proteases ADAM9, 10 or 17 or with control siRNA (labeled as C, A9 KD, A10 KD or A17 KD). All three proteases were detected by western blot in membrane preparations. The mature form of ADAM9, 10 and 17 are indicated by arrows. Calnexin served as a loading control. Cell lysates were analysed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (4B4). sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting with 4B4. The bar charts were the quantification of western blot signals. ADAMs expression levels were normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n =10, * p ‹ 0.05)

B) Knockdown of either ADAM9, 10 or 17 does not alter the protein levels of immature and mature forms of the other proteases. Membrane preparations of SH-SY5Y cells either treated with siRNA pools against ADAM9, 10, 17 or a control siRNA pool were probed for ADAM9, 10 and 17. As loading control membranes were analyzed for calnexin.

C) SH-SY5Y cells were transfected with siGENOME siRNA pools directed against MMP14 or with control siRNA (labeled as C or MMP14 KD). MMP14 expression level was detected by western blot by lysate preparations. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (14D6). The bar charts were the quantification of western blot signals. MMP14 expression level was normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 4, * p ‹ 0.05)

4.2.2 ADAM9, 10 and 17 knockdowns in HEK293 cells

The same experiments were performed using HEK293 cells to further validate the constitutive α-secretase of APP in a second cell line. siRNA transfection showed less cell toxicity in HEK293 cells even at higher concentrations in comparison to SH-SY5Y cells. Nevertheless the results perfectly fit to what was observed in the previous cell line. ADAM10 knockdown led to the reduction of sAPPα signal to ~ 10%, but the other ADAMs did not, while MMP14 was not detectable in HEK293 cells (Figure 9). The total sAPP and sAPPα+β' decreased to 60% and 40%, respectively (Figure 9). The results from HEK293 showed that ADAM10 is again the physiologically relevant constitutive α-secretase of APP.

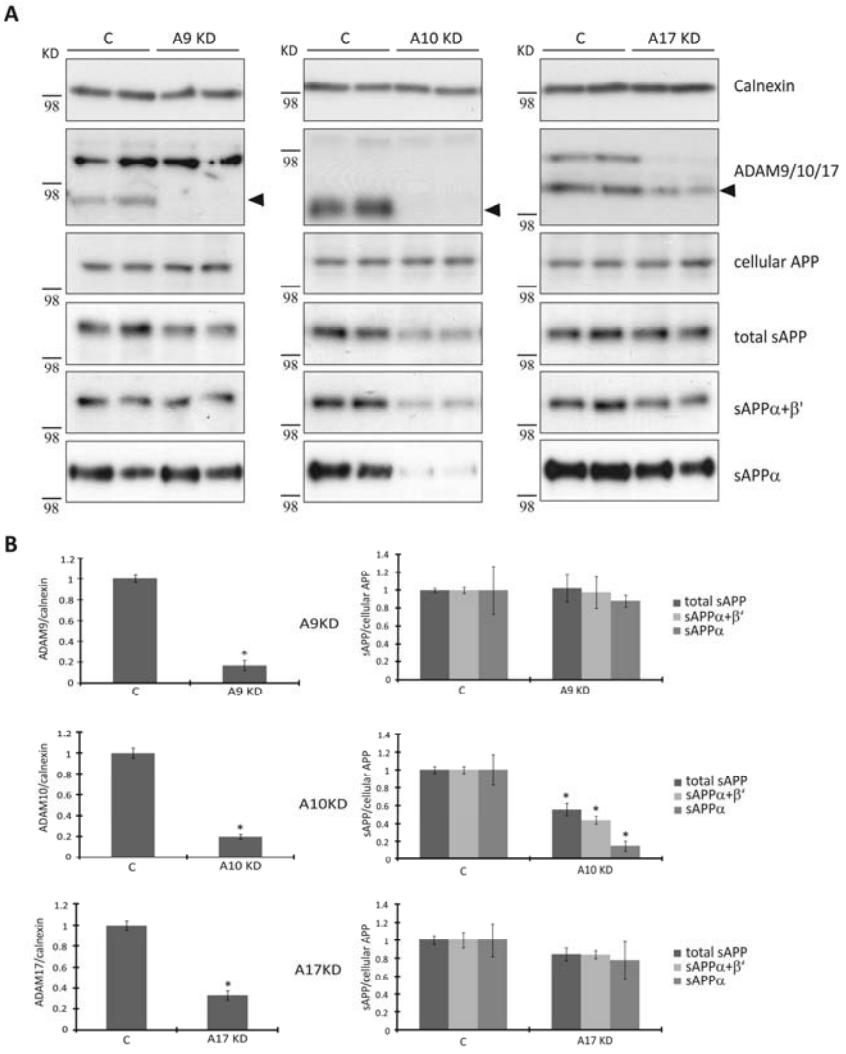

Figure 9
Transient knockdown of ADAM10 suppresses α-secretase cleavage of endogenous APP in HEK293 cells.
A) HEK293 cells were transfected with siGENOME siRNA pools directed against the proteases ADAM9, 10 or 17 or with control siRNA (labeled as C, A9 KD, A10 KD or A17 KD). All three proteases were detected by western blot in membrane preparations. The mature form of ADAM9, 10 and 17 were indicated in the blot by arrows. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (4B4). sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting with 4B4. B) The bar charts were the quantification of western blot signals. ADAMs expression levels were normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n =10, * p ‹ 0.05)

4.3 Transient knockdown of ADAM10 in APP overexpressing cells suppressed sAPPα generation

4.3.1 ADAM10 knockdown in APP-SH-SY5Y

Most previous studies about identification of APP α-secretase were performed using APP overexpressing cells due to the detection difficulty of endogenous APP. To further confirm that ADAM10 also the α-secretase of overexpressed APP, APP overexpressing cells were also used in the experiments. ADAM10 siRNA transfections were performed in stably APP overexpressing SH-SY5Y cells, followed by the APP shedding behavior analysis (Figure 10). The reduction was nearly as strong as in wild-type cells. Total sAPP, sAPPα+β' and sAPPα decreased to ~40%, 35% and 30% respectively. The higher remaining sAPP fragments could be owing to such high expression level of APP that some other proteases had to participate in the APP cleavage.

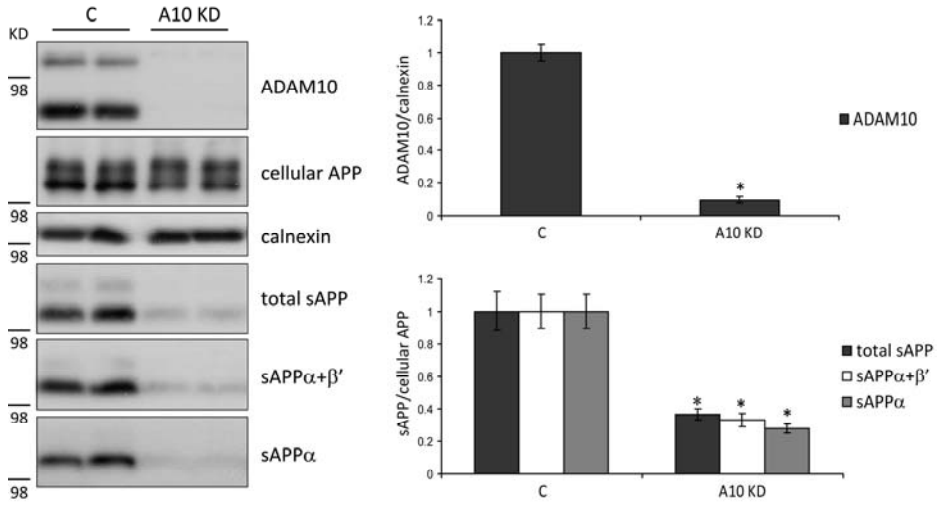

Figure 10 Transient knockdown of ADAM10 suppresses α-secretase cleavage of overexpressed APP in SH-SY5Y cells.
APP-SH-SY5Y cells were transfected with siGENOME siRNA pools directed against ADAM10 or with control siRNA (labeled as C or A10 KD). ADAM10 was detected by western blot in membrane preparations. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (4B4). The bar charts were the quantification of western blotting signals. ADAM10 expression level was normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 8, * p ‹ 0.05)

4.3.2 ADAM10 knockdown in APP-HEK293 cells

ADAM10 was also knocked down in APP overexpressing HEK293 cells (Figure 11). The results were similar to the findings from APP-SH-SY5Y cells. ADAM10 knockdown led to the strong decrease of sAPPα signal to ~ 20%. However, the reduction of total sAPP was stronger than that of sAPPα+β' signal. That could be due to the off-target effect from siRNA transfection on the APP expression vector or the promoter inside. That could also provide a clue for the explanation that in the previous research ADAM10 was not the only proteases contributed to the APP α-cleavage.

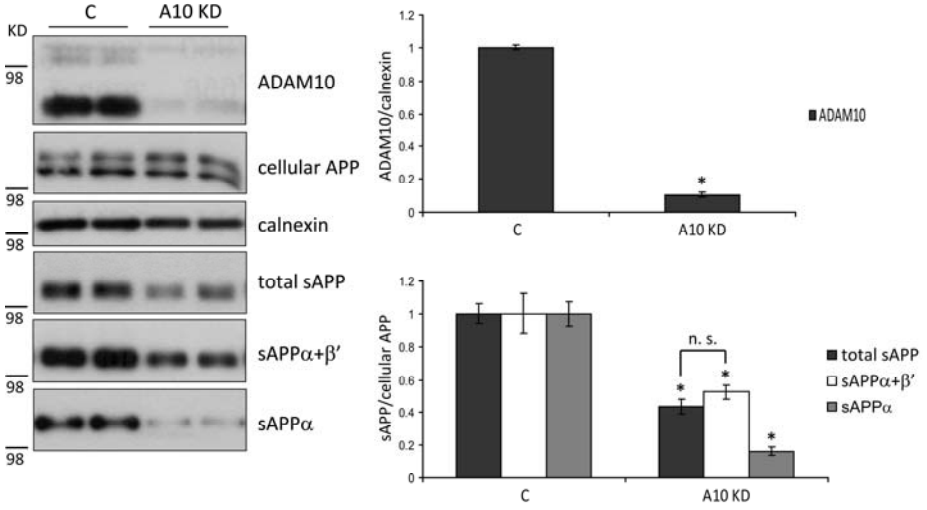

Figure 11 Transient knockdown of ADAM10 suppresses α-secretase cleavage of overexpressed APP in HEK293 cells.
APP-HEK293 cells were transfected with siGENOME siRNA pools directed against ADAM10 or with control siRNA (labeled as C or A10 KD). ADAM10 was detected by western blot in membrane preparations. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (4B4). The bar charts were the quantification of western blot signals. ADAM10 expression level was normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 8, * p ‹ 0.05)

4.4 Stable knockdown of ADAM10 in wild type cells suppressed sAPPα generation

To further validate the results from the transient knockdown of ADAM10, HEK293 cells with a stable knockdown of ADAM10 were generated using lentiviruses expressing two different shRNA sequences against ADAM10 or a negative control shRNA. It provided the evidence that in the long time period of loss of function of ADAM10, no other proteases could compensate for it and play the role of APP α-secretase. Similar to the

results from the transient ADAM10 knockdown, sAPPα decreased when ADAM10 was knocked down in HEK293 cells (Figure 12). In both shRNA transduced HEK293 cells, total sAPP, sAPPα+β', and sAPPα decreased to ~50%, ~40%, and ~15%; while sAPP and even Aβ level did not have major change due to ADAM10 knockdown compared to the control. sh7 shRNA performed more potent than sh9, represented by the ADAM expression level together with the corresponding sAPPα signal reduction. Quantification from the blots fit nicely with that of results from transient ADAM10 siRNA transfection. The similar results were obtained from SH-SY5Y cells transduced with the same shRNAs (data not shown). Results from ADAM10 transient knockdown and stable knockdown cells demonstrated that no other α-secretase candidates could compensate for the loss of ADAM10 either in short time or after long time culture period.

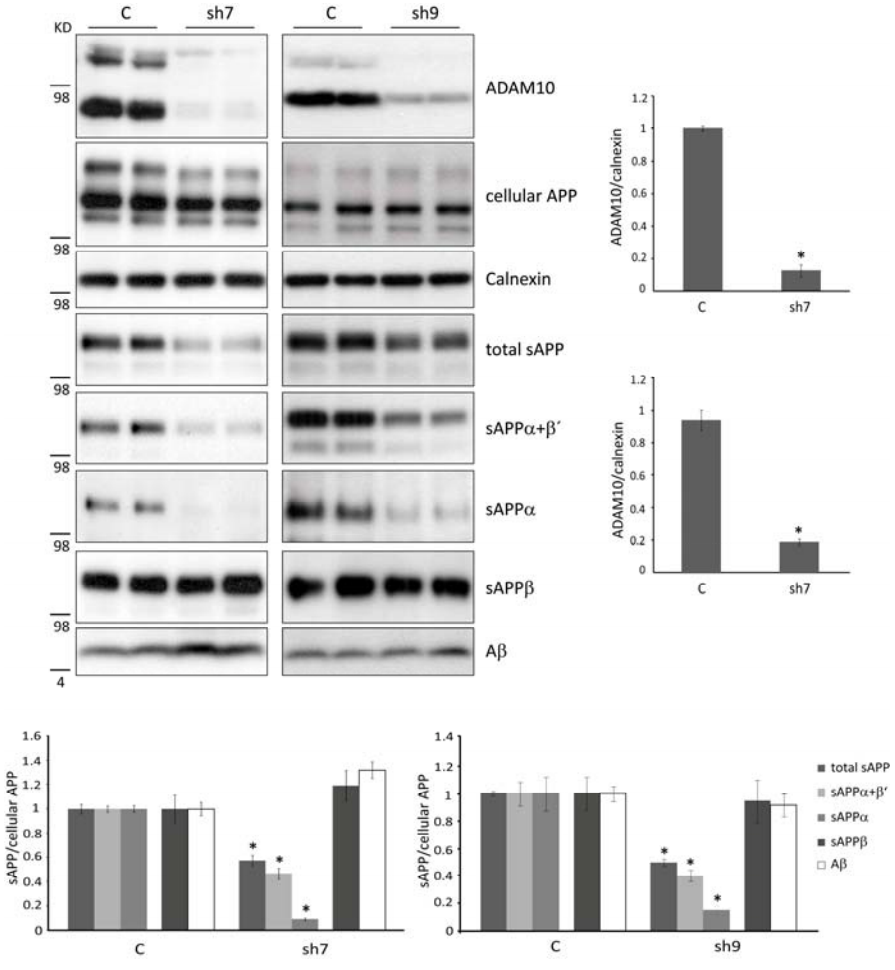

Figure 12 Suppressed α-secretase cleavage of endogenous APP in ADAM10 stable knockdown HEK293 cells. ADAM10 stable knockdown HEK293 cells were generated by shRNA viral transduction using two distinct shRNAs. In shRNA 7 and 9 transduced HEK293 cells (labeled as C, sh7 or sh9), ADAM10 was detected by membrane preparation. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (WO2), sAPPα (4B4), sAPPβ (192wt) and Aβ (2D8). sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting with 4B4. sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting with 192wt. Aβ signal was detected by 500 µl supernatant IP with 3552 and blotting with 2D8. The bar charts were the quantification of western blot signals. ADAM10 expression level was normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 6, * p ‹ 0.05)

4.5 ADAM10 knockdown in neuron-like cells suppressed sAPPα generation

In order to create a more neuronal like cellular condition and to verify the APP α-secretase in such a condition, SH-SY5Y cells were treated with all-trans retinoic acid (ATRA) in serum-free medium for 3 d and thereby differentiated into neuronal like cells. The western blot results showed that ATRA treatment and SH-SY5Y differentiation led to a mild increase in ADAM10 level, however, was not significant, together with the full length APP expression level (Figure 13 A). The APP shedding behavior of differentiated cells was not dramatically affected; secreted APP fragments, including total sAPP, sAPPα+β', sAPPα and sAPPβ, retained the similar level as the naive SH-SY5Y cells (Figure 13 A). To characterize shedding of APP in differentiated SH-SY5Y cells, siRNA transfections were performed (Figure 13 B). Upon the siRNA treatment, ADAM10 level in differentiated SH-SY5Y cells decreased to a very low level (to ~ 13%), together with the secreted APP α-cleavage fragment signals (to ~18%), similar to the results obtained in SH-SY5Y and HEK293 cells. Total sAPP and sAPPα+β' signal decreased as well but not as strong as sAPPα. Total sAPP decreased to ~50%, like the wide type SH-SY5Y cells upon ADAM10 siRNA transfection, consistent with the fact that SH-SY5Y cell differentiation did not affect the APP cleavage pattern. It implied that in the more neuronal like differentiated SH-SY5Y cells, ADAM10 played the major role of APP α-secretase as well.

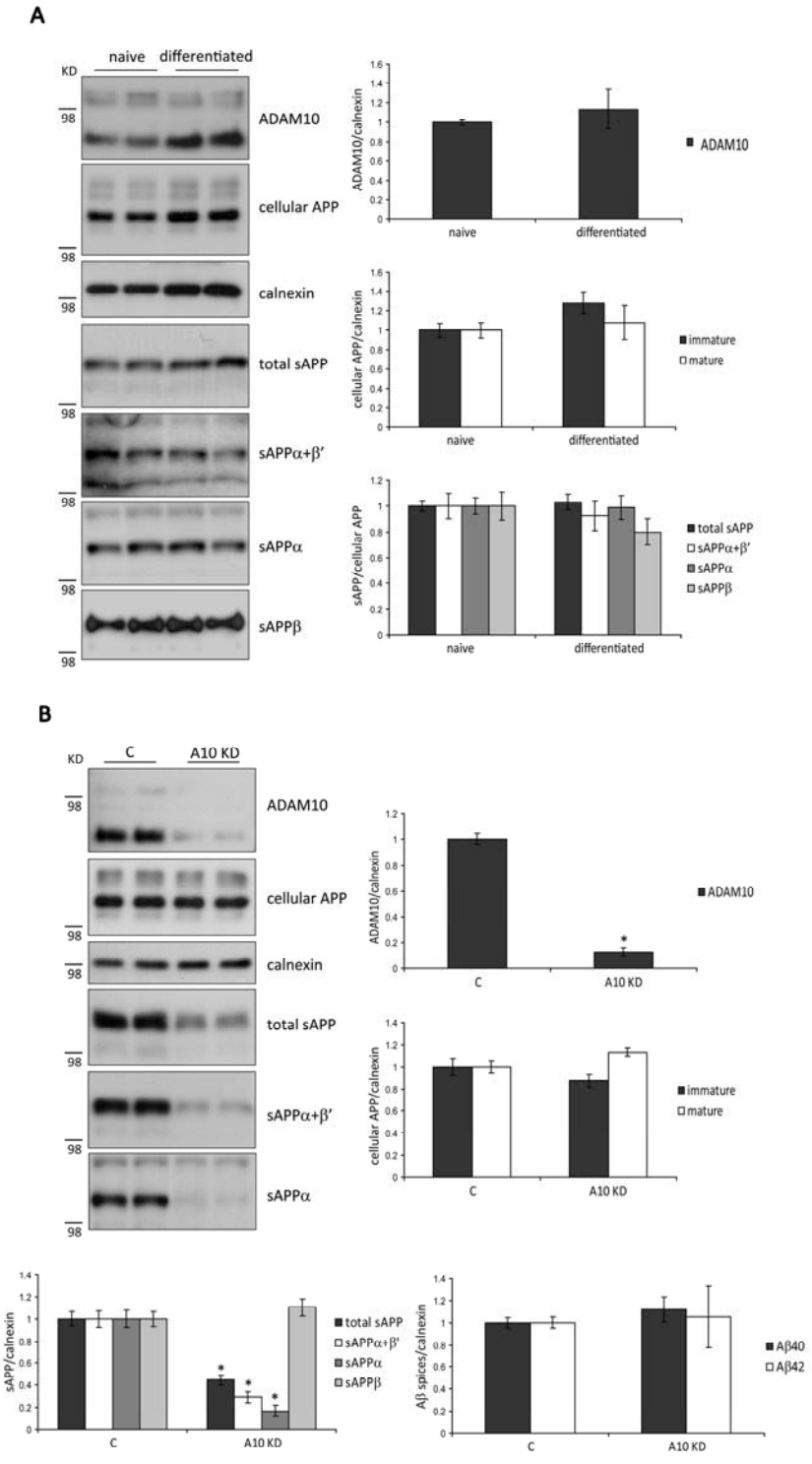

Figure 13
Transient knockdown of ADAM10 suppressed α-secretase cleavage of endogenous APP in neuron-like differentiated SH-SY5Y cells.
A) SH-SY5Y cells were treated with 25 µM ATRA in serum-free medium for 3 d to trigger the differentiation. 24 h conditioned medium was collected and western blot analysis was performed. ADAM10 was detected by membrane preparation. Total sAPP, sAPPα+β' and sAPPα signals were detected by 22C11, WO2 and 14D6 respectively. sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting by 192wt. Cellular APP was detected by lysate samples with 2C11. Calnexin was detected as a loading control. The bar charts were the quantification of western blot signals. ADAM10 expression level was normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 4, * p ‹ 0.05)
B) Differentiated SH-SY5Y cells were transfected with siGENOME siRNA pools directed against ADAM10 or with control siRNA (labeled as C or A10 KD). ADAM10 was detected by western blot in membrane preparations. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02), sAPPα (14D6) and sAPPβ (192wt). sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting by 192wt. Aβ spices were detected by Aβ triplicate ELISA. The bar charts were the quantification of western blot signals. ADAM10 expression level, cellular APP levels and sAPP fragments levels were normalized to the calnexin signal. (n = 8, * p ‹ 0.05)

4.6 ADAM10 is also APP α-secretase in serum-free medium

4.6.1 ADAM10 stable knockdown SH-SY5Y cells in serum-free medium

Serum in the cell culture media provides fundamental stimuli for the physiological signaling pathway. Thus further experiments were performed with serum-free cell culture medium to exclude a potential activating effect of APP α-secretase from serum. Because sAPPα are lower in the absence of serum (Pandiella & Massague, 1991), sAPPα in the conditioned medium was concentrated by immunoprecipitation (IP) and detected by western blot (Figure 14). The results showed that ADAM10 knockdown still led to strong reduction of sAPPα to ~10%, whose ratio was consistent with the figure obtained from normal cell culture conditions. The absence of serum seemed to only down-regulate the total APP secretion, but not affect the APP shedding pattern. It did not affect the ADAMs expression pattern in control, ADAM10 shRNA7 and 9 transduced SH-SY5Y cells, either.

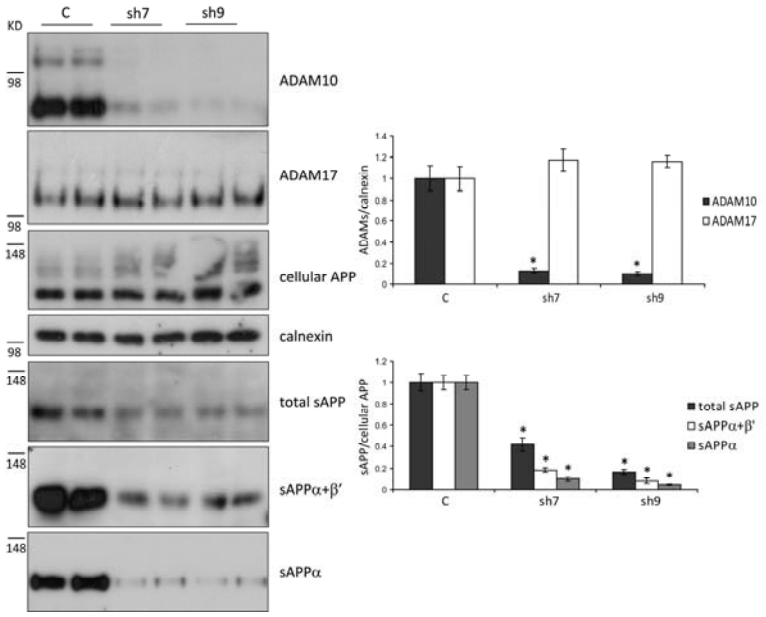

Figure 14 Suppressed α-secretase cleavage of endogenous APP in ADAM10 stable knockdown SH-SY5Y cells with serum-free medium.
ADAM10 stable knockdown SH-SY5Y cells (sh7, sh9) were pretreated with serum-free medium for 24 h, and then 24 h serum-free conditioned medium was collected. ADAM10 and 17 levels were detected by membrane preparation. Total sAPP and sAPPα+β' signals were detected by 22C11 and WO2 respectively. sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting by 14D6. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. ADAM10 and 17 expression levels were normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 6, * $p < 0.05$)

4.6.2 ADAM10 stable knockdown HEK293 cells in serum-free medium

The same experiments were also performed in HEK293 cells with a stable knockdown of ADAM10 cultured with serum-free medium (Figure 15). Similar results were achieved as in SH-SY5Y cells. In ADAM10 stable knockdown HEK293 cells, total sAPP, sAPPα+β' and sAPPα decreased, with the strongest reduction shown by sAPPα (to ~10%). Both cell lines demonstrated that ADAM10 was the physiological α-secretase of APP even without serum as a shedding trigger.

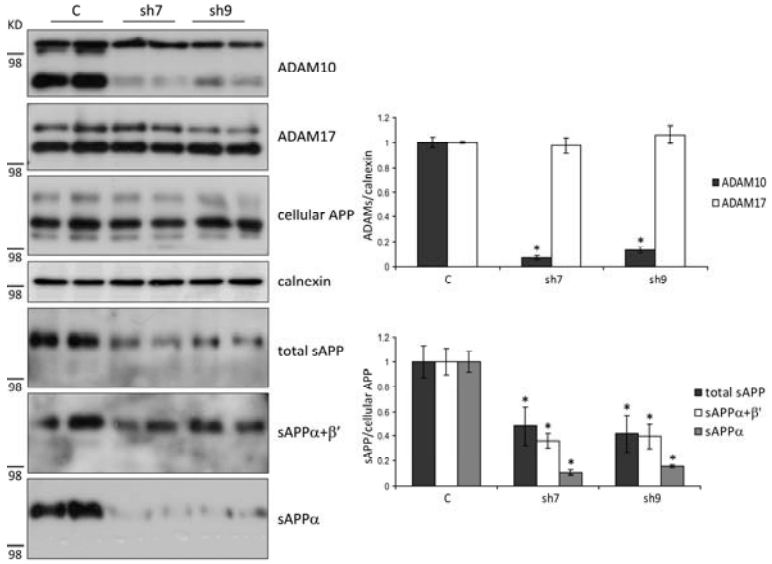

Figure 15 Suppressed α-secretase cleavage of endogenous APP in ADAM10 stable knockdown HEK293 cells with serum-free medium.
ADAM10 stable knockdown HEK293 cells (sh7, sh9) were pretreated with serum-free medium for 24 h, and then 24 h serum-free conditioned medium was collected. ADAM10 and 17 levels were detected by membrane preparation samples. Total sAPP and sAPPα+β' signals were detected by 22C11 and WO2 respectively. sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting by 14D6. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. ADAM10 and 17 expression levels were normalized to the calnexin signal. sAPP fragments levels were normalized to cellular APP signal. (n = 6, * p ‹ 0.05)

4.7 PMA induce APP α-shedding independent of ADAM10

PMA stimulates the cleavage of many cell surface membrane proteins by metalloprotease, including APP. PMA-induced shedding of APP requires ADAM17 activity, because this stimulation is abcent in ADAM17-deficient mouse embryonic fibroblasts (MEF) (Buxbaum *et al.*, 1998). Thus, whether ADAM10 was involved in the PMA stimulation of APP shedding was investigated next. To test this, wild type SH-SY5Y cells were transiently transfected with control siRNAs or siRNAs against ADAM9, 10 or 17 and

then treated with or without PMA (Figure 16). As expected, in control cells, PMA strongly increased sAPPα, in agreement with Figure 8 C. Knockdown of ADAM17 eliminated the PMA-induced increase in sAPPα production. However, neither ADAM10 nor 9 knockdown affected PMA induced APP shedding. This showed that ADAM10 was not required for PMA induction of APP shedding and suggested that under PMA-stimualtion conditions ADAM17 can directly cleave APP.

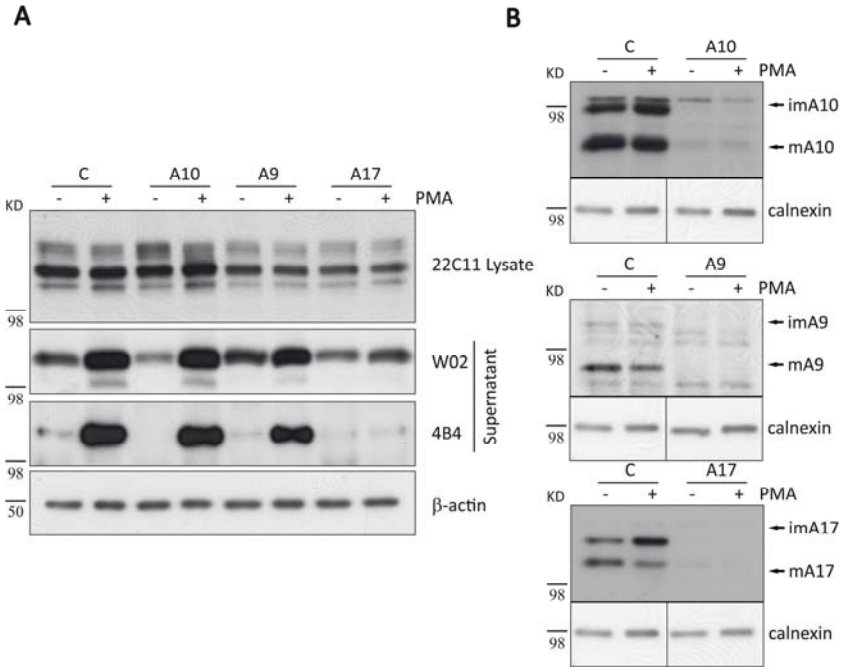

Figure 16 PMA-induced stimulation of APP shedding is independent of ADAM10, but requires ADAM17.
SH-SY5Y cells were either transfected with control siRNA pool or siRNA pools against ADAM9, ADAM10 and ADAM17; 2 d after transfection, cells were treated with 1 µM PMA (+) or ethanol (-) as solvent control for 4 h.
(A) Conditioned media were analyzed for sAPPα (4B4), sAPPα+β' (W02) and cell lysates were analyzed for cellular APP (22C11).
(B) Knockdown efficiency was analyzed by blotting against the different proteases ADAM9, 10 and 17 in membrane preparations of the respective experiments.

4.8 β-secretase does not compensate for α-secretase loss of function on APP cleavage

To study the compensatory effect of β-secretase in response of α-secretase downregulation, ADAM10 was genetically knocked down by siRNA transfection or pharmacologically inhibited. The corresponding β-secretase cleaved APP fragments were analyzed.

4.8.1 ADAM10 knockdown

As shown in Figure 12, ADAM10 knockdown led to the dramatic reduction of sAPP level. The β-secretase cleavage fragment was also detected in parallel by conditioned medium upon immunoprecipitation (IP) concentration and 192wt antibody blotting. Compared to control cells, endogenous sAPPβ and Aβ levels in HEK293 cells were unchanged for one ADAM10 knockdown construct (sh9), whereas a mild, but not significant, increase was observed for the other shRNA construct (sh7) (Figure 12). Similar results were obtained from ADAM10 knockdown experiments in differentiated SH-SY5Y cells. ADAM10 siRNA transfection led to the reduction of sAPPα signal but no upregulation of the β-secretrase cleaved fragments (Figure 13 B). Aβ levels (Aβ40, 42) showed only a trend to increase, but not statistically significant. This suggested that β-secretase did not compensate in response of α-secretase down-regulation in immortalized cell lines.

α- and β-secretases do not only generate sAPPα and sAPPβ. In parallel, they also generate the C-terminal fragments C83 and C99, respectively. Both fragments are further processed by γ-secretase, leading to a short half-life of the fragments, which makes it difficult to detect them at endogenous levels. In order to stabilize the endogenous C-terminal fragments of APP, SH-SY5Y cells were treated with the γ-secretase inhibitor DAPT (Figure 17). In control cells with normal γ-secretase activity, the α-secretase fragment C83 was clearly detected and was strongly reduced in the ADAM10 knockdown cells. Out of expectation, the β-secretase-cleavage product C99 was increased in this condition more than 2 fold upon ADAM10 knockdown. Besides, a mild increase of C89 was observed, which is the C-terminal fragment arising by BACE1 at β'-cleavage site. The increase in C99 and C89 is not consistent with sAPPβ levels, which were unchanged upon ADAM10 knockdown. The possible explanation is that C99 could also be processed by α-secretase. As ADAM10 is knocked down, together with the inhibition of γ-secretase activity, C99 was accumulated. These results from HEK293, SH-SY5Y and differentiated SH-SY5Y cells show that the strong reduction of α-cleavage does not

yield a correspondingly increased cleavage by β-secretase, which means β-secretase does not compensate for genetically loss of function of α-secretase on APP cleavage.

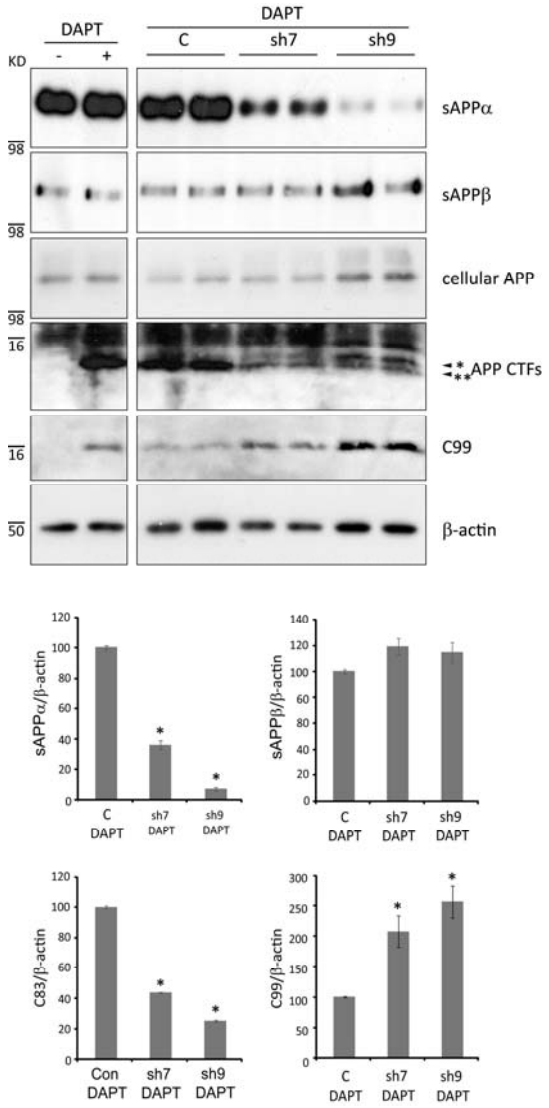

Figure 17 DAPT treatment in ADAM10 knockdown SHSY-5Y cells and APP CTFs analysis.
ADAM10 stable knockdown SHSY-5Y cells (sh7, sh9) were treated with DAPT (1 μM) for 24 h and conditioned medium was collected. sAPPα signal was detected by 500 μl supernatant IP with 5313 and blotting by 4B4. sAPPβ signal was detected by 500 μl supernatant IP with BAWT 8C10 and blotting by 192wt. APP CTFs signals were detected by 500 μl lysate IP with 6687 and blotting by 6687. C89 and C83 are indicated by * and ** respectively. C99 signal was detected by 500 μl lysate IP with 6687 and blotting by 2D8. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. All the data were normalized to the β-actin signal. (n = 6. * $p < 0.05$)

4.8.2 TAPI treatment

The metalloproteases inhibitor TAPI-1 is a kind of hydroxamate inhibitor which works as a metal chelator removing the Zn^{2+}-ion from metalloproteases resulting in the

inactivation of most of the metalloprotease (Arribas *et al.*, 1996). α-secretase at cell surface in absence of the metal co-factor can no longer cleave APP at α-site to release the soluble sAPPα. TAPI treatment in HEK293 and differentiated SH-SY5Y cells also reduced sAPPα generation without a change in β-secretase cleavage product (Figure 18). APP shedding was affected in the same pattern as observed in ADAM10 knockdown experiments. In HEK293 cells, total sAPP (22C11), sAPPα+β' (WO2), and sAPPα (14D6) fragments decreased, to ~ 59%, 36% and 23% respectively, which was consistent with ADAM10 knockdown results, while sAPPβ signal remained the same compared to the control (Figure 18 A). TAPI treatment performed similar in differentiated SH-SY5Y cells, down-regulating total sAPP, sAPPα+β' and sAPPα to ~49%, 27% and 22% respectively (Figure 18 B). Surprisingly, Aβ level was increased upon TAPI-1 treatment. In HEK293 cells, all three species of Aβ were detected by ELISA, only Aβ38 increased significantly compared to the control (Figure 18 A), although only to ~1.4 fold. While in differentiated SH-SY5Y cells, only Aβ40 was detectable and it increased even to ~2-fold compared to the control (Figure 18 B). Cellular APP and APP maturation ratio were not affected much by TAPI treatment, in both cell lines, indicating that TAPI treatment only led to the α-secretase dysfunction but did not affected APP trafficking and maturation. Taken together, these experiments suggest that β-secretase cleavage of APP does not have major change upon genetically loss of function of constitutive α-secretase. While pharmacological inhibition of α-secretase also does not affect sAPPβ production but has the tendency to increase Aβ levels, which may be because TAPI-1 also inhibited Aβ degradation enzymes activity. In words, the loss of function of α-secretase does not lead to a change of β-secretase cleavage of APP.

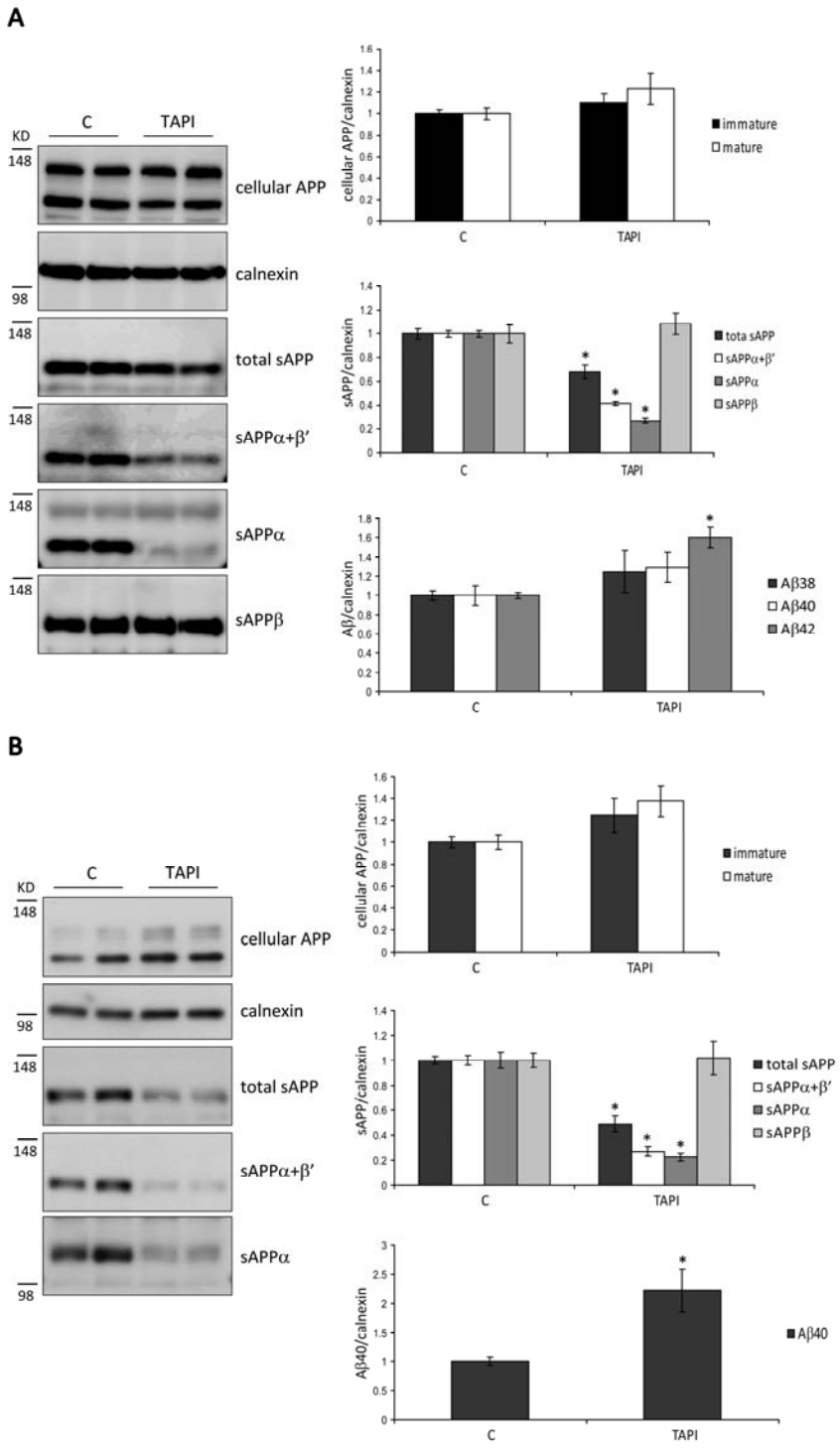

Figure 18 TAPI treatment in HEK293 and differentiated SH-SY5Y cells and APP shedding behavior analysis. A) TAPI treatment in HEK293 cells. HEK293 cells were treated with 50 µM TAPI in 1 ml medium for 24 h. DMSO was served as the control. Conditioned medium was collected and total sAPP, sAPPα+β' and sAPPα signals were detected by 22C11, WO2 and 14D6 respectively. sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting by 192wt. Aβ spices were detected by Aβ triplicate ELISA. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. All the data were normalized to the calnexin signal. (n = 8, * p ‹ 0.05) B) TAPI treatment in differentiated SH-SY5Y cells. Differentiated SH-SY5Y cells were treated with 50 µM TAPI in 1 ml medium for 24 h. DMSO was served as the control. Conditioned medium was collected and total sAPP, sAPPα+β' and sAPPα signals were detected by 22C11, WO2 and 14D6 respectively. sAPP β signal was detected by sAPPα+sAPPβ triplicate ELISA. Aβ40 level was detected by Aβ triplicate ELISA. Cellular APP was detected by lysate samples with 2C11.The bar charts were the quantification of western blot signals. All the data were normalized to the calnexin signal. (n= 6, * p ‹ 0.05)

4.9 α-secretase does not compensate for β-secretase loss of function on APP cleavage

To further test a possible compensation between α- and β-secretase of cleavage APP, now β-secretase was reduced and an effect on α-secretase cleavage of APP was analyzed, both genetically and pharmacologically.

4.9.1 BACE1 knockdown

Expression of the β-secretase BACE1 was reduced by lentiviral knockdown constructs (sh1 and sh2) in HEK293 cells (Figure 19 A). This resulted in a strong reduction of sAPPβ and Aβ generation, but not in a significant increase in sAPPα generation (~ 1.2 fold). This clearly indicates that α-secretase cleavage did not compensate for the loss of β-secretase activity on APP cleavage. Similar results were obtained from differentiated SH-SY5Y cells transiently transfected with BACE1 siRNA (Figure 19 B). BACE1 knockdown led to a mild reduction in total sAPP and sAPPα+β' fragments, and only a mild but not significant increase in the sAPPα level. In differentiated SH-SY5Y cells, Aβ and sAPPβ levels were detected by ELISA. Both signals decreased to lower than 5% compared to the control, which provided nice control evidence for the specificity of the ELISA detection.

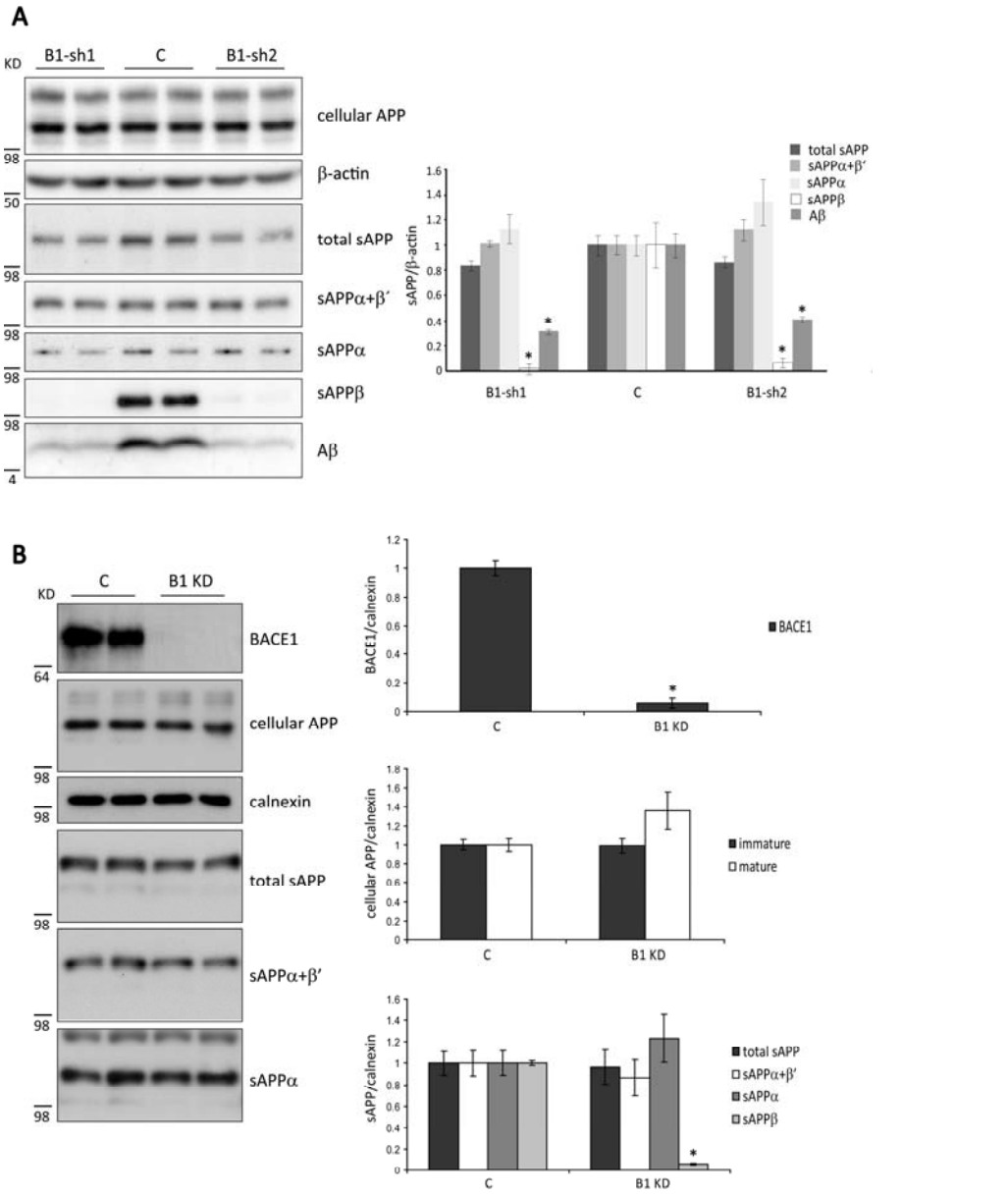

Figure 19 BACE1 knockdown in HEK293 and differentiated SH-SY5Y cells and APP shedding behavior analysis.

A) BACE1 stable knockdown HEK293 cells were generated by shRNA viral transduction. In shRNA No.1 and No.2 transduced HEK293 cells, total sAPP and sAPPα+β' signals were detected by 22C11 and WO2 respectively. sAPPα signal was detected by 500 µl supernatant IP with 5313 and blotting with 4B4. sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting by 192wt. Aβ signal was detected by 3 ml supernatant IP with 3552 and blotting with 2D8. Cellular APP was detected by lysate samples with 22C11. The bar charts were the quantification of western blot signals. All the data were normalized to the β-actin signal. (n = 6, * p ‹ 0.05)

B) Differentiated SH-SY5Y cells were transfected with Qiagen siRNA pools directed against BACE1 or with control siRNA (labeled as C or B1 KD). BACE1 was detected by western blot in membrane preparations. Calnexin was detected as a loading control. Cell lysates were analyzed for cellular APP and conditioned media for total sAPP (22C11), sAPPα+β' (W02) and sAPPα (14D6). sAPPβ signal was detected by sAPPα+sAPPβ ELISA.. The bar charts were the quantification of western blot signals. All the data were normalized to the calnexin signal. (n = 6, * p ‹ 0.05)

4.9.2 C3 treatment

The β-Secretase BACE1 is an aspartic-acid transmembrane protease which has two active site aspartate residues in its extracellular protein domain. β-secretase inhibitor C3 is a kind of statine-based BACE inhibitor which constitutes a tetrahedral, hydroxymethylene-isosteric replacement for the scissile peptide bond, mimicking the putative reaction intermediate and resulting in potent inhibition of aspartic proteases (Marcinkeviciene *et al.*, 2001). C3 treatment completely blocked the β-secretase activity in HEK293 cells, sAPPβ signal was even not detectable (Figure 20 A). Nevertheless, α-secretase cleaved APP derivative did not have any change upon the β-secretase inhibition (~ 96% compared to control). A similar result was observed in differentiated SH-SY5Y cells, too, where C3 treatment led to ~28% total sAPP reduction, strong decrease of sAPPβ but no effect on sAPPα (~ 1.1 fold compared to control) (Figure 20 B). In addition, C3 treatment in differentiated SH-SY5Y cells increased cellular APP maturation to ~1.5 fold. In conclusion, both the genetic and the pharmacological approach showed that the loss of function of β-secretase did not result to a compensation of α-secretase cleavage of APP.

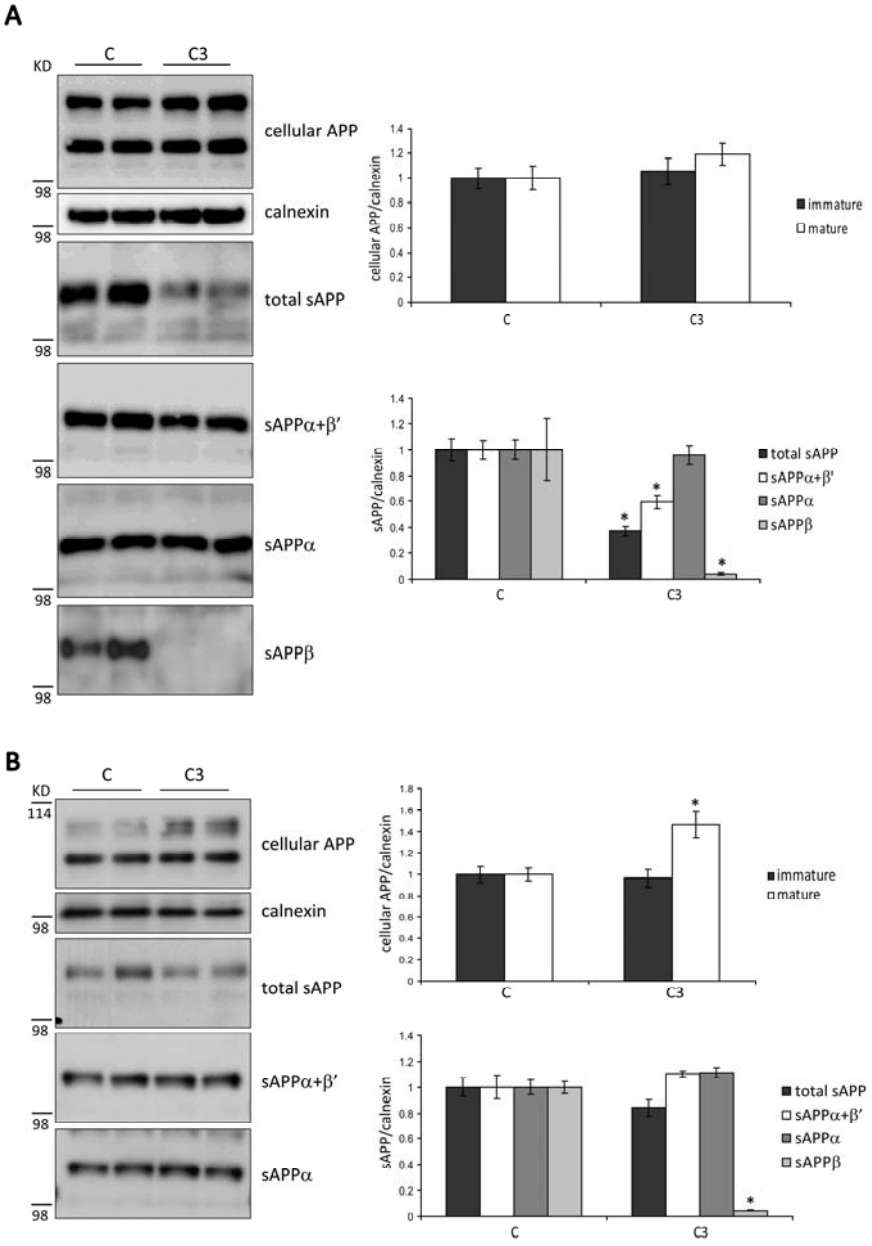

Figure 20 C3 treatment in HEK293 and differentiated SH-SY5Y cells and APP shedding behavior analysis.

A) C3 treatment in HEK293 cells. HEK293 cells pre-treated with 2 µM C3 in 500 µl medium for 2 h, followed by 24 h incubation with 1 µM C3 in 1 ml medium. DMSO was served as the control. Conditioned medium was collected and total sAPP, sAPPα+β' and sAPPα signals were detected by 22C11, WO2 and 14D6 respectively. sAPPβ signal was detected by 500 µl supernatant IP with BAWT 8C10 and blotting by 192wt. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. All the data were normalized to the calnexin signal. (n = 6, * p ‹ 0.05)

B) C3 treatment in differentiated SH-SY5Y cells. Differentiated SHSY-5Y cells pre-treated with 2 µM C3 in 500 µl medium for 2 h, followed by 24 h incubation with 1 µM C3 in 1 ml medium. DMSO was served as the control. Conditioned medium was collected and total sAPP, sAPPα+β' and sAPPα signals were detected by 22C11, WO2 and 14D6 respectively. sAPPβ signal was detected by sAPPα+sAPPβ ELISA. Cellular APP was detected by lysate samples with 2C11. The bar charts were the quantification of western blot signals. All the data were normalized to the calnexin signal. (n = 6, * p ‹ 0.05)

5 Discussion

A diverse range of membrane proteins undergoes proteolysis by a group of enzymes referred to collectively as “secretases” or “sheddases”. The cleavage generally occurs close to the extracellular surface of the membrane, releasing physiologically active protein ectodomains (Hooper *et al.*, 1997). One of the secretases is α-secretase and it is considered important in AD since it cleaves APP inside the Aβ domain and could preclude the AD-related neuro-toxic Aβ generation (see APP details in 1.2.3). The identification of the α-secretase of APP and its regulation would help to better understand APP processing and the molecular parthenogenesis of AD.

Furthermore, the α-secretase-cleaved secreted form of APP has also been considered neuroprotective and neurotrophic. From some early reports, sAPP or sAPPα was verified to have the effect to enhance the neuronal survival and neurite extension (Araki *et al.*, 1991; Milward *et al.*, 1992; Small *et al.*, 1994; Ohsawa *et al.*, 1997; Luo *et al.*, 2001a; Young-Pearse *et al.*, 2008) and play a role in functional synaptic formation (Morimoto *et al.*, 1998). Some other research demonstrated that sAPP could protect cells against toxic stress (Mattson *et al.*, 1993; Schubert & Behl, 1993; Furukawa *et al.*, 1996; Mattson *et al.*, 1999; Stein *et al.*, 2004; Gralle *et al.*, 2009). *In vivo* research further indicated that sAPP possessed the memory-enhancing effects in an AD mouse model (Meziane *et al.*, 1998). One of the APP α-secretase candidates ADAM10 was also reported to be able to increase cortical synaptogenesis (Bell *et al.*, 2008). That makes it more important and interesting to investigate the APP α-secretase in order to either understand its biological function and to evaluate its therapeutic potential.

5.1 Identification of APP α-secretase

The research about the identification of APP α-secretase has passed last decade but no homogenous results achieved. The candidates include some ADAM and MMP proteases (see details in Table 3). The ADAMs are a family of transmembrane proteins with important roles in regulating cell phenotype via their effects on cell adhesion, migration, proteolysis and signaling; The functional ADAM metalloproteinases are involved in

"ectodomain shedding" of diverse growth factors, cytokines, receptors and adhesion molecules (reviewed by Edwards *et al.*, 2008). The application of some metalloprotease inhibitors had already indicated that ADAM9, 10 and 17 were involved in the α-cleavage of APP and release of secreted APP α-fragment (Arribas *et al.*, 1996; Buxbaum *et al.*, 1998; Koike *et al.*, 1999; Lammich *et al.*, 1999; Skovronsky *et al.*, 2000; Lopez-Perez *et al.*, 2001; Slack *et al.*, 2001; Kim *et al.*, 2008). Cell free or cell based experiments indicated that ADAM9, 10 and 17 cleaved APP at the α-site between the 16th (Leu) and 17th (Lys) amino acid of Aβ domain (Lammich *et al.*, 1999; Roghani *et al.*, 1999; Koike *et al.*, 1999; Lopez-Perez *et al.*, 2001; Slack *et al.*, 2001; Hotoda *et al.*, 2002). Gene inactivation experiments revealed that APP shedding reduced to 20%-60% but was never fully suppressed (Hartmann *et al.*, 2002; Weskamp *et al.*, 2002; Asai *et al.*, 2003; Allison *et al.*, 2004; Jorissen *et al.*, 2010). Paradoxically, ADAM9, 10 or 17 knockdowns or knockouts showed no change in APP shedding in some other research (Buxbaum *et al.*, 1998; Hartmann *et al.*, 2002; Weskamp *et al.*, 2002). These heterogeneous results have led to the conclusion that ADAM9, 10 and 17 may all together contribute to α-secretase activity and that in the absence of one of them, the other proteases can still mediate APP α-secretase cleavage.

As shown in the results part (see details in 4.2 - 4.6), to reveal the exact identity of APP α-secretase, gene knockdowns of the candidates ADAM9, 10, 17 and MMP14 were performed in different cell lines upon different conditions. In the following table, the experiments carried on about APP α-secretase identification are summarized (Table 19). The results show that by using the new, sAPPα specific antibodies 4B4 and 14D6 (see details in 4.1), only the inactivation of ADAM10 but not the other metalloproteases suppressed sAPPα generation completely in immortalized cell lines, neuronal like cells and primary cultured neurons (Kuhn *et al.*, 2010). That means ADAM10 is the only physiologically relevant constitutive α-secretase of APP.

Table 19 Identification of constitutive α-secretase of APP.

	Cell lines	siRNA transfection	shRNA viral transduction
More neuronal like cells ↓	HEK293	ADAM9 KD → sAPPα -- **ADAM10 KD → sAPPα ↓** ADAM17 KD → sAPPα --	**ADAM10 KD → sAPPα ↓ (in present or absent of serum)**
	APP-HEK293	**ADAM10 KD → sAPPα ↓**	
	SH-SY5Y	ADAM9 KD → sAPPα -- **ADAM10 KD →sAPPα ↓** ADAM17 KD → sAPPα -- MMP14 KD → sAPPα --	**ADAM10 KD → sAPPα ↓ (in present or absent of serum)**
	APP-SH-SY5Y	**ADAM10 KD → sAPPα ↓**	
	Differentiated SH-SY5Y	**ADAM10 KD → sAPPα ↓**	

"↓" represents reduction; "--" represents no change.

5.1.1 ADAM10 is the only physiologically relevant α-secretase of APP

The requirement of ADAM10 in APP α-cleavage was double validated by transient and stable knockdown cells (see details in 4.2 – 4.4). This is particularly remarkable, because although ADAM10 and 17 can cleave similar peptides *in vitro* at the same peptide bonds, regarding to their distinct role in biology, they appear to have quite different substrate specificity *in vivo* (Caescu *et al.*, 2009). If compared to the other APP sheddase BACE1 which has been demonstrated as the unique protease responsible for the APP β-site cleavage (Cai *et al.*, 2001; Luo *et al.*, 2001b), it would be reasonable to assume there is only one protease responsible for α-cleavage of APP. Furthermore, cytochemical evidence shown by *in situ* hybridization supported the ADAM10 but not ADAM17 as the authentic α-secretase of APP in human cortical neurons (Marcinkeviciene *et al.*, 2001), which implied the uniquity of APP α-secretase. Results from my experiments show ADAM10 is the key player for APP α-cleavage.

The novel antibodies 4B4 and 14D6 greatly improve the analysis of APP shedding because they specifically detect the α-secretase cleaved APP without contribution of any other APP cleavage products, such as sAPPβ or sAPPβ' (see details in 4.1). Using these new antibodies, siRNAs and shRNAs against ADAM10 almost abolished the sAPPα

signal in SH-SY5Y and HEK293 cells which implied that ADAM10 is the only constitutive α-secretase in the physiological condition (see details in 4.2-4.4). ADAM9, 17 and MMP14 knockdowns lead to hardly any change in sAPPα level, meaning these three proteases do not play the role of α-secretase of APP in normal condition (see 4.2.1). Only in HEK293 cells, the ADAM17 knockdown led to a very modest decrease in total sAPP, raising the possibility that in specific cell lines ADAM17 may have a modulatory function in sAPPα generation (see 4.2.2).

In previous research, the gene inactivation could never fully eliminate the sAPPα generation. That could be due to two possibilities. On one hand, antibodies used previously also detect sAPPβ', which is the alternative derivative from BACE1 cleavage but not affected by metalloproteases inactivation. The new antibodies 4B4 and 14D6 provide accuracy and specificity for sAPPα detection, which exclude the recognition of any other APP ectodomain fragments. On the other hand, most of the previous research was performed by single siRNA or shRNA. The potential off-target effects from one single siRNA or relatively high siRNA concentration can not be ignored (Jackson *et al.*, 2003). Furthermore, I manage to use the combination of different single siRNA, to decrease the siRNA concentration to a very low level and to use the Dharmacon on-target plus siRNA to minimize the off-target effects, especially in case of the siRNA against ADAM17. The on-target plus siRNA is designed in seed region which is reported to be able to reduce miRNA-induced off-targets effects (Birmingham *et al.*, 2006; Anderson *et al.*, 2008). The abolishment of cell toxicity was achieved but no change or only a very mild reduction of sAPPα production was observed. The findings provide evidence that a physiological condition for the experiment was created, preventing as much as possible the artificial factors, to finally achieve the conclusion that ADAM10 is the only physiologically relevant α-secretase of APP.

5.1.2 Other APP α-secretase candidates do not compensate for the loss of function of ADAM10

The functional compensation among protein subtypes belonging to the same protein family seems to be important for the regulation of the homeostasis of the cell. One easy

example is the APP family which is supposed to play an important role in early development. The APP family knockout mice showed that the single knockdown of APP did not lead to major impairment of embryonic development since it could be compensated by its homologue APLP2 (Heber *et al.*, 2000; Wang *et al.*, 2005). ADAM10, as shown to be the APP α-secretase, however, seems not to be supported by other ADAM proteases under physiological conditions according to the experiments.

The studies in HEK293 and SH-SY5Y cells demonstrated that the α-secretase candidates did not compensate for each other when one of them was knocked down transiently by siRNA transfection, in which the loss of function lasted in the time period less than 72 h (see details in 4.2 and 4.3). Following, stable ADAM10 knockdown cell lines were generated (see details in 4.4). In this condition, ADAM9 or 17 did not play the role of APP α-secretase in a long-term loss of function of ADAM10, either. This confirmed that ADAM10 is the only constitutive α-secretase of APP in physiological conditions. Moreover, ADAM10 expression level did not increase as the results of the loss of function of ADAM9 and 17, which indicated that ADAMs do not compensate for each other on APP α-cleavage (see 4.2.1).

5.1.3 ADAM10 is involved in the constitutive α-cleavage of APP

APP α-secretase cleavage occurs constitutively and it is mediated by ADAM10, as shown in this thesis. In addition, a heterogeneous group of molecules can stimulate APP α-secretase shedding, which is referred to as regulated α-secretase cleavage (see details in Table 4). Actually, the serum included in the cell culture medium consists of many kinds of proteins and small molecules such as growth factors, hormones and cytokines. This combination of proteins can activate α-secretase activators (Pandiella & Massague, 1991). To exclude the effect from serum, ADAM10 stable knockdown cells were then cultured in serum-free medium (see details in 4.6). Although the total sAPP level was very low due to the absence of serum, ADAM10 still showed a major contribution to the α-secretion of APP. Serum only up-regulated the base line of APP shedding but did not affect the function of ADAM10 as the unique α-secretase.

5.1.4 ADAM17 is involved in PMA-regulated α-cleavage of APP

To further validate the findings concerning the physiological relevance of ADAM10, the question whether this secretase might also be involved in the inducible shedding was addressed. Different stimuli have been reported to activate APP α-cleavage: the PACAP (pituitary adenylate cyclase-activating polypeptide) peptide and PMA. The PACAP peptide appeared to stimulate the ADAM10 cleavage of APP (Kojro *et al.*, 2006), suggesting that ADAM10 is not only the constitutive α-secretase, but also contributes to the regulated α-secretase activity. PMA is the most frequently used APP α-secretase activator (see details in Table 5). The results showed that PMA activation does not require ADAM10, but ADAM17, which is in agreement with a previous publication using ADAM17-deficient mouse fibroblast cells (MEF) cells (Buxbaum *et al.*, 1998). These data suggest that ADAM10 plays the role under constitutive conditions but ADAM17 needs to be activated, although ADAM10 can be activated in some condition as well. Under normal physiological conditions, the constitutive APP α-shedding is only mediated by ADAM10. How do ADAM10 and 17 work under pathophysiologically relevant conditions would be interesting to be studied in the future. In that case, they may be activated in a therapeutically useful manner.

5.1.5 Cell and tissue specificity of APP α-secretase

The other problem of APP α-secretase identification is the cell or tissue specificity. At least in both SH-SY5Y and HEK293 cells, ADAM10 is the only constitutive α-secretase of APP. However, it is not always the case. It has been shown that APP α-cleavage had no reduction in ADAM10-/- MEF cells (Hartmann *et al.*, 2002). Since the processing of AD related APP shedding happens in the brain or in neurons, further validation of ADAM10 α-secretase activity in neuronal like cells becomes more meaningful.

SH-SY5Y cells were then differentiated into neuronal cells by all-trans retinoic acid (ATRA) treatment (see details in 4.5). Under these conditions, the cells develop neuronal like morphological phenotype but normal APP shedding behavior. ATRA treatment has been reported to result in the upregulation of ADAM10 expression, but only for short-time period (Koryakina *et al.*, 2009; Tippmann *et al.*, 2009). The results from

experiments showed that ADAM10 expression levels had the tendency to be slightly increased. That could be due to the time window of the treatment (3 d): ADAM10 expression level has already passed the increasing peak and is reduced to the normal level as the naive SH-SY5Y cells. Then siRNA transfection was performed to accomplish the ADAM10 knockdown. ADAM10 knockdown in neuron-like differentiated SH-SY5Y again confirmed that ADAM10 plays the major role of APP α-secretase. Further experiments were carried out using primary cultured murine neurons. ADAM9, 10 and 17 knockdowns were achieved by shRNA viral transduction (cooperated with Dr. P. Kuhn), and only ADAM10 knockdown could sufficiently abolish sAPPα production (Kuhn *et al.*, 2010), which confirms ADAM10 as the physiological relevant α-secretase in neurons.

5.2 The contribution of α-secretase cleavage of APP in cultured cell lines

Although the idea that APP undergoes two different proteolytic pathways has been widely accepted, initially it was believed that only α-cleavage was the physiological one (Esch *et al.*, 1990; Sisodia *et al.*, 1990) and it prevented amyloidogenesis under non-pathological conditions. However, later it was clearly known that not only α-cleavage, but β- and γ-secretase cleavage, including Aβ generation can also happen under physiological conditions (Haass *et al.*, 1992a; Shoji *et al.*, 1992). Indeed, Aβ peptides were detected in cerebrospinal fluid in healthy human beings (Seubert *et al.*, 1992). How do α- and β-secretases contribute to the total APP secretion? Results from different cell lines suggest that they are in a stably balanced ratio.

5.2.1 α- and β-secretase contribute to APP cleavage in a stable ratio under physiological conditions

Summarized from siRNA and shRNA transfection experiments, total sAPP was reduced to ~60% in the ADAM10 knockdown HEK293 cells compared with controls (see details 4.2.2 and 4.4). The extent of total sAPP reduction in SH-SY5Y cells by ADAM10 knockdown was to ~ 40% (see details 4.2.1 and 4.5). On the other hand, β-secretase cleavage contributes about 20% to total APP secretion in the HEK293 and SH-SY5Y

cells according to the BACE1 knockdown manipulation (see 4.9.1). β-secretase inhibition led to the similar results in SH-SY5Y cells. These results suggested that α- and β-secretases participate in APP cleavage by a stable ratio in cultured cell lines. In the primary cultured neurons, β-cleavage of APP contributes to over 90% of total sAPP which is completely different from the immortalized cell lines (Kuhn *et al.*, 2010). The contributions of α- and β-secretase cleavage of APP are shown in Figure 21.

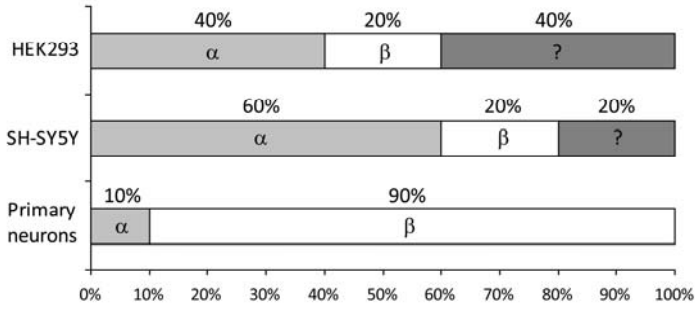

Figure 21 The contributions of α- and β-secretase cleavage of APP.
The contributions of α- and β-secretase cleavage of APP in different cell lines are shown in the bar chart. "α" represents α-cleavage of APP, mainly contributed by ADAM10; "β" represents β- and β'-cleavage of APP, mainly contributed by BACE1. There may be some other unknown proteases participating APP cleavage at different sites, represented in the figure as "?".

In HEK293 and SH-SY5Y cells, there is some percentage of APP shedding that is neither contributed by ADAM10 nor BACE1, raising up the possibility that some other unknown proteases participating in APP cleavage. In HEK293 cells, C3 treatment even led to a reduction of total sAPP to 40%. Taking away the 20% by BACE1, the rest 40% of APP shedding would be possibly from cleavage by some other proteases. One possibility is the BACE1 homologue BACE2, which can also be inhibited by β-secretase inhibitor C3.

The stable ratio of contribution by α- and β-secretase on APP cleavage also confirms that all the experiments were carried out in a physiological condition. The fact that sAPP generation suppressed upon ADAM10 knockdown indicated the participation of ADAM10 as the only physiological APP constitutive α-secretase.

5.2.2 α-secretase processes C99 together with γ-secretase

α-secretase cleavage is considered to occur directly after the action of α-secretase at the cell surface, cleaving C83 and releasing the AICD (APP intracellular domain) together

with the small p3 peptide. In the amyloidogenic pathway, APP is cleaved in the endocytosis pathway by β-secretase, and the resulting C99 is transported to the cell membrane to be cleaved by γ-secretase, leading to Aβ generation. However, C99 was found also be processed by α-secretase (see 4.8.1). When α-secretase activity was suppressed by shRNA mediated knockdown, C99 accumulated. Since the endogenous APP CTFs are cleaved by γ-secretase very quickly, the γ-secretase inhibitor DAPT was used to better analyze the CTFs processing. In accordance with the postulated APP trafficking route, the β-secretase cleavage product CTFβ could also be recycled to cell surface where it encounters both α- and γ-secretase. So as shown in Figure 23, C99 can be processed in two pathways. The first one consists of cleavage by γ-secretase leading to Aβ generation, the second one occurs by α-secretase, leading to C83 generation. For that reason, β-secretase is not the only one responsible for compensative or competitive relationship with α-secretase cleavage. The relationship between α- and γ-secretase should have been taken into account as well. However, I have to admit that the real contribution by α-secretase on C99 cleavage may be not as predominate as shown under the condition of γ-secretase inhibition. Further efforts have to be made to reveal this relationship under physiological conditions, without treatment of γ-secretase inhibitor.

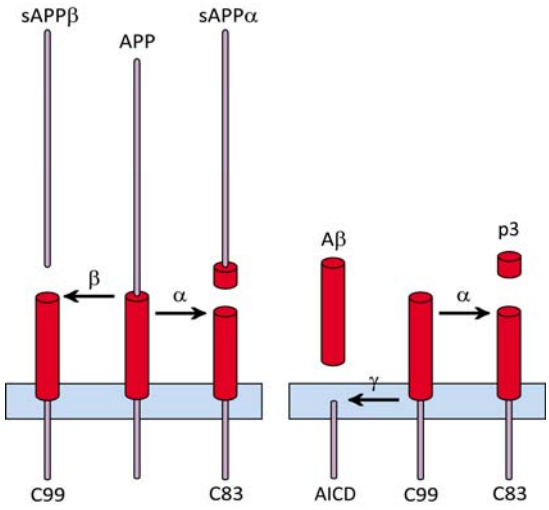

Figure 22 Relationship between α- and γ-secretase on C99 cleavage.
β-secretase cleavage product C99 could also be recycled to cell surface where it encounters both α- and γ-secretases. In case that α-secretase is activated, it cleaves not only more full length APP but also C99 competing with γ-secretase.

5.3 The relationship between α- and β-secretase on APP cleavage

The generation of sAPPα and sAPPβ/Aβ is generally thought to be inversely coupled. Multiple studies showed that an upregulation of α-secretase activity led to a reduction of APP β-cleavage (Hung *et al.*, 1993; Jacobsen *et al.*, 1994; Citron *et al.*, 1996; Buxbaum *et al.*, 1998; Koike *et al.*, 1999; Skovronsky *et al.*, 2000; Qiu *et al.*, 2001; Zhu *et al.*, 2001; Allinson et al., 2004; Postina *et al.*, 2004; Kim, 2008; Fu *et al.*, 2009) (see details in Table 5). On the basis of this assumption, the activation of α-secretase cleavage is considered a therapeutic approach in order to reduce β-secretase cleavage and therefore Aβ generation. However, contradictory studies about α-secretase activity regulation and corresponding β-cleavage behavior exist (Dyrks, 1994; Blacker *et al.*, 2002; Gandhi *et al.*, 2004) (see details in Table 5). Discrepancies in different experiments may have different reasons. On one hand, some studies are based on mutants of APP found in familial AD (Hung *et al.*, 1993; Citron *et al.*, 1996; Qiu *et al.*, 2001; Fu *et al.*, 2009), whereas others focus on the wild type protein. And these familial AD mutants are extremely rare and not found in sporadic AD. On the other hand, the experimental details between the two proteases have to be carefully specified, such as different cell lines and the ways that the regulations are achieved. In order to systematically investigate the relationship between α- and β-secretases under physiological condition, a series of experiments were performed as described in results part (see details in 4.8 and 4.9). All experiments of this thesis were based on wild type APP. Surprisingly, no obvious compensatory effect between α- and β-secretase on APP cleavage in HEK293 and differentiated SH-SY5Y cells was observed, neither when these proteases were pharmacologically inhibited nor when they were genetically knocked down. The only condition where a compensatory change was observed was in primary cultured neurons, where ADAM10 partially compensated for the loss of BACE1. This experiment was carried out by my colleague Dr. Alessio Colombo in the lab in parallel with the work shown in this thesis. These results are in accordance with data shown by a recent study that reports increased sAPPα levels upon pharmacologic inhibition of β-secretase in human (May *et al.*, 2011). The following

table summarizes APP shedding behavior changes due to activity modulation of α- and β-secretase (Table 20).

Table 20 Compensatory relationship between α- and β-secretase on APP cleavage.

More neuronal like cells ↓

Cell lines	α-secretase loss of function	β-secretase loss of function
HEK293	**ADAM10 KD** → sAPPα ↓, sAPPβ --, Aβ --	**BACE1 KD** → sAPPβ ↓, sAPPα --
	TAPI → sAPPα ↓, sAPPβ --, Aβ --	**C3** → sAPPβ ↓, sAPPα --
Differentiated SH-SY5Y	**ADAM10 KD** → sAPPα ↓, sAPPβ --, Aβ --	**BACE1 KD** → sAPPβ ↓, sAPPα --
	TAPI → sAPPα ↓, sAPPβ --, Aβ ↑	**C3** → sAPPβ ↓, sAPPα --
Neurons (in cooperation with Dr. A. Colombo, Manuscript in preparation)	*ADAM10 KD → sAPPα ↓, sAPPβ --, Aβ --*	*BACE1 KD → sAPPβ ↓, sAPPα ↑*
	TAPI → sAPPα ↓, sAPPβ --, Aβ ↑	*C3 → sAPPβ ↓, sAPPα ↑*

"↓" represents reduction; "↓" represents increase; "--" represents no change.

5.3.1 The absence of a compensatory relationship between α- and β-secretase on APP cleavage under physiological conditions

Although it is accepted that α- and β-secretase work mutually under activation or overexpression condition, this theory is based on that the unique substrate recognition of these two proteases and the identical cleavage compartment. However, the trafficking route of APP and the cleavage compartment of α- and β-secretase seem to be different (see details in 1.2.4). The surprising lack of compensatory changes leads to the rethink of the working pattern of α- and β-secretase on APP cleavage.

APP is transported through the TGN along the secretory pathway and finally reaches the plasma membrane, where α-secretase activity is found. It then enters the endocytic pathway, where the proteolytic processing by β-secretase takes place. Remaining full length APP enters a recycling pathway and is transported from the endosomal compartments back to the plasma membrane (see details in Figure 5). In immortalized cell lines, where α-secretase cleavage contributes to the majority of APP shedding (see

details in Figure 22), the loss of function of α-secretase is thus expected to lead to an increased amount of APP available for β-secretase cleavage. However, the results from experiments in immortalized cell lines, where ADAM10 activity was reduced by knockdown or TAPI treatment, did not confirm these expectations, as sAPPβ levels remained essentially unaltered. This could be due to the saturation of β-secretase activity in these cell lines. In fact, β-secretase cleavage is considered to be the rate-limiting step in Aβ generation, with a relatively slow turnover rate of BACE1 towards APP. Overexpression of PS1 does not increase Aβ generation, confirming BACE1 as the rate limiting enzyme of Aβ generation (Giliberto *et al.*, 2009). In HEK293 and SH-SY5Y cells, the amount of endogenous APP that is routed from the cell surface to the endosomal/lysosomal compartment under basal conditions may already saturate BACE1 activity, and increasing the quantity of APP molecules in this compartment after knockdown or inhibition of ADAM10 would therefore not enhance sAPPβ/Aβ production. This is in contrast to primary neurons, where BACE1 expression levels and hence its proteolytic activity are higher and more APP is cleaved by β-secretase, whereas α-secretase contributes only to a minor part of APP shedding (see details in Figure 22). According to this model, the loss of function of ADAM10 would lead to more APP available for β-secretase in primary neurons, although the expected increase of sAPPβ may be too minor to be observed.

In the opposite case, when BACE1 activity is suppressed (see details in 4.9), the amount of APP that is cleaved in the endosomal compartment decreases. Therefore more APP enters the recycling pathway and is available for the proteolytic processing by α-secretase at the cell surface. In immortalized cell lines, the additional amount of APP that is recycled after the loss of BACE1 function is only minor and may not lead to an observable change in sAPPα generation. In primary neurons however, where BACE1 represents the major sheddase of APP, the loss of function of BACE1 would lead to significant more APP available for recycling and subsequent processing by ADAM10. This could explain why the compensatory effect of α-secretase in response to BACE1 suppression was observed in primary cultured neurons, but not in immortalized cell lines.

However, the major compartment where APP is cleaved by β-secretase remains controversial. Besides the endocytic compartment, several other locations of Aβ generation have been proposed. APP has been shown to be cleaved in the TGN and ER, particularly mutant forms of APP found in familial AD, such as APP harboring the Swedish mutation (Haass *et al.*, 1995; Thinakaran & Koo, 1996; Hartmann *et al.*, 1997; Xu *et al.*, 1997). Therefore, under certain conditions, BACE1 can cleave APP already in the secretory pathway, thus before α-secretase. In this scenario, the loss of function of ADAM10 would not induce any compensatory effect, neither in immortalized cell lines nor in primary neurons. When BACE1 is knockdown or inhibited in primary cultured neurons where β-secretase takes the major job of APP cleavage, large more amount of APP is transported to cell membrane. The upregulation of substrate for α-secretase leads to the increase of sAPPα level. However, in immortalized cell lines, the loss of function of BACE1 may lead to the APP amount at cell membrane but only slightly. Since the large proportion of α-cleavage, the weak increase would not be observed.

From the performed experiments, no definitive conclusion could be drawn on the absence of a compensatory relationship between α- and β-secretase on APP cleavage. This would require a precise and quantitative estimation of the turnover rate of APP by α- or β-secretase at the plasma membrane and the endosomal compartment as well as the amount of full length APP that recycles back to the cell surface. In addition, the enzyme kinetics of both proteases has to be taken into consideration. Solid evidence has to be shown that β-secretase could also work in the TGN. The cellular trafficking of APP and therefore the access to the different secretases may differ in between immortalized cell lines and primary cultured neurons. Further research on APP trafficking would establish a solid basis for the investigation of the regulation and the compensatory relationship of APP secretases.

5.3.2 Competitive relationship of α- and β-secretase on APP cleavage under pathological conditions

Besides the compensatory relationship between α- and β-secretase on APP cleavage as discussed in 5.3.1, the existence of a competitive relationship between these two

proteases is also possible. This would implicate that upon activation or overexpression of one protease, APP cleavage by the other protease is diminished, as both proteases compete for the processing of the same substrate. Under pathological conditions, β-secretase activity may be increased, as evidence enhanced BACE1 expression levels and a concordant increase in Aβ production are found in the brains of sporadic AD patients (Yang *et al.*, 2003). If the competitive relationship between α- and β-secretase holds true, this could in turn lead to a reduction of α-secretase activity towards APP. In fact, a reduction of sAPPα generation was observed when BACE1 was overexpressed in HEK293 cells (Kuhn et al., 2010). Reduced levels of ADAM10 are observed in platelets from AD patients when compared to age-matched controls, and a quantitatively similar decrease in sAPPα is present both in thrombin-activated platelets and CSF (Colciaghi *et al.*, 2002).

5.3.3 TAPI-1 treatment inhibits α-secretase activity together with Aβ-degrading enzymes activity

What does the discrepancy of Aβ levels from the genetically and pharmacologically induced loss of function of α-secretase tell us? Aβ levels increased in both cell lines when treated with TAPI-1, but surprisingly did not change when ADAM10 was knocked down. In both conditions, sAPPβ levels remained the same when compared to the control. One explanation for this phenomenon could be the fact that TAPI-1 is a broad spectrum inhibitor for metalloproteases and could therefore inhibit most of the Aβ-degrading enzymes. Insulin degrading enzyme (IDE) (Kurochkin & Goto, 1994; Qiu *et al.*, 1998; Vekrellis *et al.*, 2000; Farris *et al.*, 2003; Leissring *et al.*, 2003), endothelin converting enzyme (ECE) (Eckman *et al.*, 2001; Eckman *et al.*, 2003), angiotensin-converting enzyme (ACE) (Tucker *et al.*, 2000; Hu *et al.*, 2001) and endopeptidase (Howell *et al.*, 1995; Iwata *et al.*, 2000; Iwata *et al.*, 2001; Kanemitsu *et al.*, 2003; Leissring *et al.*, 2003) have all been reported to be involved in Aβ degradation (Wang *et al.*, 2006) and most of them belong to the family of metalloproteases. Some matrix metalloproteinases, such as MMP2 and MMP9 were also reported to participate in the Aβ degradation (Backstrom *et al.*, 1996; Yan *et al.*, 2006; Yin *et al.*, 2006). Therefore, TAPI-1 treatment may not only inhibit α-secretase activity but also inhibit one or several enzymes involved in Aβ

degradation. To conclude, the upregulation of Aβ levels in TAPI-1 treated cells is not due to the compensatory effect of increased APP cleavage by β-secretase of APP, but presumably because Aβ degradation is inhibited.

5.4 Outlook

The activity of α-secretase towards APP can be functionally classified as constitutive or regulated. ADAM10 has been verified throughout this thesis as the only physiologically relevant, constitutive α-secretase of APP. However, the molecular identity of the physiologically relevant regulated α-secretase activity remains enigmatic. And indeed some physiological stimuli have been reported to increase α-secretase activity (see details in Table 4). Although ADAM17 has been verified as the regulated α-secretase upon PMA stimulation, PMA is a chemically synthesized compound and is possibly unable to mimic the endogenous stimuli. It would be interesting to investigate on the identity of regulated α-secretase activity using the various described physiological stimulating factors. The identification of regulated α-secretase activity could be even more meaningful from a therapeutic perspective, as the design of α-secretase activity stimulating drugs may then be tailored towards the structural features of the specific protease.

Interestingly, it was shown in this thesis that ADAM10, in addition to γ-secretase, contributes to C99 cleavage. However, due to the rapid degradation of APP CTFs by γ-secretase, inhibitors had to be used in order to ensure the detection of these APP fragments. It would be fascinating to explore the contribution of α- and γ-secretase on C99 cleavage under physiological condition, in the absence of γ-secretase inhibitors, which would be an alternative way to down-regulate Aβ generation. Further research on C99 cleavage by ADAM10 should allow a deeper understanding of the relationship between α-secretase regulation and the corresponding Aβ levels.

Furthermore, concerning to the recent finding that under pathological condition, not only β-secretase activity increases but also decrease the α-secretase activity, sAPPα would be also a potential AD biomarker. Development of sensitive sAPPα detection assay may make it easier to establish a standard biomarker screening system. Since the encoding of

two rare potentially disease-associated non-synonymous mutations (Kim *et al.*, 2009), it would be a fascinating challenge to investigate on gene manipulation of α-secretase to mimic the AD-related pathological condition and corresponding β-cleavage of APP together with Aβ generation.

6 Conclusions

The main goals of this thesis were to identify the physiologically relevant, constitutive α-secretase of APP and to valuate its potential compensatory relationship with β-secretase. Specifically, the following results were obtained.

• The newly generated sAPPα specific antibodies 4B4 and 14D6 allowed the specific detection of proteolytic APP fragments derived from α-secretase. The antibodies specifically recognized sAPPα and did not show cross reactivity with other APP fragments, including sAPPβ'. They provided the possibility to perform α-secretase research under endogenous APP expression levels, which facilitate the identification of α-secretase under the physiologically relevant condition.

• Transient and stable knockdowns of α-secretase candidates in various wild-type peripheral cells identified ADAM10 as the only physiologically relevant, constitutive α-secretase. The identity of ADAM10 as APP α-secretase was further validated in neuron-like cells and cell lines with APP overexpression. Other α-secretase candidates were unable to compensate for the loss of ADAM10 activity. ADAM10 contributed to the constitutive α-secretase activity, while the induction of APP α-shedding by PMA occurred by ADAM17, but independently of ADAM10.

• Detailed studies on the different secreted APP fragments under physiological conditions showed that α- and β-secretases contribute to APP cleavage in a stable ratio. They did not compensate for each other, when either one protease activity was lost, either genetically or inhibited pharmacologically. In addition, it was shown that both α- and γ-secretase participate in the cleavage of the β-secretase cleavage product C99.

The identification of ADAM10 as the only physiologically relevant α-secretase of APP and its relationship with β-secretase finally complete the basic understanding of APP

proteolytic processing. This provides the possibility to evaluate ADAM10 as a drug target for the therapy of Alzheimer's disease.

7 Tables and figures

8 Abbreviations

Aβ Amyloid beta peptide

Aβ40 Aβ 40-residue form

Aβ42 Aβ 42-residue form

ACE Angiotensin-converting enzyme

AChE Acetylcholinesterase

AD Alzheimer's disease

ADAM A disintegrin and metalloproteinase

AICD APP intracellular domain

APH-1 Anterior pharynx defective 1

APLP1 Amyloid-precursor like protein 1

APLP2 Amyloid-precursor like protein 2

APP Amyloid precursor protein

APS Ammonium persulfate

Asn (N) Asparagine

ATRA All-trans retinoic acid

β-ME β-Mercaptoethanol

BACE1 β-site APP cleaving enzyme 1

Bisacrylamide N-N'-Methylene-bisacrylamide

BSA Bovine serum albumin

C3 β-Secretase inhibitor IV

$CaCl_2$ Calcium chloride

ChAT Choline acetyltransferase

CTF C-terminal fragment

DAPT γ-Secretase inhibitor IX

ddH_2O Double-distilled water

DMEM Dulbecco's modified eagle medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

dNTP PCR Nucleotide Mix

DR6 Death receptor6

E. coli Escherichia coli

EB Ethidium bromide

ECE Endothelin converting enzyme

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

ER Endoplasmic reticulum

FCS Fetal bovine serum

Gln (Q) Glutamine

GluR Glutamate receptor

GPCRs G protein-coupled receptors

HCl Hydrogen chloride

HEK293 Human embryonic kidney 293 cells

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HRP Horseradish peroxidase

IDE Insulin degrading enzyme

IGF-1 Insulin-like growth factors 1

IL-1α Interleukin-1 α

IL-1β Interleukin-1 β

KCl Potassium chloride

KH_2PO_4 Potassium dihydrogen phosphate

KPI Kunitz serine protease inhibitors

Leu (L) Leucine

Lys (K) Lysine

mAChR Muscarinic acetylcholine receptor

MAPK Mitogen-activated protein kinase

MEF Mouse fibroblast cells

Met (M) Methionine

$MgCl_2$ Magnesium chloride

MMP Matrix metalloprotease

Na_2HPO_4 Disodium hydrogen phosphate

NaCl Sodium chloride

NEAA Non essential amino acids

NP-40 Nonidet P-40

P/S Penicillin/streptomycin

PACAP peptide Pituitary adenylate cyclase-activating polypeptide

PAS Protein A sepharose

PCR Polymerase chain reaction

PDBu Phorbol 12,13-dibutyrate

Pen-2 Presenilin enhancer protein 2

PI Protease inhibitor

PI3K Phosphoinositid-3-kinasen

PKC Protein kinase C

PMA Phorbol-12-myristate-13-acetate

PS1 Presenilin1

PS2 Presenilin2

RE Restriction endonucleases

RIP Regulated intramembrane proteolysis

RT Room temperature

SDS Sodium dodecyl sulfate

sAPP Secreted APP fragment

sAPPα Secreted APP α-secretase cleaved fragment

sAPPβ Secreted APP β-secretase cleaved fragment

SDS-PAGE SDS-polyacrylamide gel electrophoresis

SH-SY5Y Human neuroblastoma cells

TEMED N,N,N',N'-Tetramethylethylendiamine

TGFα Transforming growth factor

TGN Trans-Golgi network

Tris Tris hydroxymethyl aminomethane

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11 Curriculum vitae

Huanhuan Wang

Born on 4th Nov. 1983 in Hangzhou, China

Waidongshan Lane 34-3-402 310013 Hangzhou China

Huanval@gmail.com

EDUCATION & RESEARCH EXPERIENCE

• September 2008 – present **Dr. rer. nat. Candidate**

Deutsches Zentrum fuer Neurodegenerative Erkrankungen (DZNE), Munich,

Adolf-Butenandt-Institute, Department of Biochemistry,

Ludwig-Maximilians University Munich, Germany

- Research on constitutive and regulated α-secretase of amyloid precursor protein (supported by Chinese Scholarship Council and Deutsche Forschungsgemeinschaft the Collaborative Research Center "SFB 596 Molecular Mechanisms of Neurodegeneration")

• September 2006 - June 2008 **Master of Medicine in Pharmacology**

Institute of Pharmacology & Toxicology and biochemical pharmaceutics,

College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China

- Research on characteristic of memory- and motor-functioned cholinergic neurons derived from mouse embryonic stem cells and embryonic stem cells differentiating into neural cells by drug interference related with estrogen receptor pathway (supported by the National Natural Sciences Foundation of China "Interfere effects of drug involved in embryonic stem cells on their directional differentiation in vitro")

- Research on neuroprotective effect of SERM against-beta amyloid peptide induced toxicology in primary cultured neuronal cells by estrogen receptor pathway (supported by the Research League of German-National Natural Sciences Foundation of China "Effects of flavone and relative hydroxybenzene component of Chinese vegetables on preventing and curing diseases")

• September 2002 - June 2006 **Bachelor of Science in Pharmaceutical Sciences**

College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, China

- Research on protective effects of Sodium nitroprusside in hepatic ischemia-reperfusion injured rats (supported by the National Natural Sciences Foundation of China "Liver membrane mcrosomal S-glutathione transferase involved in leukotriene autocrine/paracrine loop and drug regulation")

- Research on protective effect of abstraction from a traditional Chinese medicine on chemical toxicology in primary cultured hepatocyte cells (supported by the National Natural Sciences Foundation of China "Relationship between activation of microsomal S-glutathione transferase by modification and its transform of extro-substance")

PUBLICATIONS

• **Kuhn P. H.***, **Wang H.***, Dislich B., Colombo A., Zeitschel U., Ellwart J. W., Kremmer E., Roßner S., Lichtenthaler S. F. (**2010**) ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons. *EMBO J.* **29**(17): 3020-32. (* Contributed equally to the paper)

• **Wang Z.***, **Wang H.***, Wu J., Zhu D., Zhang X., Ou L., Yu Y., lou Y. (**2009**) Enhanced co-expression of β-tubulin III and choline acetyltransferase in neurons from mouse embryonic stem cells promoted by icaritin in an estrogen receptor-independent manner. *Chem. Biol. Interact.* **179**(2-3): 375-85. (* Contributed equally to the paper)

• Wang Z., Zhang X., **Wang H.**, Qi L., Lou Y. (**2007**) Neuroprotective effect of icaritin against beta amyloid-induced neurotoxicity in primary cultured rat neuronal cells via estrogen receptor-dependent pathway. *Neuroscience* **145**(3): 911-922.

• Zhang X., **Wang H.**, Wang Z., Wu J., Zhu D., Lou Y. (**2007**) Protective effect of icaritin on apoptosis of primarily cultured rat neurons induced by Aβ25-35 peptide. *Journal of Zhejiang University (Medical Sciences)* **36**(3): 224-228.

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ADAM10 is the physiologically relevant, constitutive α-secretase of the amyloid precursor protein in primary neurons

Peer-Hendrik Kuhn[1,5], Huanhuan Wang[1,5], Bastian Dislich[1,2], Alessio Colombo[1,2], Ulrike Zeitschel[3], Joachim W Ellwart[4], Elisabeth Kremmer[4], Steffen Roßner[3] and Stefan F Lichtenthaler[1,2,*]

[1]Adolf-Butenandt-Institute, Biochemistry, Ludwig-Maximilians-University, Munich, Germany, [2]DZNE—German Center for Neurodegenerative Diseases, Munich, Germany, [3]Paul Flechsig Institute for Brain Research, University of Leipzig, Leipzig, Germany and [4]Helmholtz Zentrum München, German Research Center for Environmental Health, Institute of Molecular Immunology, Munich, Germany

The amyloid precursor protein (APP) undergoes constitutive shedding by a protease activity called α-secretase. This is considered an important mechanism preventing the generation of the Alzheimer's disease amyloid-β peptide (Aβ). α-Secretase appears to be a metalloprotease of the ADAM family, but its identity remains to be established. Using a novel α-secretase-cleavage site-specific antibody, we found that RNAi-mediated knockdown of ADAM10, but surprisingly not of ADAM9 or 17, completely suppressed APP α-secretase cleavage in different cell lines and in primary murine neurons. Other proteases were not able to compensate for this loss of α-cleavage. This finding was further confirmed by mass-spectrometric detection of APP-cleavage fragments. Surprisingly, in different cell lines, the reduction of α-secretase cleavage was not paralleled by a corresponding increase in the Aβ-generating β-secretase cleavage, revealing that both proteases do not always compete for APP as a substrate. Instead, our data suggest a novel pathway for APP processing, in which ADAM10 can partially compete with γ-secretase for the cleavage of a C-terminal APP fragment generated by β-secretase. We conclude that ADAM10 is the physiologically relevant, constitutive α-secretase of APP.

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*Corresponding author. Adolf-Butenandt-Institute, Biochemistry, Ludwig-Maximilians-University, University of Munich, Munich 80336, Germany. Tel.: +49 89 218075453; Fax: +49 89 218075415; E-mail: stefan.lichtenthaler@med.uni-muenchen.de
[5]These authors contributed equally to this work

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Introduction

The amyloid precursor protein (APP) is one of a large number of membrane proteins that are proteolytically converted to their soluble counterparts. This process is referred to as ectodomain shedding and is an important way of regulating the biological activity of membrane proteins (Pruessmeyer and Ludwig, 2009; Reiss and Saftig, 2009). APP shedding occurs constitutively by two different protease activities, called α- and β-secretases, and leads to the secretion of soluble APP (APPs) (Figure 1A). Both proteolytic cleavages are central regulatory events in the generation of the amyloid-β peptide (Aβ), which has an important function in the pathogenesis of Alzheimer's disease (AD) (Selkoe and Schenk, 2003; Haass, 2004). The α- and β-secretases are assumed to compete for APP as a substrate (Selkoe and Schenk, 2003; Postina *et al*, 2004), but have opposite effects on Aβ generation. The β-secretase is the aspartyl protease BACE1 and cleaves APP at the N-terminus of the Aβ domain, thus catalysing the first step in Aβ generation (Vassar *et al*, 1999). In contrast, α-secretase cleaves within the Aβ sequence of APP (Esch *et al*, 1990), thereby precluding Aβ generation. In addition, α-secretase cleavage generates a secreted form of APP (APPsα), which has been reported to have neurotrophic and neuroprotective properties (Furukawa *et al*, 1996; Meziane *et al*, 1998; Stein *et al*, 2004), whereas the slightly shorter form (APPsβ) generated by β-secretase seems to have a proapoptotic function (Nikolaev *et al*, 2009).

An increase of APP α-secretase cleavage is considered a therapeutic approach for AD (Fahrenholz, 2007), as it is assumed to reduce Aβ generation. However, the molecular identity of α-secretase is controversially discussed and remains to be fully established. Different metalloproteases were suggested as potential α-secretases, because their overexpression increased APP cleavage. The most frequently named ones are three members of the ADAM (a disintegrin and metalloprotease) family: ADAM9, 10 and 17 (Koike *et al*, 1999; Lammich *et al*, 1999; Slack *et al*, 2001). However, because the overexpression of a protease may artificially or indirectly increase APP α-secretase cleavage, the physiological relevance of a candidate protease needs to be shown using the corresponding protease knockdown or knockout cells. In fact, cells derived from ADAM9-, 10- or 17-deficient mice showed either no or a variable degree of reduction of APP shedding (Buxbaum *et al*, 1998; Hartmann *et al*, 2002; Weskamp *et al*, 2002). Likewise, RNAi-mediated knockdown of the individual proteases in cultured cells reduced APP shedding to different extents (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Freese *et al*, 2009; Taylor *et al*, 2009). The finding that APP shedding was never fully suppressed has led to the conclusion that ADAM9, 10 and 17 may all together contribute to α-secretase activity and that in the absence of one of them, the other proteases can still mediate APP α-secretase cleavage. This assumption is in clear contrast

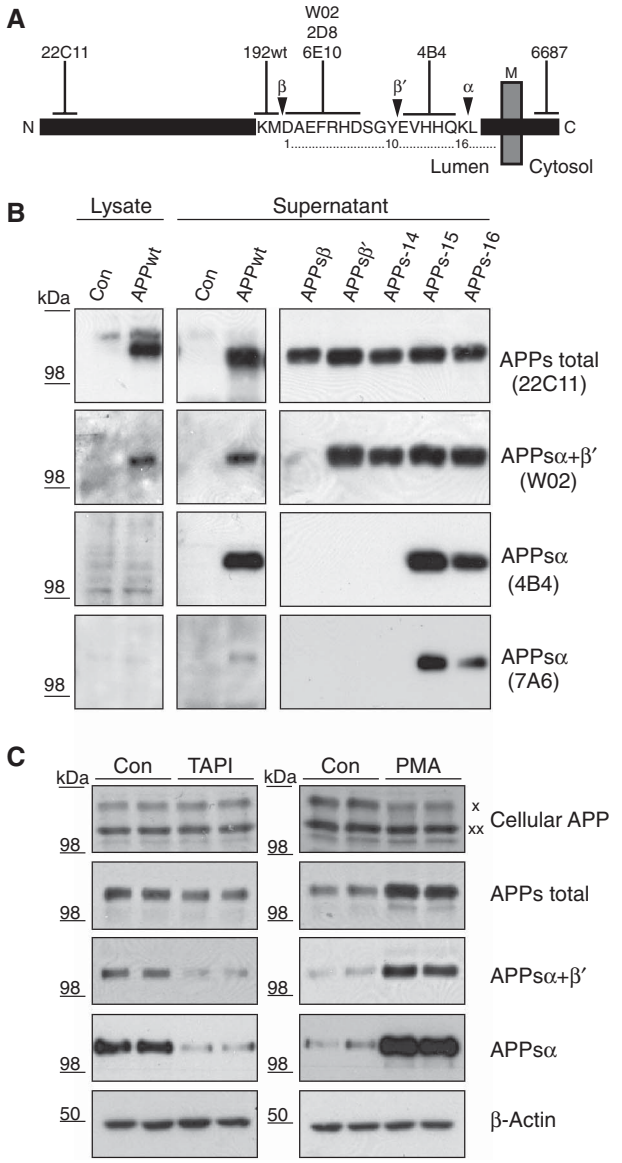


Figure 1 Characterization of newly generated APPsα-specific antibodies 4B4 and 7A6. (**A**) Schematic representation of APP. Indicated are the antibodies used in this study as well as antibody 6E10 and the APP-cleavage sites (with arrow heads) at the β-, β′- and α-secretases sites. Numbers below the sequence indicate the amino acids of the Aβ sequence. M, membrane. (**B**) Immunoblot of supernatants and cell lysates of cells expressing endogenous APP (Con) or transfected with the indicated constructs (APPwt, full-length APP; APPs, soluble APP lacking the transmembrane and cytoplasmic domain). Antibody 22C11 detects all secreted APP species, antibody W02 detects APPsα and APPsβ′, whereas antibodies 4B4 and 7A6 specifically detect APPsα (APPs-15 and APPs-16). (**C**) HEK293 cells were treated with the metalloprotease inhibitor TAPI-1 or the phorbol ester PMA. Immunoblots of conditioned media and cell lysates were probed with antibody 22C11 (APPs total), W02 (APPsα + β′) and the APPsα-specific antibody 4B4. Cellular APP is present in a lower molecular weight immature form (xx) and a higher molecular weight mature form (x) and was detected with 22C11. The β-actin blot serves as a loading control. The reduction by TAPI-1 and the increase in shedding by PMA are more pronounced when analysed with the α-cleavage-specific antibody 4B4, compared with the other antibodies.

to other ADAM protease substrates, many of which are predominantly cleaved by a single ADAM protease, such as transforming growth factor α, epidermal growth factor (EGF), the low-affinity immunoglobulin E receptor CD23 and N-cadherin (Sahin *et al*, 2004; Reiss *et al*, 2005; Weskamp *et al*, 2006; Le Gall *et al*, 2009). One aspect that makes it difficult to study APP α-secretase cleavage is the fact that APP is cleaved by distinct proteases at different peptide bonds in close proximity. For example, β-secretase has the main cleavage site at the N-terminus of the Aβ sequence, but a secondary cleavage site (termed β′-site) within the Aβ sequence close to the α-secretase-cleavage site (Figure 1A). Antibodies used in previous studies have not only specifically detected APPsα, but also the alternative β-secretase-cleavage product APPsβ′ (Figure 1A), which may have confounded the study of α-secretase cleavage. Here, we systematically address the identity of the physiologically relevant α-secretase. We generated two new monoclonal antibodies specific for the APP α-secretase-cleavage product APPsα. Using these antibodies as well as mass spectrometry, we found that ADAM10, but not ADAM9 or 17, is essential for the constitutive α-secretase cleavage of APP.

Results

Generation of an APPsα-specific antibody

To specifically detect the APP α-secretase-cleavage product APPsα, a new monoclonal antibody (4B4) was generated against a peptide comprising amino acids 11–16 of the Aβ sequence (Figure 1A). The peptide had a free C-terminus, mimicking the neoepitope generated upon α-secretase cleavage. Indeed, antibody 4B4 does not detect full-length APP in the cell lysate (Figure 1B). It specifically detects APPsα ending in amino acids 15 and 16 (APPs-15 and APPs-16), but does not detect shorter APPs species, including APPsβ′ and APPsβ (Figure 1B). In contrast, antibody W02 binds an epitope between the β- and the β′-cleavage sites and correspondingly detects both APPsα and APPsβ′, but not APPsβ. This antibody detects a similar epitope as antibody 6E10 that is frequently used for the detection of APPs (Miles *et al*, 2008). Antibody 22C11 binds to an N-terminal APP epitope and detects all APPs species tested (Figure 1B). All antibodies used specifically detect APP, because the antibodies do not detect a signal in APP knockdown cells (Supplementary Figure S1).

To further validate antibody 4B4, we tested whether conditions, which increase or decrease APPsα generation, lead to a corresponding change in the 4B4 signal. To this aim, human embryonic kidney 293 cells (HEK293) expressing endogenous APP were treated with the metalloprotease inhibitor TAPI-1 to reduce APP shedding or with the phorbol ester PMA (also known as TPA) to increase APP shedding. Both compounds did not alter the expression of APP or actin in the cell lysate (Figure 1C). TAPI-1 inhibited nearly completely APPsα generation (4B4 blot). In contrast, total APPs shedding was not as strongly reduced (22C11 blot), consistent with the fact that this antibody detects all APPs species and not only APPsα. PMA strongly stimulated total APP shedding (22C11), but the extent of the increase was much more pronounced when specifically detecting APPsα (4B4). The strong increase in APP shedding was paralleled by a reduction of the mature APP in the cell lysate (Figure 1C, marked with x). Antibody W02, which detects APPsα + APPsβ′, detected intermediate changes between 22C11 and 4B4, in agreement with the antibody detecting both APPsβ′ and APPsα. In an additional

control experiment, the β-secretase BACE1 was overexpressed, which is expected to increase β-secretase cleavage and to reduce α-secretase cleavage. BACE1 expression increased total APP shedding (22C11) in agreement with previous publications (Neumann *et al*, 2006; Schobel *et al*, 2006), but reduced as expected APPsα (4B4) (Supplementary Figure S2). Antibody W02 was not suited to detect the decrease in APPsα, because it also detects the alternative β-secretase-cleavage product APPsβ′, which was strongly enhanced upon overexpression of BACE1 (Supplementary Figure S2). Taken together, these experiments show that antibody 4B4 specifically detects APPsα, in contrast to other commonly used APP antibodies.

Knockdown of ADAM9, 10 and 17 in HEK293 and SH-SY5Y cells

Next, the three-candidate α-secretases ADAM9, 10 and 17 were transiently knocked down to evaluate their contribution to the constitutive α-secretase cleavage of endogenous APP in HEK293 and in human neuroblastoma SH-SY5Y cells. Compared with control-treated cells, the siRNA pools knocked down all three proteases with an efficiency of 75–90% (Figure 2A for HEK293; Figure 2D for SH-SY5Y; quantifications in Figure 2C and F). Levels of cellular APP as well as of the control membrane protein calnexin were not affected (Figure 2A and D). Knockdown of ADAM10 in HEK293 and SH-SY5Y cells reduced total APP shedding (22C11, normalized to cellular APP levels) to about 40% (Figure 2A, B, D and E), similar to the use of the metalloprotease inhibitor TAPI-1 (Figure 1C). In contrast, APPsα (4B4) was reduced to 10%, which corresponds to the remaining 10% of ADAM10 protease expressed in the knockdown cells. In contrast to ADAM10, the knockdown of ADAM9 did not significantly reduce total APPs or APPsα levels (Figure 2A, normalized to cellular APP levels). Knockdown of ADAM17 mildly reduced total APPs and APPsα in HEK293 cells (Figure 2A and B), but had no significant effect in SH-SY5Y cells (Figure 2C and D). The knockdown of either protease did not affect the expression level of the other proteases (Supplementary Figure S3). Taken together, expression of ADAM10, but not of ADAM9 or 17, is required for APP α-secretase cleavage. Moreover, ADAM9 and 17 did not compensate for the loss of ADAM10.

To further validate the results from the transient knockdown of ADAM10, HEK293 cells (Figure 3) and SH-SY5Y cells (Figure 4) with a stable knockdown of ADAM10 were generated using lentiviruses expressing two different shRNA sequences against ADAM10 or a negative control shRNA. In HEK293 cells, both ADAM10 shRNA sequences (sh7, sh9)

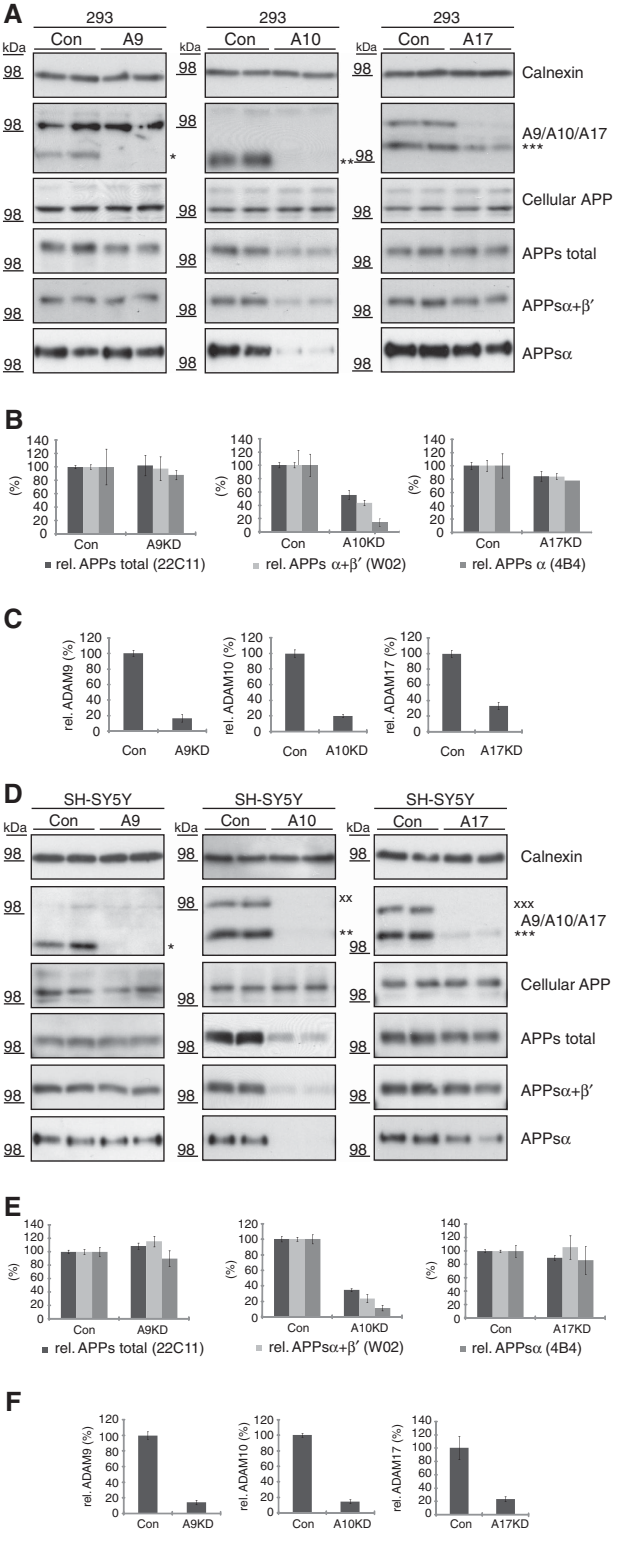
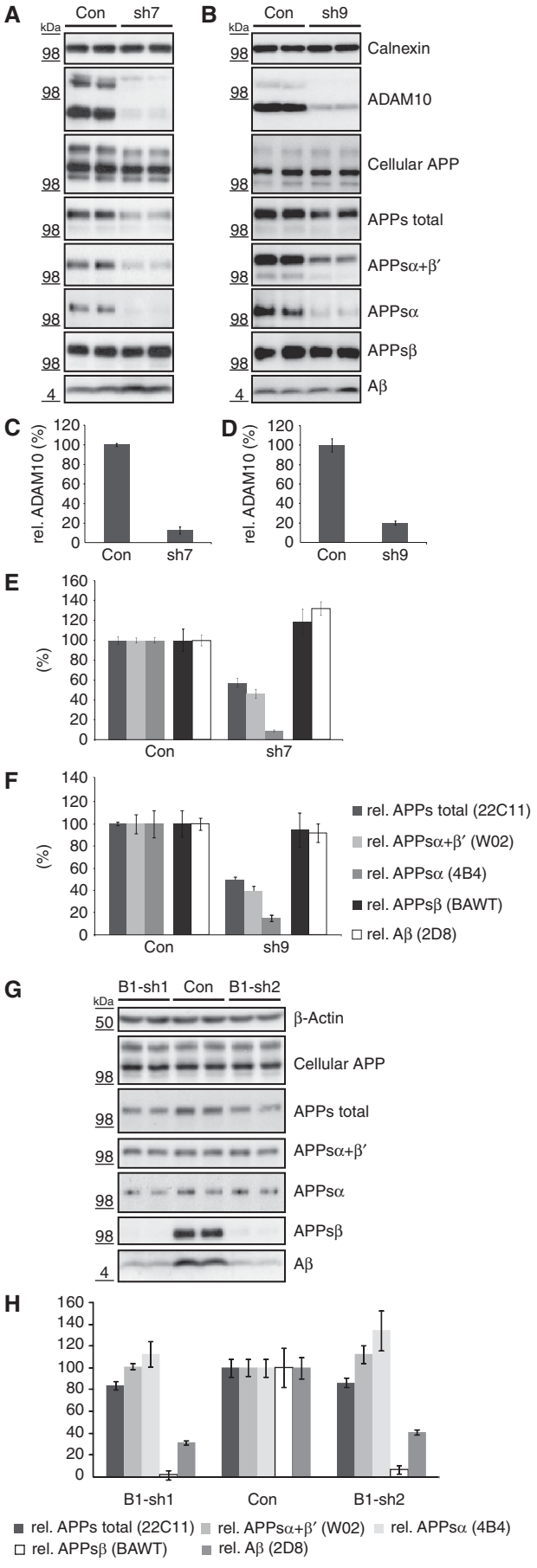


Figure 2 Transient knockdown of ADAM10 suppresses α-secretase cleavage of endogenous APP. (**A**) HEK293 cells (293) were transfected with siRNA pools directed against the proteases ADAM9 (A9), ADAM10 (A10) or ADAM17 (A17) or with control siRNA. All three proteases were detected by western blot in membrane preparations. The mature active form is indicated with * (ADAM9), ** (ADAM10) and *** (ADAM17). The immature form of ADAM17 was also detected in HEK293 cells at a higher molecular weight. Calnexin was detected as a loading control. Cell lysates were analysed for cellular APP and conditioned media for total secreted APP (APPs total, 22C11, α + β′-cleaved APP (APPsα + β′, W02) and α-cleaved APP (APPsα, 4B4). (**B**, **C**) Quantification of experiments in (**A**). APPs total, APPsα + β′ and APPsα levels were normalized to cellular APP (**B**). Quantification of the protease knockdown (KD) efficiency is in (**C**). Given are mean and standard error of eight independent experiments relative (rel.) to control. (**D**) SH-SY5Y cells were treated as in (**A**). APP fragments were detected as in (**A**). The immature protease form was not visible for ADAM9, but is indicated with xx for ADAM10 and xxx for ADAM17, whereas the mature active form is indicated with * (ADAM9), ** (ADAM10) and *** (ADAM17). (**E**, **F**) Quantification of experiments in (**D**). Given are mean and standard error of eight independent experiments.



reduced ADAM10 protease levels and APPsα levels to 10–20% of controls (Figure 3A and B; quantification in Figure 3C–F), respectively, which is similar to the transient knockdown experiments. Similar results were obtained for the stable knockdown of ADAM10 in SH-SY5Y cells (Figure 4A and B). The remaining APPsα from the SH-SY5Y cells could be fully inhibited by addition of the metalloprotease inhibitor TAPI-1 (Figure 4A), which is in agreement with the remaining ~10% of ADAM10 protease in the knockdown cells. The experiments in both cell lines show that also under stable knockdown conditions, ADAM10 is essential for APP α-secretase cleavage.

α- and β-secretase cleavage do not compete for each other in HEK293 and SH-SY5Y cells

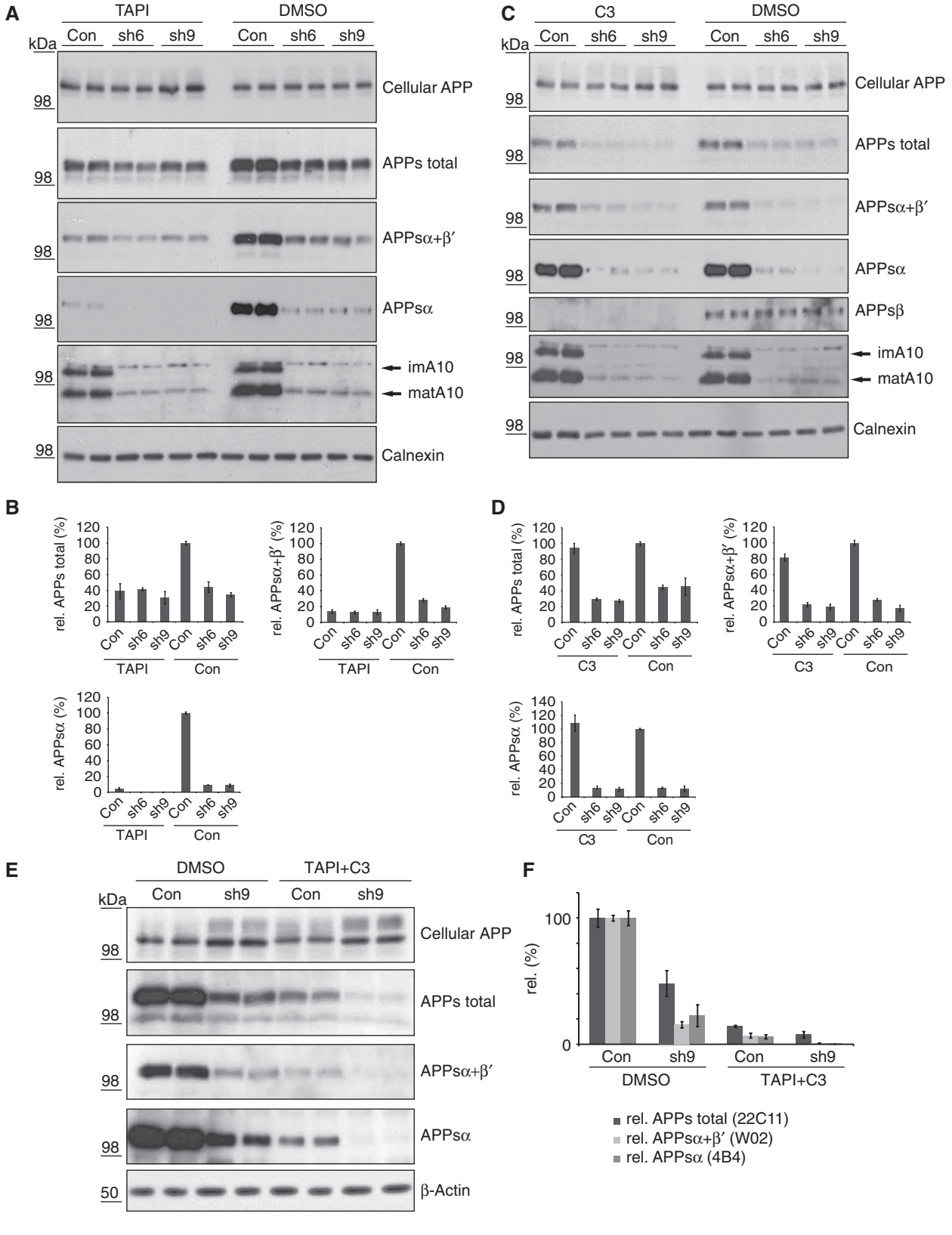
Next, we analysed whether the reduction of APPsα was paralleled by an increase in APPsβ and Aβ generation. Surprisingly, however, this was not the case (Figure 3A and B; quantification in Figure 3E and F). Compared with control cells, endogenous APPsβ and Aβ levels in HEK293 cells were unchanged for one ADAM10 knockdown construct (sh9), whereas a mild, but not significant, increase was observed for the other shRNA construct (sh7) (Figure 3E and F). These results show that the strong reduction of α-cleavage does not yield a correspondingly increased cleavage by β-secretase. To further validate this finding, the opposite experiment was carried out. Expression of the β-secretase BACE1 was reduced by lentiviral knockdown constructs (sh1 and sh2). This resulted in a strong inhibition of APPsβ and Aβ generation, but not in a significant increase in APPsα generation (Figure 3G and H). Similar results were obtained for SH-SY5Y cells. The stable knockdown of ADAM10 did not increase APPsβ levels (Figure 4C and D) and conversely, the pharmacological inhibition of BACE1 with the specific inhibitor C3 (Stachel *et al*, 2004) did not increase APPsα levels (Figure 4C and D). Thus, we conclude that under constitutive cleavage conditions, α- and β-secretases do not significantly compete for APP as a substrate in HEK293 and SH-SY5Y cells.

Contribution of α- and β-secretase cleavage to total APP secretion

Total APP shedding (22C11) was reduced to ~40% in the stable ADAM10 knockdown SH-SY5Y cells compared with

Figure 3 Analysis of APP processing in HEK293 cells with a stable ADAM10 knockdown. (**A**, **B**) HEK293 cells were infected with lentiviral vectors carrying GFP and different shRNAs: a non-targeting shRNA (Con) and two different ADAM10-targeting shRNAs (sh7 and sh9). Conditioned media of these cells were analysed with antibody 22C11 (APPs total), W02 (APPsα + β′), 4B4 (APPsα), 192wt (APPsβ) or 2D8 (Aβ), whereas cell lysates were analysed for cellular APP (22C11). Membranes were analysed for ADAM10 protein. Calnexin was detected as a loading control. (**C**, **D**) Quantification of ADAM10 knockdown from experiments in (**A**, **B**) relative (rel.) to control. (**E**, **F**) Quantification of APP fragments in (**A**, **B**). Given are mean and standard error of four independent experiments. (**G**) HEK293 cells were lentivirally infected as in (**A**). with a non-targeting shRNA (Con) or two different BACE1-targeting shRNAs (B1-sh1, B1-sh2). APP and its processing products were analysed as in (**A**). Actin was used as a loading control. (**H**) Quantification of experiments in (**G**), carried out as in (**E**) and (**F**). Given are mean and standard error of three independent experiments.

controls (Figure 4C and D). Additional inhibition of BACE1 with the specific inhibitor C3 further reduced total APP secretion to ~20%, revealing that BACE1 contributes about 20% to total APP secretion in the SH-SY5Y cells. This result also suggests that the remaining low level (<20%) of total APP secretion may result from proteases other than ADAM10 and BACE1. This was further confirmed by a combined pharmacological inhibition of α- and β-secretase cleavage in SH-SY5Y cells, which resulted in a remaining total APP secretion of 10–15% (Figure 4E and F).



ADAM10 truncates APP C-terminal fragments C99 and C89 to C83

α- and β-secretases not only generate APPsα and APPsβ, but also the C-terminal fragments C83 and C99, respectively. Both fragments are further processed by γ-secretase, leading to a short half-life of the fragments, which makes it difficult to detect them at endogenous levels. Thus, we treated SH-SY5Y cells with the γ-secretase inhibitor DAPT, which stabilizes the endogenous C-terminal fragments of APP (Figure 5A). In control cells, the α-secretase fragment C83 was clearly detected (marked with ***) and was strongly reduced in the ADAM10 knockdown cells in parallel to APPsα (quantification in Figure 5B). To our surprise, the β-secretase-cleavage product C99 was increased more than two-fold upon ADAM10 knockdown (marked with *). In addition, a mild increase of C89 was observed (marked with **), which is the C-terminal fragment arising through BACE1 at its alternative β′-cleavage site (see Figure 1A for schematic drawing). The increase in C99 and C89 is in contrast to the APPsβ levels, which were unchanged upon ADAM10 knockdown (Figure 5B). We interpret this result in the following way. C99 and C89 can be processed in two pathways. The first one consists of cleavage by γ-secretase leading to Aβ generation, the second one occurs by α-secretase, leading to C83 generation. Upon ADAM10 knockdown, the latter pathway is blocked, leading to an increase in C99 and C89 and leaving APPsβ levels unchanged.

ADAM10 is required for α-secretase cleavage in primary neurons

APP processing occurs in all cells and tissues analysed to date. However, in AD, APP processing is particularly relevant in the neurons of the central nervous system. Thus, we next investigated the contribution of ADAM9, 10 and 17 in α-secretase cleavage in primary murine cortical E16 neurons from C57/BL6 mice, expressing endogenous APP. Murine and human APP differ by three amino acids within the N-terminal half of the Aβ sequence. One of these amino-acid changes is within the peptide sequence used to generate the antibody 4B4. For this reason, we generated an additional antibody, called 7A6, which also detects the murine APPsα, but not APPsβ and APPsβ′ (Figure 1B). Using two different shRNA sequences, the lentiviral knockdown of ADAM10 reduced murine ADAM10 expression as well as murine APPsα to about 10–15% of the control (Figure 6A–C). This reveals that also in primary neurons, ADAM10 activity is required for APP α-secretase cleavage. As a control ADAM9 and 17 were also knocked down in the primary neurons. As both proteins could not be detected by immunoblot in the neuronal lysates, their expression was measured by quantitative RT–PCR (Figure 6F). Similar to the HEK293 and SH-SY5Y cells, knockdown of ADAM9 or 17 did not affect APP α-secretase cleavage (Figure 6D and E). Likewise expression of ADAM10 was not affected, as determined by both immunoblot (Figure 6D) and

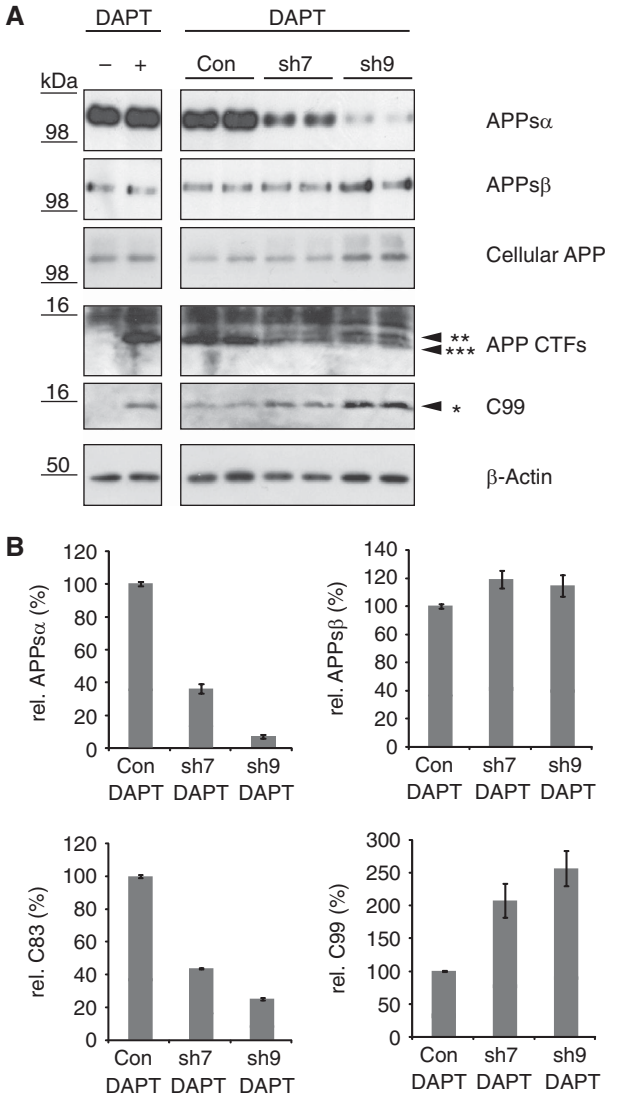
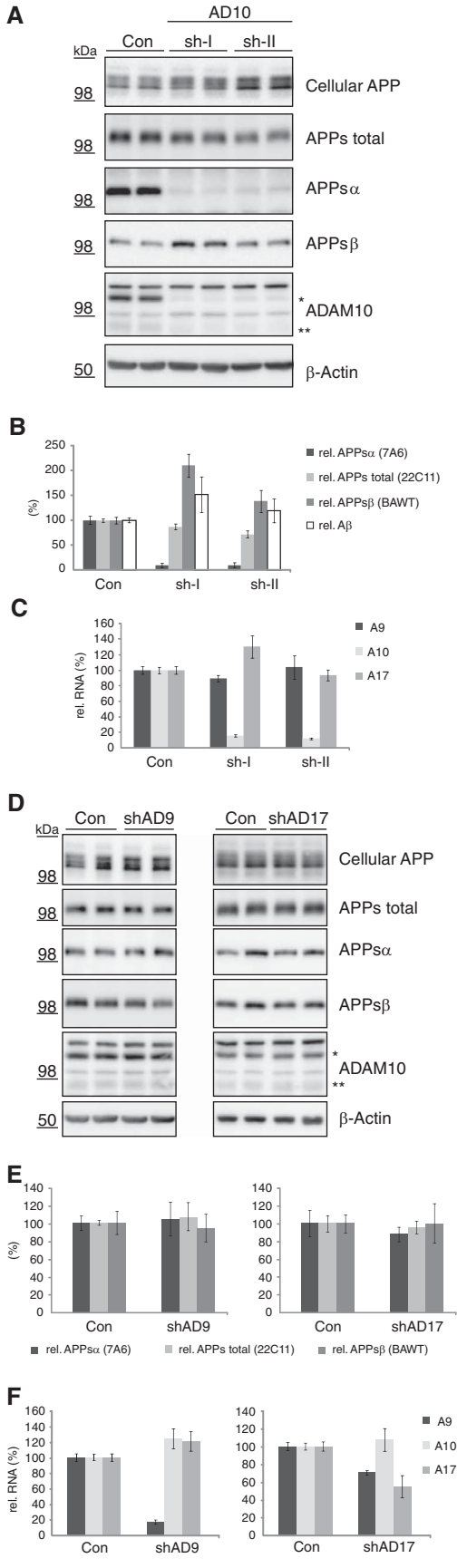


Figure 5 Absence of ADAM10 reduces C83. (**A**) Left panels: SH-SY5Y cells were treated (+) or not (−) with the γ-secretase inhibitor DAPT to stabilize APP C-terminal fragments (CTFs). Endogenous CTFs were only visible upon DAPT treatment. Right panels: SH-SY5Y cells stably expressing ADAM10 shRNAs (sh7, sh9) or control shRNA (Con) were treated with DAPT. Conditioned media were analysed for APPsα (4B4) and APPsβ (192wt). Cell lysates were analysed for cellular APP, all C-terminal fragments (6687) and specifically C99 (2D8). C99 is marked with (*), C89 with (**) and C83 with (***). (**B**) Quantification of APPsα, APPsβ, C83 and C99 normalized to cellular APP relative (rel.) to control. Given are mean and standard error of four independent experiments.

Figure 4 Analysis of APP processing in SH-SY5Y cells with a stable ADAM10 knockdown. (**A**) SH-SY5Y cells were infected with lentiviral vectors carrying GFP and different shRNAs: a non-targeting shRNA (Con) and two different ADAM10-targeting shRNAs (sh6 and sh9). Cells (Con, sh6 and sh9) were either treated with DMSO as solvent control or the metalloprotease inhibitor TAPI-1. Conditioned media of these cells were analysed with antibody 22C11 (APPs total), W02 (APPsα + β′) or 4B4 (APPsα), whereas cell lysates were analysed for cellular APP (22C11). Compared with HEK293 cells, the mature APP form is less well visible in SH-SY5Y cells. Membranes were analysed for ADAM10 protein. Calnexin was detected as a loading control. (**B**) Quantification of experiments in (**A**). APPs total, APPsα + β′ and APPsα were normalized to calnexin. Given are mean and standard error of four independent experiments. (**C**) Cells (Con, sh6 and sh9) were either treated with DMSO as solvent control or with the β-secretase inhibitor C3. Conditioned media of these cells were analysed as in (**A**). Antibody 192wt was used for the detection of APPsβ. (**D**) Quantification of experiments in (**C**), carried out as in (**B**) relative (rel.) to control. (**E**) Con and sh9 SH-SY5Y cells were treated with DMSO as a control or co-treated with the metalloprotease inhibitor TAPI-1 and the β-secretase inhibitor C3. (**F**) Quantification of experiments in (**E**), carried out as in (**B**). Given are mean and standard error of three independent experiments.

quantitative RT–PCR (Figure 6F). Together, these results show that both ADAM9 and 17 are not required for constitutive APP α-secretase cleavage in primary neurons.

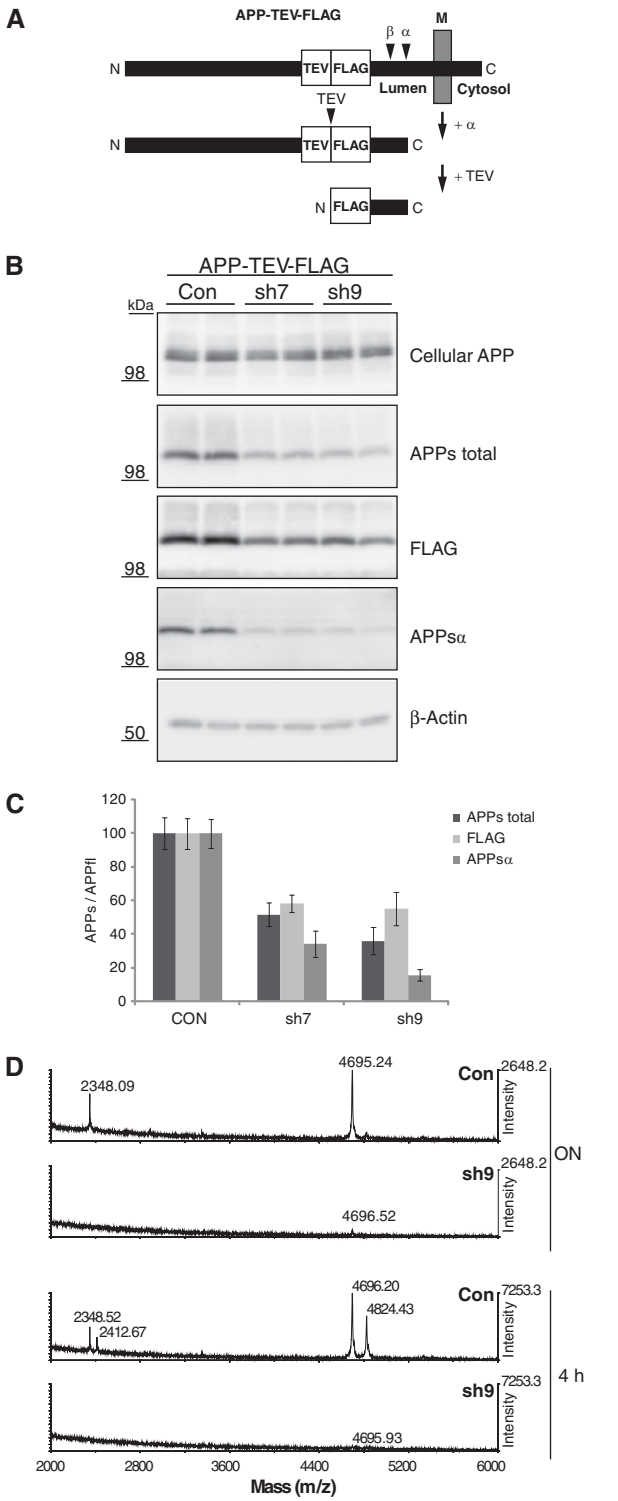


Interestingly, total APP secretion (22C11) was only mildly reduced in the ADAM10 knockdown neurons, which is most likely due to the fact that in primary embryonic neurons, α-secretase cleavage contributes only to a smaller extent to total APP secretion, compared with cell lines (Simons *et al*, 1996) (Figure 6A). Indeed, β-secretase expression is particularly high during embryonic development and in the first 2 weeks after birth, but then drops sharply (Willem *et al*, 2006). In contrast to the HEK293 and SH-SY5Y cells, the ADAM10 knockdown mildly increased APPsβ generation (1.4–2-fold) for both shRNAs tested. Aβ followed a similar trend. However, only one of the shRNA sequences (sh-I) significantly increased Aβ, whereas the other sequence (sh-II) did not. This suggests that at least in embryonic neurons with their high expression of β-secretase, a reduction of ADAM10 may increase Aβ levels.

Mass-spectrometric analysis of APP α-secretase-cleavage products

The experiments above relied on the use of cleavage site-specific antibodies for the detection of APPs and in particular of APPsα. Next, we used mass spectrometry as an independent method to detect APPsα and to investigate the reduction of APPsα in ADAM10 knockdown cells. To this aim, the following strategy was used. APP is *N*- and *O*-glycosylated at different positions within its ectodomain, which leads to a broadening of the peaks obtained for APPs by mass-spectrometric analysis, making identification of specific cleavage sites difficult. To avoid this situation, two short peptide tags were included into the APP ectodomain between the most C-terminal glycosylation site and the N-terminus of the Aβ sequence (Figure 7A). One of the two peptide sequences encodes a TEV protease-cleavage site, the other one encodes a FLAG tag. This mutant APP construct (APP-TEV-FLAG) was stably expressed in SH-SY5Y cells. The secreted form of APP-TEV-FLAG was immunoprecipitated from the conditioned medium with an anti-FLAG antibody and then digested *in vitro* with TEV protease. This leads to the removal

Figure 6 ADAM10 is essential for α-secretase cleavage of APP in primary cortical neurons. (**A**) Primary cortical neurons were prepared at E16 from C57/BL6 mice and infected with purified lentiviral particles carrying GFP and either a non-targeting control shRNA (Con) or two distinct murine ADAM10-targeting shRNAs (sh-I, sh-II). Four days before harvest, the medium was changed. Media were analysed for total secreted APP (antibody 22C11), APPsα (7A6) and APPsβ (BAWT). Cell lysates were analysed for cellular APP, ADAM10 and β-actin (loading control). Immature ADAM10 is indicated with (*) and mature ADAM10 with (**). Protein levels of ADAM10 were reduced by both shRNAs to about 10% of control (not shown). (**B**) Quantification of total secreted APP, APPsβ and APPsα, relative (rel.) to control. Aβ was measured by ELISA. Given are mean and standard error of eight independent experiments. (**C**) Quantitative RT–PCR shows that ADAM10 mRNA levels are strongly reduced, whereas levels of ADAM9 and 17 mRNA were not affected. Given are mean and standard error of four independent experiments. (**D**) Lentiviral knockdown of ADAM9 and 17 in primary neurons was carried out as in (**A**). (**E**) Quantification of results in (**D**) was performed as in (**B**). Both knockdowns did not affect APP processing. Given are mean and standard error of five independent experiments. (**F**) Quantitative RT–PCR shows efficient knockdown of ADAM9 and 17, whereas ADAM10 levels were not affected. Knockdown of ADAM17 partially reduced ADAM9 mRNA levels. Given are mean and standard error of four independent experiments.



E

Peptide	Charge	Measured Mass (Da)	Calculated Mass (Da)	Deviation (Da)	Sample
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+1	4695.24	4695.11	+0.13	ON Con
" "	+2	2348.09	2349.06	−0.97	ON Con
" "	+1	4696.52	4695.11	+1.41	ON sh9
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQK	+1	4824.43	4823.21	+1.22	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+1	4696.20	4695.11	+1.09	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQK	+2	2412.67	2413.11	−0.44	4 h Con
GDYKDDDDKSGLTNIKTEEISEVKMDAEFRHDSGYEVHHQ	+2	2348.52	2349.06	−0.54	4 h Con
" "	+1	4695.93	4695.11	+0.82	4 h sh9

of the glycosylated part of the APP ectodomain, resulting in ~5 kDa peptides having the FLAG tag at their new N-terminus and C-terminally ending at the peptide bond, where APP shedding occurs by the secretases (Figure 7A). First, we verified that APP-TEV-FLAG was processed in a manner similar to the wild-type, endogenous APP. Stable knockdown of ADAM10 in APP-TEV-FLAG-expressing SH-SY5Y cells reduced APP-TEV-FLAG shedding to a similar extent (Figure 7B; quantification in Figure 7C) as observed for the endogenous, wild-type APP in SH-SY5Y cells (compare with Figure 4). Total APPs (22C11) was reduced to 40–50%, whereas APPsα (4B4) was reduced to 15–30% (Figure 7C). Taken together, APP-TEV-FLAG is processed similar to wild-type APP.

For the mass-spectrometric measurements, APP-TEV-FLAG cells were incubated overnight. Secreted APP-TEV-FLAG was immunoprecipitated from the conditioned medium and processed by TEV protease. A major peak at 4695.24 Da was identified, which corresponds to a peptide having glutamine 15 of the Aβ sequence as its C-terminal amino acid (Figure 7D and E). This is in agreement with the C-terminus of APPsα isolated from human brain (Pasternack *et al*, 1992). In addition, a less intensive peak was observed at 2348.09 Da. This is exactly half the molecular weight of the more prominent peak and corresponds to the same peptide, but with a double-positive charge instead of a single-positive charge. Importantly, upon ADAM10 knockdown, both peptide mass peaks were nearly completely suppressed, which is consistent with the reduction in α-secretase cleavage observed in the immunoblots (Figure 7C). Peptides with a C-terminus at the β- or β′-cleavage sites were not detected. As these cleavages make up a small proportion of total APP shedding (see C3-inhibitor treatment in Figure 4C), it is likely that these peptides were below the detection limit in our analysis or were not stable enough during the isolation procedure for the mass-spectrometric analysis. When the APP-TEV-FLAG-expressing cells were incubated for 4 h instead of overnight, a second mass peak at 4824.43 Da was observed (Figure 7D). This corresponds to the peptide having amino-acid lysine 16 of the Aβ sequence as its C-terminal amino acid (Figure 7E). The heterogeneity of one amino acid at the C-terminus is in agreement with the finding that *in vitro* ADAM10 cleaves between lysine 16 and leucine 17 (Lammich *et al*, 1999) and may then be followed by an as yet unidentified carboxypeptidase cleavage, removing lysine 16 (Esch *et al*, 1990). The knockdown of

Figure 7 Mass-spectrometric analysis of APP α-secretase cleavage in stable ADAM10 knockdown SH-SY5Y cells. (**A**) Schematic representation of the APP-TEV-FLAG construct, its cleavage by α-secretase and the further processing with TEV protease to generate small peptides for mass-spectrometric analysis. M, membrane. (**B**) SH-SY5Y cells stably expressing APP-TEV-FLAG were analysed by immunoblot for APPs total (22C11), APPsα (4B4) and FLAG immunoreactivity in the conditioned medium and for cellular APP and β-actin in the cell lysate. (**C**) Quantification of APPs total, FLAG reactivity and APPsα of three independent experiments. Given are mean and standard error. (**D**) APP-TEV-FLAG was immunoprecipitated from medium after overnight (ON) or 4 h culture, digested with TEV-protease and analysed by mass spectrometry. Medium was used from control (Con) or ADAM10 knockdown cells (sh9). (**E**) Table containing peptide sequence, charge, measured masses (from **D**) and calculated masses and deviation of the measured peptides in Da.

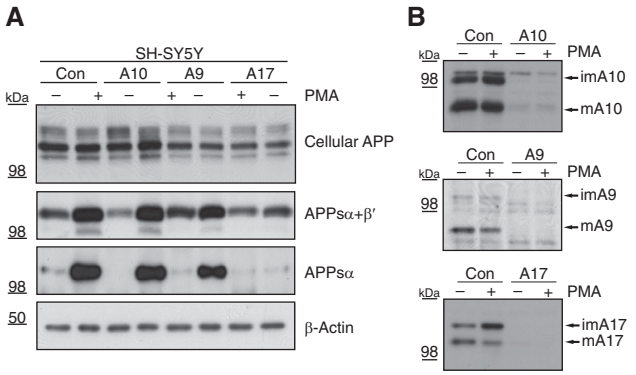


Figure 8 PMA-induced stimulation of APP shedding is independent of ADAM10, but requires ADAM17. SH-SY5Y cells were either transfected with control siRNA pool (Con) or siRNA pools against ADAM9 (A9), ADAM10 (A10) and ADAM17 (A17); 2 days after transfection, cells were treated with 1 μM PMA (+) or ethanol as solvent control (−) for 4 h. (**A**) Conditioned media were analysed for APPsα (4B4), APPsα + β′ (W02) and cell lysates were analysed for cellular APP (22C11). (**B**) Knockdown efficiency was analysed by blotting against the different proteases ADAM9, 10 and 17 in membrane preparations of the respective experiments.

ADAM10 also suppressed the generation of the longer peptide (Figure 7D).

Taken together, the mass-spectrometric analysis—in addition to the use of the cleavage site-specific antibody 4B4—provides a second, independent method to show that ADAM10 is essential for α-secretase cleavage of APP.

PMA stimulates ADAM17 cleavage of APP independently of ADAM10

The phorbol ester PMA stimulates the metalloprotease cleavage of many cell surface membrane proteins, including APPs (Figure 1; Buxbaum *et al*, 1998). PMA-induced shedding of APP requires ADAM17 activity, because this stimulation is lost in mouse embryonic fibroblasts deficient in ADAM17 (Buxbaum *et al*, 1998). Thus, we next investigated, whether the PMA stimulation of APP shedding also required ADAM10. To test this, SH-SY5Y cells expressing endogenous APP were transiently transfected with control siRNAs or siRNAs against ADAM9, 10 or 17 and then treated with or without PMA (Figure 8A and B). In control-transfected cells, PMA strongly increased APPsα (4B4) (Figure 8A), in agreement with Figure 1. knockdown of ADAM17 suppressed the PMA-induced increase in APPsα production. However, when ADAM10 or 9 were knocked down, PMA-stimulated APPsα generation occurred as in wild-type cells (Figure 8A). This shows that ADAM10 is not required for PMA induction of APP shedding and suggests that under these conditions ADAM17 can directly cleave APP.

Discussion

The α-secretase is an important proteolytic activity with the ability to prevent Aβ generation. In this study, we systematically evaluated the contribution of ADAM9, 10 and 17 to α-secretase cleavage of APP. Using a new, APPsα-specific antibody and two different cell lines as well as primary neurons expressing endogenous APP, we found that ADAM10, but not ADAM9 or 17, is essential for α-secretase cleavage. The requirement for ADAM10 was further validated by mass-spectrometric determination of APP-cleavage products. From this we conclude that ADAM10 is the physiologically relevant, constitutive α-secretase of APP and that ADAM9 and 17 are not redundant for this cleavage. This is particularly remarkable, because ADAM10 and 17 appear to have a broad substrate specificity and can cleave similar peptides *in vitro* at the same peptide bonds (Caescu *et al*, 2009). The clear specificity in cells suggests the existence of additional, as yet unknown factors, which control the protease specificity in the cellular environment.

Different metalloproteases, most notably ADAM9, 10 and 17 have previously been suggested as candidate α-secretases, because they cleave APP-derived synthetic peptides *in vitro* and because their overexpression in cells or mice increases APP shedding (Koike *et al*, 1999; Lammich *et al*, 1999; Roghani *et al*, 1999; Slack *et al*, 2001; Postina *et al*, 2004). However, data resulting from overexpression studies do not prove that a particular protease is the physiologically relevant protease for a given substrate. In fact, experiments using cells with a knockout or a knockdown of the corresponding proteases gave less clear results about their involvement in APP α-secretase cleavage. RNAi-mediated knockdown of ADAM9, 10 or 17 reduced APP shedding by 20 to 60% (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Freese *et al*, 2009; Taylor *et al*, 2009). In contrast, knockout cells deficient in ADAM9, 10 or 17 showed no change in APP shedding (Buxbaum *et al*, 1998; Hartmann *et al*, 2002; Weskamp *et al*, 2002). Only in a subset of ADAM10-deficient fibroblasts, APP α-secretase cleavage was altered to a variable degree (Hartmann *et al*, 2002). The reason for this variability is not yet clear. Importantly, because ADAM10 knockout mice die embryonically and ADAM17 knockout mice die perinatally, only embryonic fibroblasts, but not neurons, from these animals have been analysed for APP shedding. Taken together, the finding, that APP shedding was never fully abolished, was taken as evidence that all three proteases may have redundant functions with regard to APP α-secretase cleavage. In contrast to the previous studies, we used the novel antibody 4B4 that specifically detects the α-secretase cleaved APP (APPsα) without a contribution of other APP-cleavage products, such as APPsβ or APPsβ′. Using this new antibody, siRNAs and shRNAs against ADAM10 or treatment with the metalloprotease inhibitor TAPI almost completely blocked APPsα generation in HEK293 and SH-SY5Y cells and in primary neurons. ADAM9 and 17 were not required for APPsα formation. Only in HEK293 cells, the ADAM17 knockdown led to a very modest decrease in total APP secretion, raising the possibility that in specific cell lines ADAM17 may have a modulatory function in APPsα generation. Our knockdown data for ADAM9 and 17 complement the previous finding that ADAM9-deficient primary hippocampal neurons and ADAM17-deficient mouse embryonic fibroblasts do not show evidence of an altered α-secretase cleavage compared with their corresponding wild-type control cells (Buxbaum *et al*, 1998; Weskamp *et al*, 2002). At present, it is unclear, why additional studies using siRNAs against ADAM9 or 17 reported a moderate reduction of APP α-secretase cleavage. However, because those previous studies used only one siRNA per target gene or no control siRNA or relatively high siRNA concentrations (Asai *et al*, 2003; Allinson *et al*, 2004; Camden *et al*, 2005; Taylor

et al, 2009), it seems possible that earlier conclusions about ADAM9 and 17 as APP α-secretase may have been due to off-target effects (Jackson *et al*, 2003), but not due to the specific reduction of ADAM protease expression.

Interestingly, a cleavage by both ADAM10 and 17 was also suggested for the Notch1 receptor. Proteolytic Notch cleavage and signal transduction are required for cell differentiation processes. Recent work established that ADAM10 is the relevant protease for the physiological ligand-induced Notch1 cleavage and signalling, but that under certain ligand-independent conditions, including disease-linked Notch1 mutations, Notch1 cleavage can also be mediated by ADAM17 (Cagavi Bozkulak and Weinmaster, 2009; van Tetering *et al*, 2009).

APP α-secretase cleavage occurs constitutively, which requires ADAM10, as shown in this study. In addition, a heterogeneous group of molecules can stimulate APP α-secretase shedding (Bandyopadhyay *et al*, 2007), which is referred to as regulated α-secretase cleavage. Two stimuli activating APP α-cleavage are the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) and the phorbol ester PMA. The PACAP peptide appears to stimulate the ADAM10 cleavage of APP (Kojro *et al*, 2006), suggesting that ADAM10 is not only the constitutive α-secretase, but also contributes to the regulated α-secretase activity. In addition, ADAM17 can act as regulated APP α-secretase activity, at least upon stimulation with the phorbol ester PMA. We found that this activation does not require ADAM10, but ADAM17, which is in agreement with a previous publication using ADAM17-deficient MEF cells (Buxbaum *et al*, 1998). Future studies need to address whether ADAM17 cleavage of APP also occurs under physiologically or pathophysiologically relevant conditions other than upon treatment with the synthetic phorbol ester PMA.

Previous studies, which will be discussed below, suggested that α- and β-secretases compete for APP as a substrate, such that a change in β-secretase cleavage results in the corresponding opposite change in α-cleavage and vice versa. On the basis of this assumption, an activation of α-secretase cleavage is considered as a therapeutic approach to reduce β-secretase cleavage and Aβ generation. Although this competition seems to be clearly the case for the regulated component of the α-secretase cleavage (see below), we show here that this is not always the case for the constitutive α-secretase cleavage of APP. The knockdown of ADAM10 did not significantly increase APPsβ levels in HEK293 and SH-SY5Y cells. Likewise the β-secretase inhibitor C3 blocked APPsβ generation in SH-SY5Y cells, but did not increase APPsα levels, which is in agreement with a recent study using a different β-secretase inhibitor in CHO cells (Kim *et al*, 2008). The reason for this uncoupling of α- and β-secretase cleavage under constitutive conditions is not yet clear. The cellular APP levels may not be rate limiting for α- and β-secretase cleavage, such that a reduction of one cleavage does not increase the other cleavage. Alternatively, it may reflect that α- and β-secretase cleavage occur in different cellular compartments (described below), such that a reduced α-secretase cleavage would not necessarily increase the endosomal APP levels available for β-secretase cleavage. In contrast to the cell lines, the knockdown of ADAM10 induced a mild increase in β-secretase cleavage in the primary neurons. This effect was more pronounced for APPsβ than for Aβ. The difference between the neurons and the cell lines may result from the different β-secretase expression levels in both cell types. Although the β-secretase BACE1 is ubiquitously expressed, its expression is particularly high in neurons during embryonic development (such as the E16 neurons used here) and in the first 2 weeks after birth and then drops sharply (Willem *et al*, 2006). Future studies need to address whether a knockdown of ADAM10 still increases β-secretase cleavage in adult neurons, where BACE1 levels are reduced. In fact, a lack of competition between constitutive α- and β-secretase cleavage in the adult brain comes from a study, where a dominant-negative ADAM10 mutant decreased APPsα, but did not alter APPsβ levels in a transgenic mouse brain (Postina *et al*, 2004).

The α-secretase cleavage predominantly occurs at the plasma membrane (Sisodia, 1992), but also in the *trans*-Golgi network (TGN), at least upon stimulation with PMA (Skovronsky *et al*, 2000). In contrast, β-secretase cleavage of wild-type APP occurs mainly in the endosome and to a lower extent in the TGN (Koo and Squazzo, 1994; Vassar *et al*, 1999). Previous studies reported a competition between α- and β-secretases for APP as a substrate. This was typically the case when the corresponding protease was overexpressed or when an APP mutant was used or when α-secretase cleavage was activated above its constitutive level. Under these conditions, α- or β-secretase cleavage mostly occurred in a cellular compartment where the constitutive cleavage does not take place to the same extent. For example, overexpression of the β-secretase BACE1 strongly reduced α-secretase cleavage (this study and Vassar *et al*, 1999), presumably because overexpressed BACE1 artificially cleaves APP in early compartments of the secretory pathway before APP has access to α-secretase at the plasma membrane. A second condition, where a competition between α- and β-secretase cleavage was observed, is the Swedish mutant form of APP (SweAPP), which is linked to a familial form of AD. Compared with wild-type APP, the SweAPP is more efficiently cleaved by β-secretase and is processed to more Aβ and less APPsα, presumably because the SweAPP is already cleaved by β-secretase in the TGN before it has access to α-secretase (Haass *et al*, 1995). As another example, PMA increased APPsα and reduced APPsβ and Aβ in APP-transfected CHO cells (Skovronsky *et al*, 2000). The authors argued that PMA shifts APP α-secretase cleavage away from the plasma membrane towards the Golgi/TGN, such that APP is cleaved earlier in the secretory pathway and less APP is available for β-secretase cleavage. This shows that the regulated component of α-secretase (i.e. the increase of α-secretase cleavage above its constitutive level) can compete with β-secretase and consequently reduces Aβ generation, in agreement with the idea that a pharmacological activation of α-secretase may be a therapeutic approach to AD (Fahrenholz, 2007).

Another outcome of our study is that the ADAM10 knockdown increased the levels of C99 in SH-SY5Y cells, although the amount of β-secretase cleavage (measured by APPsβ levels) was unchanged. From this finding, we conclude that C99 can principally be processed in two pathways. In the first one, C99 is directly cleaved by γ-secretase leading to Aβ generation. In the competing pathway, C99 is first cleaved by α-secretase, leading to C83 generation, which may prevent Aβ generation. Upon ADAM10 knockdown, the latter

pathway is blocked, leading to an increase in C99, while leaving APPsβ levels unchanged. As C99 is short-lived, we used a γ-secretase inhibitor to visualize it. The increase in C99 upon ADAM10 knockdown is unlikely to occur to the same extent in the absence of a γ-secretase inhibitor. If that were the case, we would expect an increase in Aβ, as it is the direct cleavage product of C99. This, however, was not the case. From this we conclude that the direct C99 cleavage by γ-secretase is the predominant pathway for C99 processing under normal conditions. However, when γ-secretase cleavage is blocked, the competing pathway by α-secretase becomes more prominent resulting in C99 turnover to C83. A cleavage of C99 by α-secretase is consistent with a previous study, showing that overexpressed C99 can be converted to C83 in neurons (Cupers *et al*, 2001). A possible competition between α- and γ-secretases for C99—even under conditions, where γ-secretase is not inhibited—is supported by a recent study, which detected N-terminal Aβ fragments, such as Aβ1–15 and Aβ1–16, that seem to result from α-secretase cleavage of C99 (Portelius *et al*, 2009).

Our new antibodies, which are specific for APPsα and do not detect APPsβ′, may be helpful in the search for biomarkers of AD. Previous studies reported that APPsα levels are significantly decreased in the CSF of sporadic AD patients compared with controls, but there was not a complete separation between both groups (Sennvik *et al*, 2000; Fellgiebel *et al*, 2009). Potentially, the use of APPsα-specific antibodies, such as 4B4 may help to separate AD and control subjects more clearly. In addition, changes in APPsα levels may help to identify individuals with mutations in ADAM10. Mutations in the prodomain of ADAM10, which reduce ADAM10 protease activity, have recently been genetically linked to an increased risk for late-onset AD in seven distinct families (Kim *et al*, 2009).

In summary, our study defines ADAM10 as the physiologically relevant, constitutive α-secretase for APP and will allow to further explore the function of ADAM10 in AD.

Materials and methods

Reagents, plasmids and shRNA

The following antibodies were used: FLAG M2 (Sigma), ADAM10 (Calbiochem-422751), ADAM17 (Chemicon), ADAM17 (Oncogene), ADAM9 (Cell Signaling), HRP-coupled anti-rabbit, anti-mouse (DAKO), HRP-coupled anti-rat (Santa Cruz), Calnexin (Stressgen), β-actin (Sigma), monoclonal antibody (mAb) 22C11 (anti-APP ectodomain) and mAb W02 (against amino acids 5–8 of Aβ) from Konrad Beyreuther; polyclonal antibody (pAb) 5313 (anti-APP ectodomain), pAb 6687 (against APP C-terminus), pAb 3552 (against Aβ) and mAb 2D8 (against Aβ1–16) from Christian Haass and pAb 192Wt from Dale Schenk. Rat mAb 4B4 (IgG2a, APPsα specific), mAb 7A6 (IgG2a, APPsα specific; detects also murine APPsα) and rat mAb BAWT (IgG2a, APPsβ specific, used for immunoprecipitation) were generated against peptides EVHHQK-COOH (amino acids 11–16 of Aβ), YEVHHQ-COOH (amino acids 10–15 of Aβ) and ISEVKM-COOH (amino acids directly preceding the β-secretase-cleavage site), respectively (Ullrich *et al*, 2010). The following reagents were used: metalloprotease inhibitor TAPI-1, BACE inhibitor C3 and Dodecyl maltoside (DDM) from Calbiochem; siRNA pools siGenome against ADAM9, 10 and 17 and siRNA pool on target plus against ADAM17 and corresponding controls from Dharmacon. Lipofectamine 2000, RNAimaxx and TEV protease from Invitrogen. Cloning of plasmids is described in the Supplementary data section. shRNA sequences are listed in Supplementary Table 1.

Cell culture, transfections, RNAi, sample preparation, immunoblot, Aβ measurements

HEK293-T cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% foetal calf serum (FCS/Gibco) and G418 (Invitrogen) to maintain the large-T antigen. SH-SY5Y cells were cultured in F12/DMEM (Lonza) supplemented with 15% FCS (Gibco) and non-essential amino acids (PAA). knockdown of ADAM9, 10 and 17 in HEK293T and SH-SY5Y cells was performed transfecting 10 nM of siGenome pool targeting ADAM9, 10, 17 and corresponding controls or transfecting 10 nM of OnTarget plus pool targeting ADAM17 and corresponding controls (in SH-SY5Y). One day after transfection, medium was replaced. After overnight incubation, conditioned medium and cell lysate (in 150 mM NaCl, 50 mM Tris pH 7.5, 1% Nonidet P-40) were collected. For detection of ADAM9, 10, 17 and calnexin, cell membranes were prepared as described (Sastre *et al*, 2001). Detection of secreted and cellular APP was as described (Schobel *et al*, 2008). For precipitation of APPsβ, 500 μl of conditioned medium were incubated with 30 μl protein G sepharose and 50 μl of BAWT antibody for 2 h on a rotary shaker. APPsβ was detected with 192wt antibody. For precipitation of APPsα, 500 μl of conditioned medium were incubated with 30 μl protein A sepharose and 3.3 μl of 5313 antibody. APPsα was detected with 4B4 antibody. Inhibition of α- and β-secretases was performed with 50 μM TAPI-1 and 1 μM C3, respectively, for 24 h. Endogenous human Aβ was immunoprecipitated with antibody 3552 and detected with rat mAb 2D8 as described (Page *et al*, 2008). Murine Aβ40 was measured in conditioned media of murine primary cortical neurons, which were diluted 25-fold before analysis with an ELISA-kit (IBL, JP27720) according to the instructions of the manufacturer.

Lentivirus production and transduction

Lentiviruses were generated by transient cotransfection of HEK293T cells with the plasmids psPAX2, pCDNA3.1 (−)-VSV-G and as transfer vector pLVTHMmod or pLKO2mod-EGFP-WPRE for gene knockdown or FU-ΔZeo for gene overexpression using Lipofectamine 2000. For transduction of cell lines, medium was replaced by fresh antibiotic-free medium 1 day after transfection. Overnight conditioned medium was filtered through 0.45 μm sterile filters and directly added to the target cells. After 6 h, incubation medium was exchanged against cell type-specific growth medium of the target cells. Lentiviral particles for infection of murine primary cortical neurons were concentrated by one run of ultracentrifugation for 2 h at 22 000 r.p.m. in a SW28 rotor (Beckman) of the overnight conditioned medium 48 h after transfection. Following ultracentrifugation, the supernatant was removed and the viral pellet carefully resuspended in TBS-5 (50 mM Tris, 130 mM NaCl, 10 mM KCL, 5 mM $MgCl_2$, 5% (w/v) BSA) after a 4 h incubation period at 4°C. Lentiviral stocks were stored at −80°C until use.

Preparation and lentiviral transduction of neurons

Primary neuronal cultures were obtained from the cerebral cortex of E16 C57/BL6 mouse embryos, incubated with 200 U of papain (Sigma Aldrich) (30 min at 34°C) and subsequently mechanically dissociated. All experimental procedures on animals were performed in accordance with the European Communities Council Directive (86/609/EEC). Neurons were plated in six-well plates ($\sim 1.5 \times 10^6$ cells/well) pre-coated with 25 μg/ml poly-D-lysine (Sigma Aldrich). Plating medium was B27/neurobasal (Gibco) supplemented with 0.5 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Neurons were infected with lentiviruses at 3 DIV. Cell lysates and supernatant were collected at 8 DIV.

Mass spectrometry of APP-cleavage sites

SH-SY5Y cells were infected with FUΔ-Zeo-DsRed-UAS-APP-TEV-FLAG and FUΔ-Zeo-Gal4-VP16. Infected cells were subsequently FACS sorted according to their DsRed expression to obtain a homogenous-expressing population. Afterwards, cells were infected with PLVTHM encoding shRNAs against ADAM10 sh7 and sh9 or a control shRNA. Secreted APP-TEV-FLAG was immunoprecipitated with FLAG-M2 agarose. Immunoprecipitation was followed by three washes with STED (NaCl 150, Tris 50 mM, EDTA 2 mM, 0,5% DDM), three washes with STE and three washes with ddH_2O. Precipitated APP-TEV-FLAG ectodomain was eluted with 40 μl 100 mM glycine pH 2.5. The eluate was subsequently neutralized with 200 μl 100 mM Tris pH 8.0 and supplemented with 0.5 mM EDTA and 1 mM DTT and addition of 0.5 μl TEV protease. Protease

digest was incubated at 4°C overnight on a rotary shaker. The digest was diluted with 15 ml of PBS. To precipitate the digested FLAG peptide, FLAG M2 agarose was added and incubated at 4°C for 2 h on a rotary shaker. Agarose was washed three times with PBS and three times with ddH_2O. Afterwards, peptides were eluted in a 1/20/20 mixture of trifluoracetic acid/acetonitrile/ddH_2O saturated with α-Cyano matrix. A total of 1 µl was spotted on a hydrophobic target and measured with a Voyager DestR in linear mode. The MALDI-TOF mass spectrometer was externally calibrated with a peptide standard mixture (Sequazyme calibration mixture III).

Quantitative real-time PCR

Total RNA was extracted using RNeasy Mini kit (Qiagen) from primary neurons following the manufacturer's instructions. Concentrations and purities of total RNA were spectrophotometrically assessed at 260 and 280 nm. Total RNA was reverse transcribed into cDNA in a 20 µl reaction volume, using high-capacity cDNA Reverse Transcription kit (Applied Biosystems/ABI). Real-time PCR reaction was carried out on a 7500 Fast Real-Time PCR machine (ABI) with the POWER SYBR®-Green PCR Master Mix (ABI) based on a modification of the manufacturer's recommended protocol. Reactions were performed in duplicate in 96-well plates (ABI) according to the following protocol: pre-incubation at 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Used primers including ADAM9, 10 and 17 and the three reference genes Actb, GapDH and Tbp are listed in Supplementary Table 2. Validation experiments were performed to verify the amplification efficiency of each gene, which consistently ranged from 1.8 to 2.2. The statistic analysis was performed by the ΔCt value method. The relative expression of ADAM9, 10 and 17 was normalized to all three reference genes.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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