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Detection of the Alzheimer´s disease soluble amyloid precursor protein and modulation of its proteolytic processing

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Declaration

I hereby declare that this thesis is the result of my original work under the supervision of Prof. Stefan Lichthenthaler. All data presented in the thesis were performed independently. The thesis is complete with references from scientific literature that has been used as a background for the projects. Sources of cited information have been listed in the "Reference" section.
The results have been partially published in the paper " A new sandwich immunoassay for detection of the α-secretase cleaved, soluble amyloid precursor protein in cerebrospinal fluid and serum" (Taverna M., Straub T., Hampel H., Rujescu D., Lichtenthaler S.F., 2013, Journal of Alzheimer´s disease, in press).
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 this thesis began in October 2008 and it was performed in the laboratory of Prof. Stefan Lichtenthaler at Deutsches Zentrum fuer Neurodegenerative Erkrankungen (DZNE), Munich and the Adolf-Butenandt Institute, Ludwig-Maximilians University Munich, Germany.

Munich, on

Mara Taverna

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ABSTRACT

Alzheimer´s disease (AD) is the most prevalent form of dementia worldwide. The major pathological hallmarks of the disease are neuronal loss, extracellular insoluble deposits of amyloid β-peptide (Aβ), and intracellular neurofibrillary tau tangles. In particular the amyloid β peptide (Aβ) is a small hydrophobic peptide, resulting from the sequential cleavage of the amyloid precursor protein (APP) catalysed by β-secretase BACE1 and the γ-secretase complex. In contrast, APP cleavage by the α-secretase ADAM10 prevents Aβ formation by cleavage within the Aβ domain. Since the precise aetiology of the disease is still uncertain, diagnosis of Alzheimer´s disease is not trivial, especially in the early stage of the disease, when disease-modifying drugs could be applied to significantly slow the progression of the disease. Besides neuropsychogical tests and imaging techniques, biomarkers reflecting these neuropathological changes, namely Aβ42, tau and phospho-tau levels, are measured in cerebrospinal fluid of demented patients. These biomarkers can support the diagnosis of Alzheimer´s disease, but they do not allow a complete separation between demented and non-demented elderly people. Thus, research on potential AD biomarkers is focused on finding new proteins or protein fragments, which could better correlate with the disease and reflect the pathological changes, ideally at the early stage of the disease. APPsα is the product of α-secretase cleavage, and it reflects the metabolic processing of APP. It has been proposed as a potential biomarker for AD, but with poor results. Several studies reported divergent results about changes in APPsα levels in CSF from AD patients, mainly because of the use of antibodies which did not exclusively detect APPsα, but they cross react with other product of APP metabolism. Here, a new APPsα ELISA-like sandwich immunoassay was developed using the new 14D6 antibody, which specifically detects APPsα in cell culture supernatant, human CSF and serum. APPsα levels in CSF and serum from Alzheimer´s disease patients and non-demented controls were measured. A moderate, but significant increase in APPsα levels in CSF was detected in AD patients in comparison to controls, whereas APPsα levels were significantly decreased in serum. The new 14D6 immunoassay performed better than the commercially available assay for the detection of the α-secretase product of APP. Therefore, this new assay is broadly applicable for specific APPsα measurement in cell culture media, CSF and serum.

The second part of the thesis relates to the modulation of APP cleavage by α- and β-secretases. In fact, detection of APP soluble fragments is not only important for diagnostic purposes, but also to gain further knowledges about the regulation of APP proteolytic processing. For example, little is known about the role of kinases in this process. APP intracellular domain contains several phosphorylation sites, making kinases an attractive target of study to better understanding the molecular mechanisms at the basis of Alzheimer´s disease. Moreover, kinases could be putative drug-targets, with the goal of reducing Aβ generation and tau phosphorylation. Dysregulation of a number of different kinases has been described to play a role in the pathology of AD, but only few of them have been extensively studied. Since an unbalanced phosphorylation rate of APP and related pathways could trigger AD pathology, the focus of the second part of this thesis is to identify new kinases, which modulate the proteolytic processing of APP. An RNA-interference high-throughput screening was performed to knock-down the human kinome (720 genes) in neuroblastoma cells expressing endogenous APP. Different validation strategies generated a list of putative hits, which were further investigated in murine embryonic neurons. Among these candidates, the kinase STK39 was identified as new modulator of β-secretase cleavage of APP. STK39 knock-down showed a decrease in APPsβ shedding, Aβ generation and BACE1 protein level in both neuroblastoma cells and primary neurons. These new findings show that inhibition of STK39 may be beneficial to prevent Alzheimer´s disease, and STK39 could be a putative interesting drug target.

ZUSAMMENFASSUNG

Die Alzheimer-Krankheit (AD) ist die häufigste Demenzform weltweit. Die wichtigsten pathologischen Kennzeichen der Krankheit sind der Verlust von Nervenzellen, extrazelluläre Ablagerungen des unlöslichen Amyloid β-Peptides (Aß) und intrazelluläre, neurofibrilläre tau Ablagerungen. Das Amyloid-β-Peptid (Aß) ist ein kleines hydrophobes Peptid, welches aus der sequentiellen Spaltung des Amyloid-Vorläufer-Proteins (APP) durch die Proteasen β-Sekretase BACE1 und den γ-Sekretase-Komplex resultiert. Im Gegensatz dazu verhindert die Ektodomänenspaltung innerhalb der Aβ-Domäne von APP durch die α-Sekretase ADAM10 die Aβ-Bildung. Aufgrund der noch unsicheren Ätiologie der Alzheimererkrankung ist die Diagnose der Alzheimer-Krankheit nicht einfach, insbesondere in der frühen Phase der Erkrankung, wenn krankheitsmodifizierende Medikamente noch angewendet werden können, um das Fortschreiten der Krankheit aufzuhalten. Neben neuropsychologischen Tests und bildgebenden Verfahren, welche diese neuropathologischen Veränderungen nachweisen können, werden Biomarker wie z.B. Aβ42, tau und phospho-tau im Liquor von dementen Patienten gemessen. Diese Biomarker können die Diagnose der Alzheimer-Krankheit unterstützen, erlauben aber keine 100%-ige Spezifität bei der Diskriminierung zwischen dementen und nicht-dementen älteren Menschen. So konzentriert sich die Alzheimer Biomarker Forschung auf die Suche nach neuen Proteinen oder Proteinfragmenten, die besser mit pathologischen Veränderungen, idealerweise in einem frühen Stadium der Krankheit korrelieren. APPsα resultiert durch die α-Sekretase-Spaltung von APP und spiegelt damit die metabolische Verarbeitung von APP wider. Es ist als potentieller Biomarker für die AD vorgeschlagen worden, jedoch mit sehr variablen Ergebnissen. Mehrere Studien zeigen divergierende Ergebnisse über die Veränderungen in den Spiegeln von APPsα im CSF von AD-Patienten, vermutlich vor allem wegen der Verwendung von Antikörpern, die nicht ausschließlich mit APPsα, aber auch mit einem anderen Produkt des APP Stoffwechsels reagieren können. Deshalb wurde hier ein neuer APPsα ELISA-ähnlich-Sandwich-Immunoassay, unter Verwendung des neuen 14D6 Antikörper, welcher spezifisch APPsα im Zellkulturüberstand, menschlichem CSF und Serum nachweisen kann, entwickelt. Die APPsα Spiegel im Serum und Liquor von Alzheimer-Patienten und nicht-dementen Kontrollen wurden gemessen. Eine mäßige,

aber signifikante Zunahme des APPsα Spiegels im CSF von AD-Patienten wurde im Vergleich zu den Kontrollen nachgewiesen, während die APPsα Spiegel im Serum deutlich sanken. Damit ist der neue 14D6 Immunoassay besser als kommerziell erhältliche Assays zum Nachweis des α-Sekretase-Produktes von APP und könnte somit eine breite Anwendung für die Bestimmung von APPsα im Zellkulturmedium, Serum und Liquor finden.

Der zweite Teil der Arbeit bezieht sich auf die Modulation der APP-Spaltung von α- und β- Sekretase. In der Tat ist die Detektion von löslichen APP Fragmenten nicht nur wichtig für diagnostische Zwecke, sondern auch für ein tiefergehendes Verständnis der Regulation der APP-Proteolyse. Zum Beispiel ist wenig über die Rolle von Kinasen in der APP Proteolyse bekannt. Die APP intrazelluläre Domäne enthält jedoch mehrere Phosphorylierungsstellen, wodurch Kinasen ein attraktives Studienziel für ein besseres Verständnis der grundlegenden molekularen Mechanismen der Alzheimer-Krankheit sind. Darüber hinaus können Kinasen pharmakologische Targets sein, um die Bildung von Aβ und Tau-Hyperphosphorylierung zu verringern. Die Dysregulation verschiedener Kinasen ist im Rahmen der Alzheimer Demenz beschrieben worden und könnte damit eine Rolle in der Pathologie der AD spielen. Jedoch wurden nur wenige von ihnen ausgiebig untersucht. Da eine unausgewogene Phosphorylierung von APP und verwandter Signalwege die Pathologie von AD auslösen könnte, steht die Studie neuer Kinasen, die die proteolytische Prozessierung von APP modulieren, im Mittelpunkt des zweiten Teils dieser Arbeit. Um das menschliche Kinom (720 Gene) in Neuroblastom-Zellen, die endogenes APP exprimieren, in der Expression zu unterdrücken, wurde ein RNA-Interferenz Hochdurchsatz-Screening durchgeführt. Verschiedene Validierungsstrategien führten zu einer Liste von Treffern, die weitergehend in murinen embryonalen Neuronen untersucht wurden. Unter diesen Kandidaten wurde die Kinase STK39 als neuer Modulator der β-Sekretase-Spaltung von APP identifiziert. Der Knock-down von STK39 zeigte eine Abnahme in der Bildung von APPsβ, Aβ und des Proteinlevels von BACE1 in beiden Neuroblastom-Zellen und primären Neuronen. Diese neuen Ergebnisse zeigen, dass die Hemmung von STK39 vorteilhaft sein kann, die Alzheimer-Krankheit zu verhindern, womit STK39 ein mutmaßliches interessantes pharmakologisches Target sein könnte.

1 INTRODUCTION

1.1 Alzheimer's disease (AD)

1.1.1 History

Progressive mental deterioration in the elderly has been described throughout history. However, only in 1906 the German Physician Dr. Alois Alzheimer, specifically identified symptoms and histopathological hallmarks of a dementia type which nowadays is known as Alzheimer´s disease. In a clinical report, Dr. Alzheimer carefully described the symptoms and the anatomical characteristics of his patient Auguste Deter. At the beginning of the disease, the 51-year-old woman showed unjustified jealousy towards her husband. Soon she was affected by a rapid loss of memory, disoriented in time and space, and hallucinated. Her cognitive capabilities were seriously impaired, leaving instead motoric capabilities unaltered. She was able to walk independently and to use hands equally well. After 4 and a half years of increasing imbecility, the patient died.
The post mortem analysis of the brain showed an evenly atrophic brain, with neurofibrillary tangles impregnated of Bielschowsky´s silver staining and "minute military foci which are caused by deposition of a special substance in the cortex", nowadays known as amyloid plaques (Alzheimer, 1907; Alzheimer et al., 1995).

A B

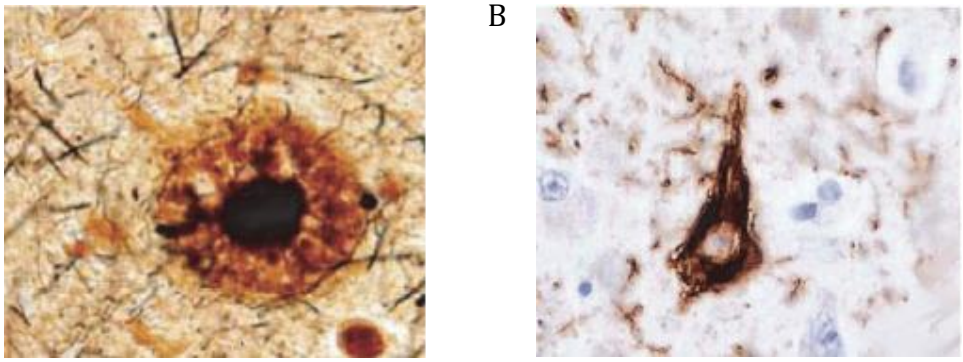

Fig. 1: Histopathological changes in Alzheimer´s disease brain. A) extracellular β-amyloid plaque B) intracellular neurofibrillary tangle (from Sanchez, 2011).

1.1.2 Epidemiology

Alzheimer´s disease (AD) is the most common cause of dementia in the elderly, accounting for up to 75% of all dementia cases. Since AD is a progressive neurodegenerative disease, its impact to public health and social care systems is having great resonance considering the worldwide phenomenon of population aging (Reitz et al., 2011). The number of people older than 65 years is expected to increase from 7% to 12% by 2030, with peaks in developing countries. Currently, 25 million of those people are affected by dementia, and most of them suffer from AD. People with dementia are estimated to double every 20 years. People with AD were estimated to be 4.5 million in 2000 in US, and by 2050 this number is predicted to be quadruple. Thus, enormous resources are needed for appropriate care of AD and dementia patients, considering that the worldwide overall societal costs of dementia in 2005 in US were more than $315 billion, without calculating the unpaid health care network like families and friends (Reitz et al., 2011; Wimo et al., 2011).

1.1.3 Pathology

The most obvious macroscopic characteristics of an Alzheimer´s disease brain is the weight decrease from approximately 1300-1700 g of the normal adult brain to 800-1000g of the AD one. The shrinking affects usually both hemispheres in a symmetrical manner, mostly in the cortical area and hippocampal regions (Sanchez, 2011).

Microscopically, the main lesions associated with Alzheimer´s disease are senile plaques, neurofibrillary tangles and neuronal loss, in particular cholinergic neurons.
Senile plaques are abnormal amyloid deposits, firstly described by Alois Alzheimer, where the main component is the amyloid β peptide (Aβ), a 4 kDa hydrophobic peptide prone to aggregation (Glenner and Wong, 1984; Masters et al., 1985a; Masters et al., 1985b). Aβ peptide is the normal product of the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretase. After β-secretase cleavage, γ-secretase can cleave at two different positions, producing the 40 and 42 amino acid long Aβ40 and Aβ42. Both peptides are found in amyloid plaques, with prevalence of the Aβ42, due to its higher rate of fibrillization and insolubility (Serrano-Pozo et al., 2011). In pathological conditions, Aβ42 accumulates and aggregates in extracellular deposits, called amyloid plaques.

Growth and deposition of amyloid plaques are partially linked to disease progression and this process can be followed in vivo in patients using the Pittsburg-Compound-B (PIB) associated with a position emission tomography (PET) analysis (Braak and Braak, 1997; Braak and Braak, 1991; Verhoeff et al., 2004). Another approach to follow and categorize the plaques is the Thioflavin-S staining (a dye specific for the β-pleated sheet conformation). In this case, Thioflavin-S negative diffuse plaques are not harmful for the neuronal environment, and they are found in brains of cognitively normal elderly people, whereas Thioflavin-S positive dense-core plaques are concomitant to neuronal loss in many patients with AD dementia (Bussiere et al., 2004; Urbanc et al., 2002; Vehmas et al., 2003). Amyloid plaques accumulate preferably in the cortex (Braak and Braak, 1991). Another main histopathological hallmark of AD are the neurofibrillary tangles (NFTs). They were described for the first time by Alois Alzheimer, and they are mainly made of paired helical filaments which are also called fibrils (Alzheimer et al., 1995). The major component of neufibrillary tangles is the microtubule-associated protein tau, a 68 kDa protein that is hyperphosphorylated and aberrantly misfolded in diseased conditions (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b; Iqbal et al., 1986). In healthy neurons, tau is associated with microtubules and involved in the promotion of microtubule formation (Gallyas, 1971). NFTs can be seen with silver staining methods described also as Gallyas technique (Braak and Braak, 1991) or phospho-tau specific antibodies, such as AT8 and PHF1 (Braak et al., 2006). The intracellular location of the NFTs is thought to be the main cause of breakdown of axons and dendrites (Serrano-Pozo et al., 2011).

Several clinicopathological studies have established a correlation between the distribution and amount of NFTs and the duration and severity of dementia (Bierer et al., 1995). In particular, the distribution of the neurofibrillary tangles matches with the neuropsychological profile found in different progressive stages of AD dementia.

1.1.4 Familial and sporadic Alzheimer´s disease

Alzheimer´s disease is a heterogeneous dementia characterized by the following genetic and clinical features.

The familial form of AD (FAD) consists of barely 5% of the total AD patients´ population. The cognitive impairment in FAD is associated with neurological symptoms such as spasticity, ataxia and motor control disorders at early age, between 25 and 60

years old, so that the familial form of AD is also called early onset AD (EOAD). The first evidence of genetic correlation in AD cases was the discovery of the triplication of the amyloid precursor protein (APP), which is located in the 21q21 locus, in patients with Down Syndrome and concomitant cognitive impairment by Glenner and Wong (Glenner and Wong, 1984). APP gene was cloned in 1987 (Kang et al., 1987; Tanzi et al., 1987), leading to linkage studies in families with subjects affected by FAD.

Many missense mutations have been so far discovered in APP gene (http://www.molgen.ua.ac.be/ADMutations). These mutations impair APP processing via different mechanisms. For example, the Swedish mutation KM670/671NL is located at the β-cleavage site of Aβ sequence. Since this mutation provides a better substrate to β-secretase activity, Aβ40 and Aβ42 production is increased (Citron et al., 1992; Mullan et al., 1992). Other mutations are localized C-terminally to the Aβ sequence, such as the Austrian, Iranian, French, German, London, and Florida mutations. They are linked to an increased production of Aβ42, which has the property to aggregate faster (Suzuki et al., 1994). The Arctic and Dutch mutations are located in the mid region of Aβ (Levy et al., 1990; Nilsberth et al., 2001). They change the structure of the Aβ peptide, increasing the aggregation property and enhancing its toxicity (for details concerning the structure of APP, see chapter 1.2.1, Fig.3; for APP processing, see chapter 1.2.2, Fig.4). The inheritance of these mutations is autosomally dominant.

Other mutations have been found in presenilin1 and presenilin2 genes. Presenilins are the major components of the γ-secretase complex, the proteolytic activity of which is the rate-limiting step for the production of Aβ. To date, several missense mutations, small deletions, insertions or splice mutations have been identified in PSEN1 and PSEN2 genes (http://www.molgen.ua.ac.be/ADMutations). These mutations are responsible of increasing the relative ratio between Aβ42 and Aβ40 (De Strooper, 2007).

The sporadic form of AD is the most common cause of dementia in subjects older than 60 years, and it comprises more than 90% of all AD patients.

The genetic component of the sporadic form is less clear in comparison with the early onset: several susceptibility genes have been so far reported, wherein Apolipoprotein E (ApoE) genotype is the strongest risk factor of this group (Myers et al., 1996). Several single nucleotide polymorphisms have been found in ApoE gene (Nickerson et al., 2000). The following most common three SNPs are responsible for the production of the three main ApoE isoforms: the APOE allele ε4 is reported to be the risk factor for AD,

whereas the allele ε2 shows a protective role; allele ε3 is the neutral common allele (Corder et al., 1994; Corder et al., 1993; Strittmatter et al., 1993). The different combination of these alleles alters ApoE function and structure (Mahley and Huang, 2006). The APOE ε4 allele colocalizes with amyloid plaques (Schmechel et al., 1993; Wisniewski et al., 1994). The increased risk of developing AD carrying one ε4 allele is estimated to be three-fold higher in comparison to the ε3 allele, and 12-fold when carrying both copies (www.alzgene.org). The underlying mechanisms how ApoE might modulate Aβ accumulation and aggregation are still under debate. Nevertheless, studies so far suggest that ApoE influences in one hand Aβ clearance across the blood brain barrier and thus Aβ seeding and oligomerization (Castellano et al., 2011; Jiang et al., 2008).

1.1.5 The amyloid cascade hypothesis

The amyloid cascade hypothesis postulates that Aβ aggregation is the first and crucial step at the beginning of Alzheimer´s disease pathology (Hardy and Selkoe, 2002). This hypothesis was supported by the presence of Aβ as main constituent of amyloid plaques, and the discovery of FAD mutations in PSEN1 and PSEN2, which increase the production of Aβ42, accelerating the onset of Alzheimer´s disease (Ertekin-Taner, 2007; Glenner and Wong, 1984; Jonsson et al., 2012)

According to this hypothesis (Fig.2), unbalanced production/degradation of highly aggregating Aβ42 peptides leads to the accumulation and subsequent aggregation of this β-amyloid peptide. The abundantly produced Aβ peptide forms first oligomers, than protofibrils, fibrils and finally amyloid plaques (Martins et al., 2008; Rijal Upadhaya et al., 2012). Aβ-mediated neurotoxicity is actually more linked to Aβ oligomers, rather than to monomers or fibrils, as it was thought at the beginning of AD research (Dahlgren et al., 2002; Walsh et al., 2002). Oligomers were found to promote calcium dysregulation and membrane disruption (Demuro and Parker, 2005).

In this hypothesis, tau hyperphosphorylation and tau tangles were not taken in consideration. Only recently it has been shown that Aβ oligomers foster also tau hyperphosphorylation (Jin et al., 2011; Ryan et al., 2009).

The amyloid cascade hypothesis was first postulated by John Hardy in 1992 (Hardy and Higgins, 1992) and then revised considering new findings in 2002 (Hardy and Selkoe, 2002).

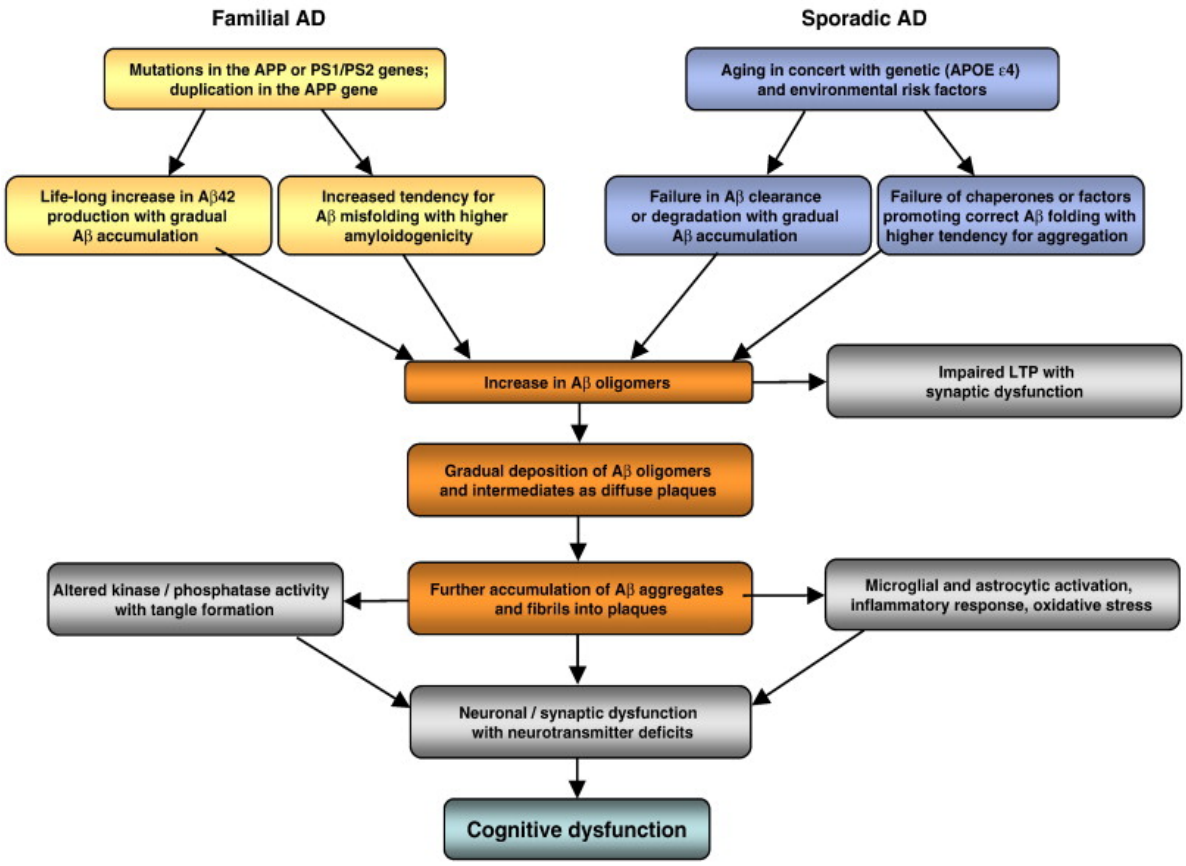

Fig. 2: The modified amyloid cascade hypothesis. The sequence of pathogenic events leading to Alzheimer´s disease dementia is caused by the toxicity of Aβ oligomers. tau hyperphosphorylation and tangles are downstream events that contribute to neuronal degeneration and cognitive impairment (from Hampel et al., 2010b).

The amyloid cascade hypothesis had so far great influence not only in driving research for better understanding the onset of AD, but also in the development of new drugs as treatment for AD in pharmaceutical industries. However, treatment approaches based on this hypothesis failed in phase III clinical trials, since treated patients were in the advanced stage of the disease.

1.2 Amyloid precursor protein (APP)

The amyloid precursor protein (APP) belongs to a family of conserved type I membrane proteins which includes also APP like protein 1 (APLP1) and 2 (APLP2) in mammals, as well as homologue proteins like APL-1 in C. elegans and APPL in Drosophila. These proteins have several conserved motifs in common, like the extracellular domains E1 and

E2 and the YENPTY sequence, involved in the endocytosis of these proteins. Aβ sequence is present only in the APP sequence (Zheng and Koo, 2011).

APP is constitutively expressed in the body, with prominent expression in brain, especially in neurons. The human APP gene is located on Chromosome 21, spanning approximately 240 kb (Zheng and Koo, 2011), and it was cloned for the first time in 1987 (Kang et al., 1987). APP undergoes alternative splicing, resulting in the production of several mRNA and subsequently in isoforms ranging from 695 to 770 residues. The major isoforms are APP695, APP751, and APP770. The longest and widely expressed proteins, APP751 and APP770, contain the extracellular serine-protease inhibitor homolog sequence called Kunitz-type domain. APP695 is instead the isoform predominantly expressed in neurons, and it lacks the Kunitz domain (De Strooper and Annaert, 2000; Zheng and Koo, 2011).

1.2.1 Structure of APP

APP is a type 1 transmembrane protein, characterized by a large N-terminal ectodomain, which undergoes shedding by α- and β- secretases, a transmembrane domain, partially containing the amyloid β sequence, and a short C-terminal intracellular domain, probably involved in intracellular signalling (Haass, 2004; Kang et al., 1987).

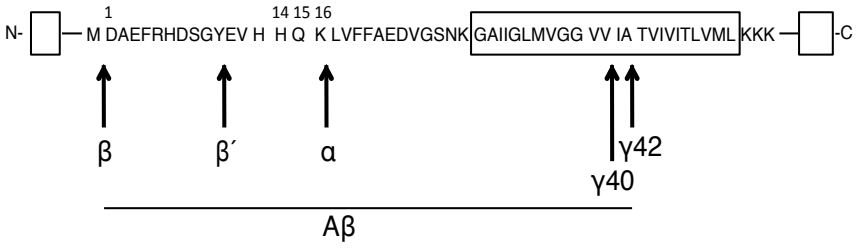

Fig. 3: Scheme of amyloid precursor protein (APP). A) The N-terminal ectodomain, the transmembrane domain (box) and the C-terminus are depicted. The β-amyloid peptide results from the cleavage by β- and γ-secretases at two different positions (Aβ40 and Aβ42). The alternative β´site at amino acid position 11 of the Aβ sequence generates shorter Aβ species, whereas the α-cleavage prevents Aβ formation, by cleaving within the Aβ sequence.

1.2.2 Proteolytic processing of APP: α-, β-, γ- secretases

Full length APP undergoes post- translational modifications like N- and O- glycosylation, phosphorylation and tyrosine sulfation (Haass et al., 2012; Lichtenthaler et al., 2011). Following these modifications, the sequential proteolytic processing of APP by α-, β-, and γ-secretase takes place in the secretory pathway. The cleavage of the nearly entire extracellular domain is accomplished by α- and β-secretases, and it results in the production of the soluble APP APPsα and APPsβ, respectively (Fig.4). The remaining membrane-tethered fragments, called α- and β- carboxyterminal fragments or CTFα and CTFβ, are further cleaved by γ-secretase at different positions generating either the p3 peptide from CTFα or Aβ peptides from CTFβ. The cleavage by γ-secretase leads to the production of the APP intracellular domains, AICD, in both cases. Since the α-secretase cleavage occurs within the Aβ sequence, preventing the formation of the toxic species, this pathway is also referred as the non-amyloidogenic pathway. In the opposite, the β-secretase driven pathway is called amyloidogenic pathway (Lichtenthaler et al., 2011).

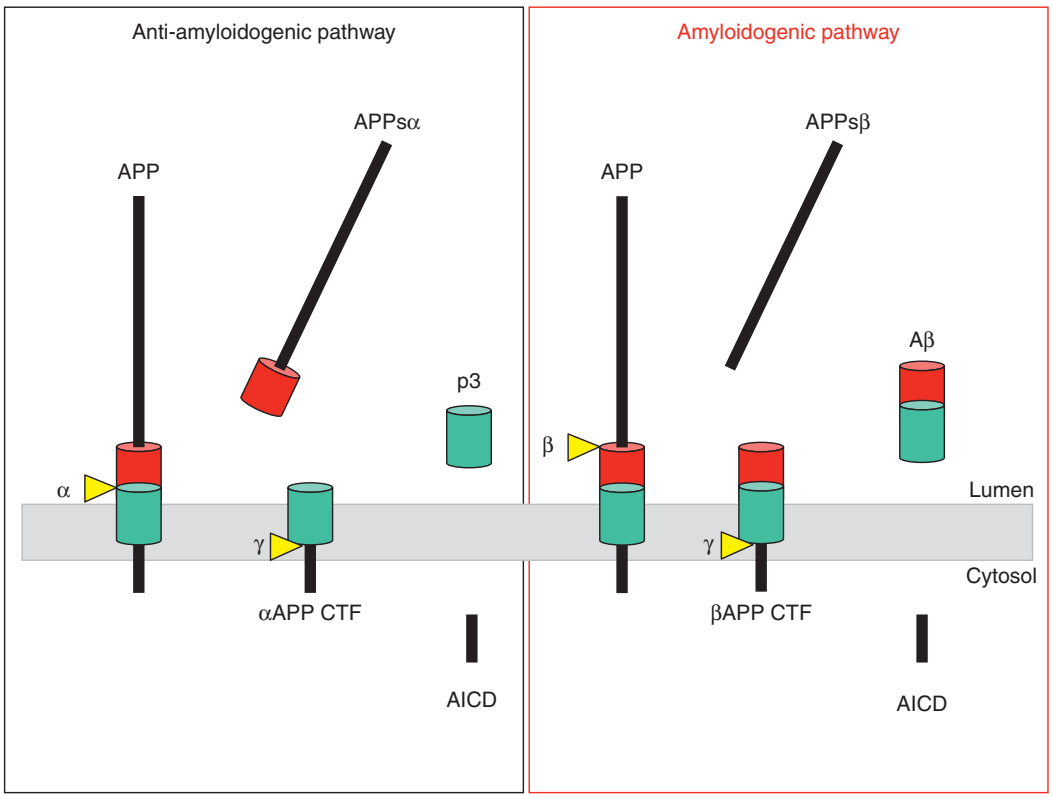

Fig. 4: Proteolytic processing of amyloid precursor protein (APP). In the anti-amyloidogenic pathway, α-secretase cleaves within the Aβ peptide, preventing the generation of toxic Aβ species. APP cleavage by β- and γ-secretases leads to the amyloidogenic pathway, which generates Aβ peptides. APP: amyloid precursor protein; APPsα: soluble amyloid precursor protein α; APPsβ: soluble amyloid precursor protein β;

Aβ: amyloid β peptide; βAPP CTF: C-terminal fragment β; αAPP CTF: C-terminal fragment α; AICD: APP intracellular domain; p3: p3 peptide (from Haass et al., 2012).

The β-secretase identity has been revealed in 1999: the transmembrane aspartyl protease BACE1 (β-site APP cleaving enzyme 1) is responsible for the cleavage of APPsβ at the β-site Met 671-Asp672 (Vassar et al., 1999) and the production of the CTFβ, which can be either cleaved by γ-secretase liberating Aβ, or, as recently discovered, it can be further cleaved by α-secretase (Portelius et al., 2011b).

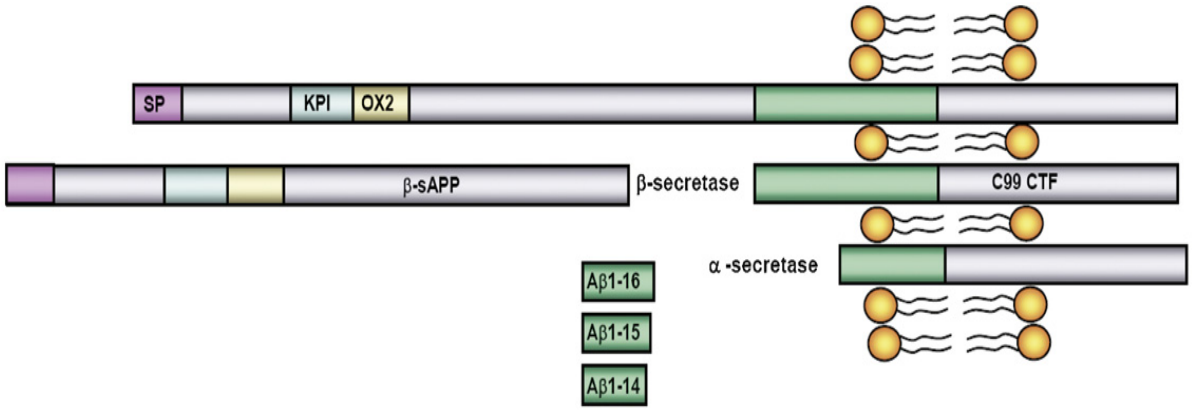

Fig. 5: Alternative processing of APP. The cleavage by β-secretase leads to the production of the soluble APPβ fragment (β-sAPP) and the membrane bound CTFβ (C99 CTF), which can be still sequentially cleaved by α-secretase. This cleavage prevents generation of longer and highly aggragable Aβ species, and produces shorter Aβ peptides, such as Aβ1-16, Aβ1-15, Aβ1-14 (from Portelius et al., 2011b).

Additionally, BACE1 can cleave APP at the β`site, a secondary cleavage site located at Glu11 of the Aβ sequence, generating the CTF89. This secondary processing generates truncated forms of Aβ (Vassar et al., 1999).

In contrast, many zinc metalloproteinases from the ADAMs family (a disintegrin and metalloproteinase domain containing protein), such as ADAM10, ADAM9 and ADAM17, were investigated for years as putative α-secretases for APP. Only recently, the identity of the APP constitutive α-secretase in neurons was assigned to ADAM10 in our laboratory (Kuhn et al., 2010; Lichtenthaler, 2011). RNAi mediated depletion of the three candidates showed that ADAM10 was indeed the only constitutive α-secretase in murine embryonic neurons. ADAM10 cleaves APP between Lys16 and Leu17 of the Aβ sequence (Kuhn et al., 2010).

The CTFs produced after cleavage by ADAM10 and BACE1 are substrates of the γ-secretase complex. Four main subunits are responsible for γ-secretase activity: presenilin, nicastrin, APH1 and PEN2 (Edbauer et al., 2002; Edbauer et al., 2003). The GxGD

aspartyl protease presenilin (composed by Presenilin 1 and 2) is the catalytic subunit of the γ-secretase complex (Haass and Steiner, 2002) and it cleaves the CTFβ within the transmembrane domain generating several Aβ peptides and truncated forms of Aβ (Fukumori et al., 2010; Portelius et al., 2011a; Steiner et al., 2008), where the prominent species is Aβ40. The highly aggregating Aβ42 consists of only 10% of the total amount (Selkoe, 2004)

Notably, all three proteases have been described to cleave other transmembrane proteins besides APP, such as growth factors, cytokines, cell surface receptors and corresponding ligands in the case of ADAM10 (Haass et al., 2012). Moreover, BACE1 is involved in the cleavage of neuregulin-1 (Fleck et al., 2013; Willem et al., 2006), and other several subtrates (Kuhn et al., 2012; Zhou et al., 2012). Similarly, γ-secretase is reported to cleave more than 50 transmembrane proteins, such as the important Notch intracellular signaling cascade (Wolfe, 2010; Wolfe and Kopan, 2004).

1.3 Regulation of APP shedding

The proteolytic cleavage in the ectodomain of the amyloid precursor protein (APP shedding) is regulated at two main levels: the trafficking of APP from cell compartments and the plasma membrane, and the regulation of secretases´activity responsible for its cleavage.

1.3.1 Intracellular transport of APP

The intracellular transport and localisation of APP plays an important role in promoting either the amyloidogenic or the non-amyloidogenic pathway, thus being an important factor for neurodegeneration.

APP is translated in the endoplasmatic reticulum, and transported through the Golgi apparatus to the trans-Golgi-network. N- and O-glycosylations, as well as phosphorylation and tyrosin sulphation, take place in the Golgi apparatus (Haass et al., 2012; Zheng and Koo, 2011).

In neurons, the highest intracellular concentration of APP is found in the TGN at a steady state. TGN-derived vesicles transport APP to the cell surface, where α-cleavage can

occur. Alternatively, APP can be re-internalized via the endosomal/lysosomal degradation pathway (Koo et al., 1996).

When APP full-length and APP shedding products were found in clathrin-coated vesicles, the involvement of the endocytic pathway in the amyloid generation became of great interest (Koo et al., 1996). Different approaches, mainly by inhibiting APP internalization impairing clathrin-dependent endocytosis with compounds or removing the APP internalization motif YENPTY contained in its cytoplasmic tail, could show that endocytosis of APP is necessary for Aβ production, resulting in increased full-length APP at the cell surface and APPsα secretion. The cell surface bound full-length APP is internalised in a clathrin-mediated manner and transported to endosomes, where it is cleaved by BACE1. In fact, BACE1 has an optimal activity at low pH of the endosomal compartments and TGN, and then either returned to the cell surface where CTFβ is cleaved by γ-secretase generating Aβ, or trafficked to the lysosomes and degraded (Koo et al., 1996; Sannerud and Annaert, 2009). Endosomally located APP can also be re-transported to the TGN and Golgi, and thereby being still processed by β- and γ-secretase (Thinakaran and Koo, 2008; Vetrivel and Thinakaran, 2006).

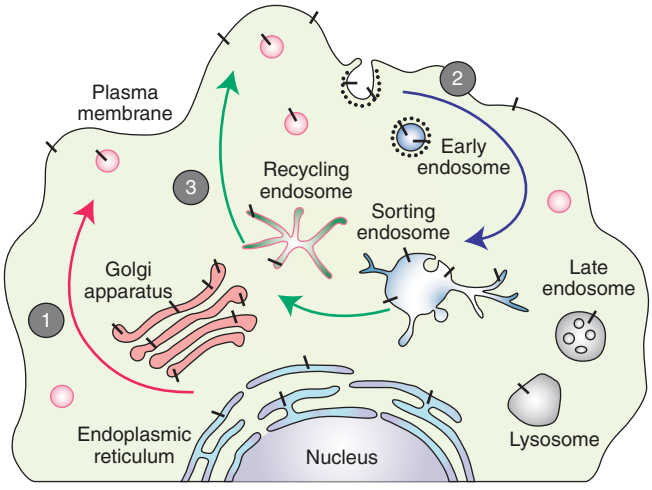

Fig. 6: APP trafficking within the cell. APP (black bars) is translated in the endoplasmatic reticulum, undergoes maturation through the Golgi apparatus and it is transported to the cell surface through the constitutive secretory pathway (1). Once APP reaches the plasma membrane, it is rapidly internalized (2) and trafficked to endosomal and recycling compartments, to the trans-Golgi apparatus or again to the cell surface (3). APP degradation takes place in the lysosome. Anti-amyloidogenic processing of APP mainly occurs at the cell surface, whereas the amyloidogenic pathway is prominent in the endocytic organelles (from Haass et al., 2012).

A number of other trafficking factors have been of research interest due to their role in modulationg APP localisation and Aβ generation. For example, the cytoplasmic tail of APP was reported to interact with X11 (mint1, mint2, mint3) family members, Fe65, Fe65L1 and Fe65L2. Namely, the interaction between APP and X11 stabilize APP intracellularly, affecting both amyloidogenic and non-amyloidogenic pathway (Saito et al., 2011). Another member of the endocytic pathway, the GTP-binding protein Rab6, has been found to interfere with APP processing (Zhang et al., 2011). Also other components of the endocytic pathway, namely Clathrin, Dynamin I, Adaptor Protein-2, can regulate the production of Aβ (Sorkin and von Zastrow, 2009). In particular, in the presence of overexpression of the dominant-negative Dynamin 1 mutant, decreased Aβ production is associated with increased APPsα shedding (Carey et al., 2011)

Moreover, the neuronal highly expressed protein SorLA/LR11 (a type I membrane protein) is reduced in patients with Alzheimer´s disease, and it is involved in APP transport between the plasma membrane, endosomes and Golgi. Overexpression of SorLA results in the accumulation of APP into the Golgi apparatus (Andersen et al., 2005; Schmidt et al., 2007). Additionally, SorLA/LR11 knock-out mice have increased levels of Aβ (Rogaeva et al., 2007).

1.3.2 Regulation of the secretases´ activity

The activity of the two secretases ADAM10 and BACE1 is regulated at different levels, namely at the trascription, translation, post-traslational modifications and compartments localisation. In these processes, colocalisazion of APP with the secretases and its interaction with cellular factors are important for APP shedding modulation (Lichtenthaler et al., 2011).

The transcription of ADAM10 as well as the one of BACE1 is controlled by different transcriptional factors, which bind to the promoter region (Rossner et al., 2006; Tippmann et al., 2009). Moreover, the translation of ADAM10 mRNA is regulated via a region at the 5´-UTR, which act as a repressor, as well as for BACE1 (Lammich et al., 2010; Lammich et al., 2011). Additionally, the stimulation of receptors with neurotransmitters or growth factors can increase ADAM10 activity, as result of the activation of different signaling pathways (Pitulescu and Adams, 2010). Also lipid distribution and composition at membrane level can play a role in the modulation of BACE1 activity (Ehehalt et al., 2003). Additionaly, natural anti-sense RNAs and

microRNAs have been described to control BACE1 translation (Faghihi et al., 2008; Hebert et al., 2008)

As already mentioned, the intracellular localisazion of BACE is critical for its activity: the enzyme is especially active in acidic compartments like endosomes and TGN. As seen for ADAM10, also BACE activity is influenced by the cholesterol rate of the membrane enviroment, as well as the lipid distribution within the membrane (Dislich and Lichtenthaler, 2012).

In contrast, the activity of γ-secretase is very much dependent on the correct assembly and maturation of the γ-secretease complex. A first immature complex is assembled in the ER, and then transported to the Golgi, where it undergoes glycolsylation and other post-translational modifications. The correct delivery of the functionally mature complex to the endosomes/lysosomes and to the plasma membrane, as well as the rate of the CTFs produced from the other secretases, are limiting steps for the activity of the γ-secretase (Lichtenthaler et al., 2011).

1.4 Biomarkers

“ Biomarkers are objective measures of a biological or pathogenic process that can be used to evaluate disease risk or prognosis, to guide clinical diagnosis, or to monitor therapeutic interventions” (Blennow, 2010; Blennow et al., 2012).

Research on biomarkers for the diagnosis and treatment of Alzheimer´s disease is still a controversial field. In the recent years, advances in monitoring the progression of the disease have been achieved. Based on the amyloid cascade hypothesis (Hardy and Selkoe, 2002), which considers the imbalance between the production and clearance of Aβ as initiating event of the disease, body fluids like cerebrospinal fluid (CSF) and plasma have been analysed for products of APP metabolism. Neuroimaging techniques are nowadays a helpful tool for identifying brain dysfunctions. These clinical investigations give information about the biochemical profile, morphological changes in specific brain regions, and they support the tracking of the presence as well as the entity of Aβ plaques.

Many anti-Aβ drug candidates, including Aβ immunotherapy, secretase inhibitors, and Aβ aggregation inhibitors, are currently tested in clinical trials (Blennow, 2010; Lee et al., 2011).

The neuroclinical symptoms of Alzheimer´s disease show up when the homeostasis of the brains is already compromised. For this reason, it is of major importance to find

biomarkers and to develop drugs that are effective at the very early stages of the disease. A systematic screening of subjects with pre-dementia symptoms, together with administration of disease modifying drugs would be the ideal goal for effective AD treatment (Blennow et al., 2012).

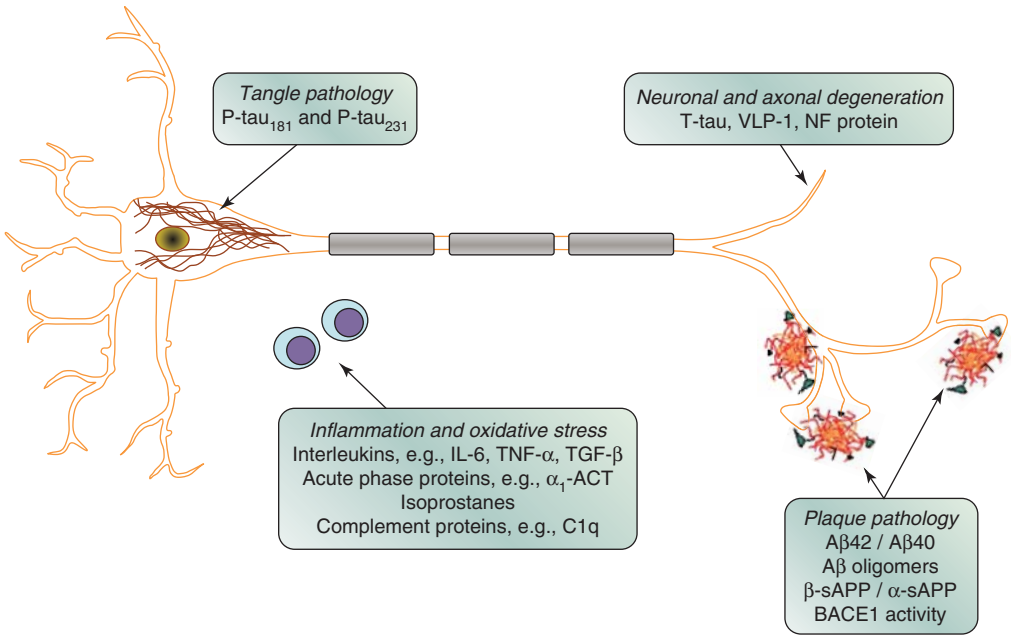

Fig. 7: Scheme of a neuron with extracellular β-amyloid plaques and intracellular neurofibrillary tangles. Some of the investigated CSF biomarkers from putative pathological processes in Alzheimer´s disease are given (from Blennow et al., 2012). P-tau: phosphorylated tau; T-tau: total tau; VLP-1: visinin protein-like 1; NF: neurofilament; IL-6: interleukin 6; TNF-α: tumor necrosis factor α; TGF-β: transformimg growth factor β; ACT: antichymotrypsin; C1q: C1 complex subcomponent; Aβ: amyloid β peptide; β-sAPP: soluble amyloid precursor protein β; α-sAPP: soluble amyloid precursor protein α; BACE1: β-secretase.

1.4.1 Cerebrospinal fluid biomarkers

CSF is considered to be a particularly important source for Alzheimer´s disease biomarkers, being in close contact with the extracellular space in the brain where the major biochemical changes occurs in AD pathological conditions. CSF reflects changes in Aβ aggregation metabolism and the formation of neurofibrillary tangles. For this reason, levels of Aβ42, tau and phosphorylated tau have been extensively investigated as potential biomarkers.

Levels of Aβ42 in the CSF reflect the balance between aggregation and clearance of Aβ in the brain. Decreased levels of approximately 50% in CSF Aβ42 are reported in AD

patients, probably because the aggregation rate into plaques is very high (Blennow, 2004; Fagan et al., 2009; Tolboom et al., 2009).

Levels of total tau are a measure of neuronal damage and degeneration. A marked increase in total tau levels correlate with a number of clinical acute manifestation of brain disorders (Hampel et al., 2010a; Zetterberg, 2008a). In AD-type of dementia, CSF total tau increases of around 3-fold in a disease progression manner from mild cognitive impairment (MCI) to AD (Blennow, 2004; Sunderland et al., 2006). Rapid increase in CSF total tau levels well correlates with rapid cognitive decline (Hampel et al., 2010a). Together with total tau, CSF phosphorylated tau is measured to monitor the phosphorylation state of tau protein that is at the basis of the tangle formation. Tau can be phosphorylated mainly at amino acid residues 181 and 231 (Brunden et al., 2009). Like tau, also phosphorylated tau181 levels increase in MCI and AD cases, well correlating with the severity of the disease and the conversion between early MCI, MCI and AD (Blennow, 2004; Hampel and Blennow, 2004; Hampel et al., 2010a).

Recently, several studies have proven the diagnostic potential of the combination of these three biomarkers in the AD diagnosis (Hansson et al., 2006; Maddalena et al., 2003). Nowadays, simultaneous quantification of Aβ42, tau and phospho-tau provide a partial discrimination between AD patients and non-demented controls, with still extensive overlap between the groups of control subjects and AD patients (Blennow, 2004; Hansson et al., 2006; Hansson et al., 2010; Mattsson et al., 2010). Moreover, the combination of these biomarkers may also differentiate AD from other type of Taupathies, even if the heterogeneity of these types of dementia and the partial overlap of clinical presentations render the diagnosis difficult (Hampel et al., 2004; Schraen-Maschke et al., 2008).

To improve the pre-clinical diagnosis of Alzheimer´s disease, especially at the individual levels, other biomarkers have been investigated in CSF. For example, other APP processing products such as APPsα and APPsβ have been measured as putative biomarkers, since they are directly secreted into the extracellular space and diffused into the CSF. Controversial results have been reported in the literature, where CSF levels of these two proteins have been found to be slightly increased or unaltered (Gabelle et al., 2010; Lewczuk et al., 2010; Olsson et al., 2003; Rosen et al., 2012; Zetterberg and Blennow, 2008). Therefore, more studies are needed to fully evaluate the potential of APPsα and APPsβ as new biomarkers.

For this reason, new specific biomarkers represent a first priority´s challenge for the accurate diagnosis of AD and for monitoring the effect of new drugs.

1.4.2 Plasma biomarkers

Plasma biomarkers for AD diagnosis have been investigated with little success so far.
Aβ has been examined as a peripheral biomarker for AD. Studies reported slightly higher level of plasma Aβ40 and Aβ42 in AD patients, although with large overlap between controls and AD patients (Graff-Radford et al., 2007). In addition, contradictory results have been published concerning the Aβ42 and the Aβ42/Aβ40 ratio as an indicator for AD risk (Sunderland et al., 2006; Zetterberg, 2008b; Zetterberg and Blennow, 2008; Zetterberg et al., 2010). The lack of correlation between plasma Aβ species and the status of the disease is explained from different hypothesis: first of all, APP is expressed ubiquitously, and it is difficult to isolate the ratio of Aβ derived from the brain; second, Aβ is a hydrophobic peptide, thus very likely to be bound to plasma carrier proteins, which make the measurement difficult and less reliable.
Since research of Aβ as a peripheral marker for AD did not help the already difficult diagnosis of AD, other methods have been developed for finding more suitable biomarkers for AD. For example, one study (Ray et al., 2007) identified 18 novel plasma proteins involved in signaling and inflammation. Using this panel of new biomarkers, it was possible to differentiate between MCI, AD and control subjects with close to 90% accuracy. Changes involving increased expression levels of other plasma proteins, like complement factor H and alpha2-macroglobulin, were found in AD patients in another study (Hye et al., 2006). A full blood based algorithm for screening of AD patients was recently developed for testing plasma and serum, with high accuracy in diagnosing AD (O'Bryant et al., 2011).
The easy sampling of plasma makes this body fluid an interesting source of biomarkers for AD. Therefore, more efforts in finding significant and reproducible changes to correlate different stages of AD are urgently needed.

1.4.3 Role in diagnosis and clinical trials

A biomaker is a valuable tool not only to diagnose AD more precisely, but also to evaluate the effects of a new drug. They can be especially helpful for monitoring the effect of new drugs at different disease stages, and for early diagnose of AD, when symptoms are yet lacking or indistinct. Moreover, because AD is a very heterogeneous disease at clinical and neuropathological levels, accurate and specific biomarkers would have a role to create a better stratification of AD subtypes, increasing the chance to develop targeted treatments to prevent the worsening of the neurological conditions, and also to distinguish Alzheimer´s disease from other similar dementia.
Nowadays, CSF biomarkers like Aβ42, tau and phsphorylated tau are considered informative for the diagnosis of AD. Nevertheless, these biomarkers still rely on invasive or expensive procedures like lumbar puncture, and this analysis is performed only when patients show clear sympthoms of dementia. In the opposite, disease modifying drugs are more effective if they are administered in the earliest stage of AD, before the histopathological changes of the disease become prevalent (Blennow et al., 2012)

1.4.4 Therapeutic approaches to AD

Different approaches have been developed in order to find treatment strategies for an effective treatment of Alzheimer´s disease. Many drugs have been tested in the recent years, maily encountering failure in clinical trials. The currently approved drugs used for the symptomatic treatment of AD are cholinesterase inhibitors and the NMDA receptor antagonist memantine (Mangialasche et al., 2010; Salomone et al., 2012). Unfortunately, these drugs do not affect the rapid progression of the disease, highlighting the need of development of disease modifying drugs. For this reason, β- and γ-secretase inhibitors have been used to reduce Aβ production. The major problem of these compounds is the potential non-specific effect, being these two secretases involved in key pathways of the cellular system, like for example intramembrane cleavage of N-Cadherin, Notch receptor, ErbB4 and others (Salomone et al., 2012).
Since oligomers are considered to be the neurotoxic species in AD pathology, drugs like tramiprosate that prevent Aβ aggregation have been developed, without determining major clinical improvements in Phase III clinical trials (Aisen et al., 2011). Others are currently tested in Phase II and III clinical trials.

In the opposite, drugs promoting Aβ clearance are considered a promising approach for AD treatment. Active immunization is used to induce the self-production of antibodies against Aβ, whereas passive immunization is based on antibodies produced in vitro. Active immunization enhances Aβ clearance via promoting the phagocytosis by microglia of the complex Aβ-antibody. Unfortunately, this kind of immunotherapy led to brain inflammation, and passive immunization has been favoured as an alternative strategy. The most promising Aβ targeting antibody is bapineuzumab (Kerchner and Boxer, 2010), even if it seems to have adverse effect on the subset of AD patients carrying ApoE ε4 allele.

Since a number of new findings recently correlated tau and Aβ in the onset of AD pathology (Ittner and Gotz, 2011), increasing pharmacological strategies are nowadays focused on inhibiting tau-phosphorylation, preventing tau aggragation, and promoting tau disassembly. A target for the inhibition of tau phosphorylation is the kinase GSK-3β, which is the main enzyme involved in this process. Drugs targeting GSK-3β have shown so far many adverse effects (valproate) (Lonergan and Luxenberg, 2009) or no clear effect (litium) (Hampel et al., 2009).

1.5 Kinases as modulators of APP shedding

Kinases have been described in the past to be altered in AD brains, and to have a role in the regulation of APP processing. In fact, the cytoplasmic domain of APP contains a number of possible phosphorylation sites that can modify the protein structure, thereby having an important role in the regulation of its function, trafficking and metabolism.

1.5.1 Kinases altered in AD

In 2002, Manning and colleagues (Manning et al., 2002) published the full human kinome, comprehensive of 518 kinases. However, only a small fraction of them have been studied in the context of AD. Aberrant tau and APP phosphorylation seem to correlate with AD status, making kinases and phosphateses an interesting research topic and putative candidates for drug target. Cyclin dependent kinase 5 (cdk5) and glycogen synthase kinase (GSK3) have been studied as first candidates for AD pathogenesis (Drewes, 2004; Noble et al., 2005). In fact, they phosphorylate disease-associated

residues on tau, and they colocalize with tau tangle in brains of AD patients (Shelton and Johnson, 2004).

In vivo studies showed that GSK3 and cdk5 regulate Aβ generation. Concerning the further, GSK3α and GSK3β isoforms have been investigated to have a role by linking amyloid and tau pathology. GSK3β is abundantely expressed in the nervous system, whereas GSK3α is mainly expressed in non-neuronal tissues. Several evidences link activation of GSK3β to tau pathology, Aβ synthesis, and apoptotic neuronal death. In particular, GSK3 phosphorylation not only enhances BACE1 activity and subsequently it increases Aβ production, but it also regulates Aβ production by direct phosphorylation of presenilin1 and APP, thereby interfering with γ-secretase cleavage of APP. Moreover, GSK3 phosphorylation downregulates α-cleavage of APP by inhibiting constitutive and PKC-induced α-cleavage. Additionally, Aβ accumulation can act as a feedback loop, inducing further GSK3 activation via Aβ-mediated neuroinflammation. Together with its role in tau hyperphosphorylation, these mechanisms can be at the basis of neuronal damage, ultimately leading to cognitive impairment in Alzheimer´s disease patients. GSK3β represents currently a putative therapeutic target for AD (Cai et al., 2012).

In the latter, cdk5 directly phosphorylates not only tau and neurofilaments, but also APP. Abnormal activation of cdk5 leads to tau and APP hyperphosphorylation, enhancing the formation of neurofibrillary tangles and increasing Aβ production. Cdk5 is activated by p35 and p39. Under stress conditions, both activators are cleaved by calpain. In particular, the resulting active p25 binds cdk5 and it is responsible of tau and APP hyperphosphorylation (Shukla et al., 2012). Moreover, overexpression of p25 leads to increased BACE mRNA levels, and enhanced Aβ production (Wen et al., 2008). Increased levels of hyperactivated cdk5 and p25 have been reported in AD brains, making this kinase an interesting drug target as well.

1.5.2 APP phosphorylation

The APP cytoplasmatic domain is characterized by several phosphorylatable amino acid residues. APP physiological phosphorylation state has been investigated in different cell lines, namely neurons and dividing cells. The mature form of APP is found to be phosphorylated in neurons, whereas the immature form is maily phosphorylated in dividing cells. In both case, the major phosphorylation site in the cytoplasmic region of the protein is at Thr668 (numbering for APP695 isoform). Kinases like cdk5 (cyclin-

dependent kinase-5) and GSK-3β (glycogen synthase kinase-3β) are described to phosphorylate APP at Thr668 in neurons (Aplin et al., 1996; Iijima et al., 2000). In dividing cells, the cdk5 homologue CDK1/CDC2 kinase phosphorylates APP at the same residue. Under stress conditions, JNK is also capable of APP phoyphorylation at the Thr668. The phosphorylation at this residue induces important conformational changes in the cytoplasmatic region of the protein and it interferes with APP binding partners (Suzuki and Nakaya, 2008).

Additionally, the APP C-terminus contains the internalization motif YENPTY, spanning from amino acid 682 to amino acid 687 (numbering from APP 695). This motif is normally phosphorylatable and it regulates binding of interaction partners involved in cell signaling. Within APP YENPTY motif, Tyr682 and Tyr687 are alternatively phosphorylated by a number of different kinases, such as Abl and Src kinases (Tamayev et al., 2009). This alternative phosphorylation may have a role in regulating APP CTFs processing, by directing APP to different cellular compartments, promoting or inhibiting the amyloidogenic pathway of APP (Schettini et al., 2010). Additionally, phosphorylation of Thr668 and Thr682 are increased in AD brains (Lee et al., 2003).

1.6 High- throughput screening by RNA interference

RNA interference is an endogenous and natural mechanism broadly used for studying gene function. It has been discovered by Fire and colleagues (Fire et al., 1998) in Ceanorhabditis elengans, as a tool to manipulate the expression of an endogenous gene and to find its relevance in a specific pathway. Through this process, the expression of a specific gene can be knocked down, with a high degree of specificity and selectivity, triggering sequence specific degradation of mRNA transcripts in the cytoplasm.

Silencing of gene expression can be regulated via 3 major categories of small RNAs: miRNA, siRNA and shRNA. miRNA are endogenous regulators of the cell homeostasis, and they function as repressors of expression of heterologous genes. siRNA and shRNA can be endogenous and exogenous molecules that are either artificially inserted into the cell or normally produced in the nucleus for silencing the genes from which they are derived.

Concerning the shRNA pathway, long double stranded RNAs (typically shRNA and pre-miRNA) are exported into the cytoplasma by Exportin, where they are processed by the endonuclease Dicer to generate 21-23 bp double-stranded siRNAs. These molecules bind

to the Argonaute complex (Ago2 in mammals). They normally have a 3´overhang, which identifies the guide strand that is selected by the Ago2 for being incorporated into the RISC complex (RNA-induced silencing complex). The guide strand- RISC complex targets the complementary mRNAs, determining the cleavage and the degradation of the specific mRNA. The corresponding complementary strand undergoes degradation by exonucleases. Accidently, also the complementary strand can be integrate into the RISC, originating non-specific knock-down (Martin and Caplen, 2007; Rao et al., 2009a; Rao et al., 2009b). Synthetic double-stranded siRNAs artificially delivered into the cytoplasm do not need to be shortened by of unrelated genes, but they are immediately processed by the Argonaute-RISC complex to achieve the knock down of the specific mRNA (Rao et al., 2009a; Rao et al., 2009b). The match between siRNA and mRNA is usually perfect, and this prerequisite is required for cleavage of mRNA.

Differently, miRNA are transcribed by RNA polymerase II and undergo post-trascriptional modifications like polyadenylation and capping. The miRNA transcripts contain hairpin structures, and they are firstly processed in the nucleus by the enzyme Drosha (Mendell, 2005). Afterwards, pre-miRNAs are transported into the cytoplasm via Exportin, where they are also processed in the same way as siRNA via Dicer and the Argo/RISC complex. miRNA match with mRNA is imprecise, allowing miRNA to target hundreds of mRNA (Fabian et al., 2010; Sigoillot and King, 2011).

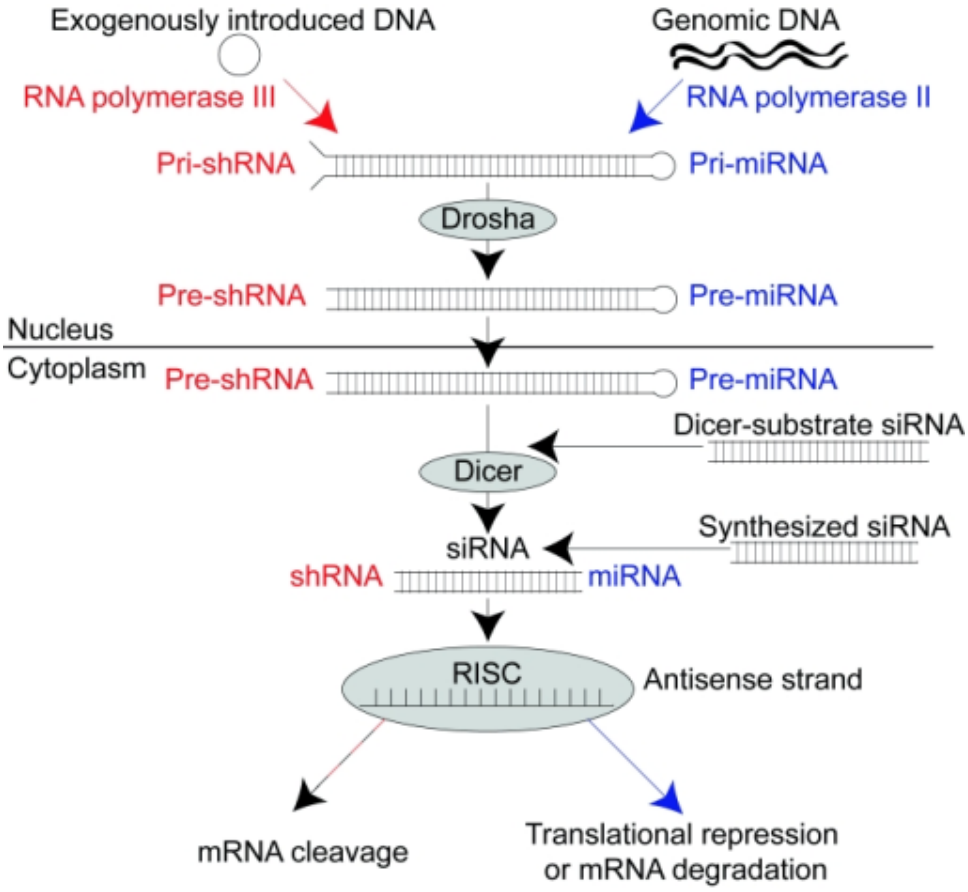

Fig. 8: RNA interference pathways in mammalian. Dicer processes endogenous or exogenous long dsRNA or siRNA into 21-23 nucleotides dsRNA duplexes. Most shRNAs and siRNAs have precise complementary with the target mRNA and their binding to Ago2-RISC complex mediates mRNA cleavage, thereby initiating RNA interference (from Cohen and Xiong, 2011).

RNAi-based knock-down is a powerful tool to conduct functional studies in cultured cells and organisms. Nevertheless, since both siRNA and miRNA pathways use the endogenous RNAi machinery in the cellular system, unspecific repression of transcripts can occur, causing off-target effects. These off-target effects can be divided into two main categories: the sequence-dependent and the sequence independent off-target effects. Concerning the sequence-dependent off-target effects, the choice of the siRNA or miRNA strand which is loaded into the RISC complex is very much dependent on the thermodynamic stability of the dsRNA ends. If the complementary strand is loaded into the RISC, unwanted mRNA which shares homology to the strand sequence will be silenced. Moreover, the miRNA-induced off-target effect can also arise from the imprecise match which is at the basis of the miRNA mediated translational repression. While siRNA knock-down is based on the perfect complementary of the siRNA with the corresponding mRNA, miRNAs usually require only few nucleotides of homology in the

seed region at the 5′ end of the guide strand (from nucleotides 2 to 8) to target the desired mRNA. Even extended regions are described to mediate the same off-target effect, and these target sites are mostly located to the 3`UTRs of target genes (Bartel, 2009).

The sequence-independent off-target effects are based on general impairment of the homeostasis of the cell (Jackson and Linsley, 2010). For example, prolonged expression of shRNA can saturate the endogenous regulation of gene expression, which is necessary to keep the physiology of the cellular system. Moreover, synthetic siRNA can activate the endogenous miRNA response from the cell, which interferes with the normal expression of many other genes. This miRNA-mediated response to exogenous siRNA is nowadays well documented via transcriptional profiling (Khan et al., 2009). Along with the physiological response of the cell against exogenous agents, also other types of immune and stress response can be induced by siRNA or viruses for the expression of shRNA (Sledz and Williams, 2004).

Taking advantage from the availability of sequenced genomes from many organisms, classical genetic screening have been slowly substituted by RNAi screening. This approach is rapid and allows systematic screening of either the whole genome or pathways of interest, thereby increasing the probability to find genes responsible for lethal or weak phenotypes (Boutros and Ahringer, 2008; Martin and Caplen, 2007).

Nevertheless, off-target effects can have strong impact on the output of an RNAi high-throughput screening, leading to the production of false-positive hits. It is necessary to control unwanted targeting of RNAi molecules, as well as building strong validating approaches for the newly identified modulators of a specific pathway.

2 AIM OF THE WORK

Alzheimer´s disease is the most common form of elderly dementia worldwide, and it is characterized by histopathological hallmarks such as β-amyloid depositions and neurofibrillary tangles. The amyloid precursor protein (APP) contains the Aβ sequence, the highly aggregating toxic peptide found in β-amyloid plaques. The Aβ peptide is generated through the sequential cleavage of APP by β- and γ-secretases. A third protease, called α-secretase, cleaves APP within the Aβ peptide, preventing Aβ production.

The aim of this thesis is to address two major topics in Alzheimer´s disease:

- Investigation of products of APP metabolism as new potential biomarkers in AD.

Cerebrospinal fluid biomarkers such as Aβ42, tau and phospho-tau are used for helping Alzheimer´s disease diagnosis. Nevertheless, a precise individual diagnosis of the disease is yet not possible, due to the lack of 100% specificity of these and other biomarkers. Products of APP metabolism like APPsα and APPsβ have been detected in cerebrospinal fluid and plasma as putative biomarkers for increasing the accurancy of AD diagnosis. Specifically, APPsα has been proposed as a potential biomarker for AD. APPsα ectodomain results from the α-secretase cleavage of APP, which corresponds to the non-amyloidogenic pathway. Several studies investigated APPsα levels in CSF and plasma of healthy subjects and AD patients, but with poorly consistent results. Here, a new antibody for more specific detection of APPsα will be used to establish an ELISA-like immunoassay. The performance of the new immunoassay will be tested in cerebrospinal fluid and serum, and a group of AD patients will be analysed in comparison to control subjects.

- RNAi- screening of the human kinome to identify new modulators of APP shedding.

Detection of APPsα and APPsβ is not only important from a diagnostic point of view, but also as a read out to better understand how APP processing is regulated. In particular,

kinases´ activity plays a role in controlling this process. In fact, abnormal regulation of kinases´ activity has been linked to the etiology of Alzheimer´s disease. Hyperphosphorylated tau and differential APP phosphorylation are key mechanisms for the onset of tau tagles and amyloid plaques. So far, only few kinases have been extensively studied for their role in Alzheimer´s disease. Here, a RNAi high-throughput screening of the human kinome will be performed to identify new kinases involved in the control of APP shedding and consequently in Aβ generation. The screening will be performed in neuroblastoma cells expressing endogenous APP, in order to select for kinases implicated in the physiological processing of APP. Best candidates will be validated with different strategies to exclude false-positive hits due to RNAi-mediated off-target effects. Neuronally expressed genes will be further selected and tested in murine embryonic neurons expressing endogenous APP.

3 Materials and methods

3.1 Material

3.1.1 General material and equipment

Table 1: General material and equipment

Material	Manufacturer
Analytical balance (200-0,0001 g)	Ohaus
Autoclave (Tuttnauer 3850 EL)	Systec
Balance (2000-0,01 g)	Ohaus
Falcon tubes	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
Multichannel pipette (300 µL)	Eppendorf
pH electrode	Schott
pH-meter	WTW
Pipettes	Eppendorf/Gilson
Accu-Jet	Brand
Pipette tips	Sarstedt
Thermomixer	Eppendorf
Centrifuge (-4°C- Room temperature)	Eppendorf
Table centrifuge (Biofuge pico)	Heraeus
Microwave	Sharp
Incubator (37°C)	Heraeus
Vortex VF2	IKA Labortechnik

3.1.2 Chemical reagents

Table 2: Chemical reagents

Chemical reagent	Manufacturer
Calcium chloride	J.T. Baker
Disodium hydrogen phosphate	Sigma
Dimethyl sulfoxide	Merk
Ethylenediaminetetraacetic acid	Sigma
Ethanol	Merck
2-Propanol	Merck
Glycerol	Sigma
Hydrogen chloride	Merck
Magnesium chloride	Merck
Potassium chloride	Merck
Potassium dihydrogen phosphate	J.T. Baker
Sodium chloride	Roth
Tris hydroxymethyl aminomethane	Biomol
Triton X-100	Merck
NP-40	Sigma
Tween 20	Merck
B-Mercaptoethanol	Merck

3.1.3 Molecular Biology

Table 3: Material for molecular biology techniques

Material	Manufacturer
7500 Fast Real-Time PCR	Applied Biosystems
Agarose Electrophoresis	PeqLab
Agarose Images (CCD Video Camera)	MS
Printer for Agarose Images	Mitsubishi
Nanodrop	Implen
UV-photometer	Jasco

Table 4: Reagents used for molecular biology techniques

Material	Manufacturer
1 kb-DNA ladder	Gibco Invitrogen
Agarose NA	Amersham biosciences
Ampicillin	Roth
DEPC	Sigma
HEPES	Biomol
High-capacity cDNA reverse transcription kit	Applied Biosystems
NucleoBond AX500 Kit	Macherey-Nagel
NucleoSpin Extract Kit	Macherey-Nagel
NucleoSpin Plasmid Kit	Macherey-Nagel
Oligonucleotides	Sigma and Thermo
Power SYBR-Green PCR Master Mix	Applied Biosystems
QIAshredder	Qiagen
RNeasy RNA extraction Kit	Qiagen
Restriction enzymes	New England Biolabs
T4 DNA Ligase	Roche
T4 DNA Ligase Buffer	Roche

3.1.4 Cell culture

Table 5: Material for cell culture

Material	Manufacturer
Bunsen	Heraeus
Sterile pipettes	Sarstedt
Sterile falcon tubes	Sarstedt
Filters (0.45 mm)	VWR
Poly-D-Lysine pre-coated plates	BD Biosciences
Cell colture plates and flasks	Nunc
Waterbad	GFL

Liquid nitrogen tank	Messer Griesheim Chronos
Counting chamber Neubauer	Optik Labor
Incubator	Heraeus
Sterile hood	Heraeus
Centrifuge Megafuge 1.0	Heraeus

Table 6: Reagents for cell colture

Material	**Manufacturer**
DMEM	Gibco
DMEM-F12	Invitrogen
Optimem	Gibco
Neurobasal	Invitrogen
DMEM-High Glucose	Gibco
B27	Invitrigen
FCS	Gibco
Penicillin/Streptomycin	Gibco
Poly-L-Lysine	Sigma
Poly-D-Lysine	Sigma
Trypsin-EDTA (0,05%)	Gibco
LipofectamineTM 2000	Invitrogen
LipofectamineTM RNAiMax	Invitrogen

3.1.5 Biochemistry

Table 7: Material for biochemistry

Material	**Manufacturer**
Aβ triplex assay kit	MSD
APPsα/APPsβ assay kit	MSD
Plate reader Sector Image	MSD
Whatmann paper	Macherey-Nagel
Nitrocellulose membrane (Potran)	Schleicher & Schuell
PVDF membrane (Immobilon-P)	Millipore
SDS-PAGE Electrophoresis	Bio-Rad

SDS-PAGE glasses and combs	Bio-Rad
Transfer	Bio-Rad
Films SuperRX	Fujifilm
Western Blot developer (LAS-4000)	Fujifilm

Table 8: Reagents for biochemistry

Material	**Manufacturer**
Acrylamid (19:1/ 40% w/v)	Bio-Rad
Ammonium-Persulfate (APS)	Roche
BCA-Assay Kit Uptima	Interchim
BSA Uptima	Interchim
ECL Western Blot Detection Reagent	Amersham Biosciences
ECL plus Western Blot Detection Reagent	Amersham Biosciences
I-Block	Tropix
Milk powder	Frema
Protein marker See Blue Plus 2	Invitrogen
MSD Read Buffer	MSD
MSD standards for APPsα/APPsβ Assay	MSD
MSD standards for Aβ Triplex Assay	MSD
Protease Inhibitor Mix	Sigma
Protein Sepharose A	Sigma

3.1.6 Buffers and Media

Solutions were dissolved in double-distilled water (ddH_20).

Table 9: Buffers and Media

Buffer or Medium	**Composition**
4X DNA Sample Buffer	30% Glycerin, 10 mM EDTA, 0,05% OrangeG
4X SDS Sample Buffer	4 ml 20% Glycerin, 4 ml 20% (w/v) SDS, 1 ml β-mercaptoethanol, 1.25 ml 1 M Tris pH 6,8, 10 µL 10% Bromophenolblue
Ampicillin solution	100 mg/ml Ampicillin in 70% Ethanol

Antibody solution for MSD Assays	TBS, 0,05% Tween 20, 1% BSA
Bloking solution for APPsα/APPsβ Assay	TBS, 0,05% Tween 20, 3% BSA
Bloking solution for Aβ Triplex Assay	TBS, 0,05% Tween 20, 1% BSA
Bloking solution for Western Blot	% Milk pouder, PBS, 0,05% Tween 20; or 2 g I-Block, 500 ml PBS-Tween
Freezing medium	FCS, 10% DMSO
Electrophoresis buffer for SDS-PAGE	25 mM Tris, 0.2 M Glycin, 0,1 % SDS
LB-medium	1% Trypton, 0,5% Yeast extract, 0,5% NaCl pH 7
Lysis buffer	50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40 ot Triton, 2 mM EDTA, PI-Mix
PBS	140 mM NaCl, 10 mM Na2HPO4, 1,75 mM KH2PO4, pH 7,4
Antibody solution for Western Blot	Antibody in optimal concentration, 0,25% w/v BSA, 0,05% Tween, PBS
siRNA buffer	300 mM KCl, 30 mM HEPES pH 7,5, 1 mM MgCl2
SOB-medium	2% Trypton, 0,5% yeast extract, 0,5% NaCl, 10 mM MgCl2, 10 mM MgSO4, 2,5 mM KCl
STE	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA
STEN	50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0,2% NP40
STEN-NaCl	STEN, 175 mM NaCl
STEN-SDS	STEN, 0,1% SDS
TAE Buffer (50X)	2 M Tris, 1 M Acid Acetic, 50 mM EDTA
TBS	50 mM Tris-HCl ph 7,4, 150 mM NaCl
Transfer Buffer for Western Blot	25 mM Tris, 0,2 M Glycin
Washing solution for MSD assays	TBS, 0,05% Tween-20

3.1.7 Antibodies

Table 10: List of antibodies

Antibody	Epitope	Specie	Dilution	Source
14D6	APPsα	Rat	1:1000	Developed together with E. Kremmer
5G11	APPsα	Rat	1:10	Developed together with E. Kremmer
192wt	APPsβ	Rabbit	1:100	Elan
22C11	APP N-terminus	Mouse	1:5000	K. Beyreuther
APLP1	APLP1	Rabbit	1:1000	Proteintech
Sez6	Sez6	Rabbit	1:2000	Jenny Gunnersen
Calnexin	Calnexin	Rabbit	1:2000	Stressgene
STK39	STK39	Rabbit	1:500	Sigma
ADAM10	ADAM10 C-terminus	Rabbit	1:1000	Calbiochem
BACE1 (3D5)	BACE1	Mouse	1:2000	B. Vassar
5313	444-592 of APP sequence	Rabbit	1:1000	C. Haass
Anti-goat-HRP	Goat IgG	Donkey	1:5000	Santa Cruz
Anti-mouse-HRP	Mouse IgG	Goat	1:5000	Promega
Anti-rabbit-HRP	Rabbit IgG	Goat	1:5000	Promega
Anti-rat-HRP	Rat IgG	Goat	1:5000	Santa Cruz

3.1.8 siRNAs, shRNAs, plasmids

siRNAs were synthesized by Dharmacon (siGenome) and Qiagen, and provided in pools or single sequences. The siGenome kinase library was provided by Dharmacon. shRNA were cloned in the lab with the cloning strategy of Dr. Kuhn (Kuhn et al 2010).

Table 11: shRNA

Target	Target sequence 5´-3´
STK39-1	CAAGAACGCGTAGCCATAAAG
STK39-2	GCCCAACCAAACGCTAATGAA
STK39-3	GTCCTAGAAGAGGCGATAATT
STK39-4	GGTGATGTTACACGGAATAAA
STK39-5	ACTATTCTTAGCAGTCAATTT
STK39-6	GACGGCCATGATGTAGTTATA
STK39-7	GCTCAGTACAAATAGCAGATT
GAK-1	GAGTATGCATTAAAGCGATTA
GAK-2	CCAAACAGCAAGACTTAATAT
GAK-3	GCTAAGCATCCAGGACATTAT
GAK-4	AGATACAGACCCACTCAAATT
GAK-5	CTGTCACAGTTTGTGATTTAC
TTBK2-1	GCACCTTTCTTGACCATATTT
TTBK2-2	TGGCACTATGATGACGAATAT
TTBK2-3	TTGGCTTGGCTCGACAATTTA
TTBK2-4	AGCCAGGCTACGCAGATATAA
TTBK2-5	ATTGCGCAGACATTCAAATAT
TTBK2-6	TTCGAGGGACAGTTCGTTATG
TTBK2-7	GCACCTTTCTTGACCATATTT
TTBK2-8	CCAGCTTCTAACATCCGTGTT
TTBK2-9	CCAACTGCCTTGGAGAGAAAT
MAPK6-1	GCGTGATTCCAGTTTACATTA
MAPK6-2	GACAAGTTAAACGACTTGAAT
MAPK6-3	GAACACCTACACCAGCTATTT

Table 12: List of plasmids

Name	Usage
pLKO2mod-EGFP-WPRE-mmSPAK shRNA20	STK39 Knock-down
pLKO2mod-EGFP-WPRE-mmSPAK shRNA43	STK39 Knock-down
pLKO2mod-EGFP-WPRE-control shRNA	Control (from P.H. Kuhn)
pCEP4/AβPP751	provided by K. Beyreuther
peak12/AβPP695	provided by S. Lichtenthaler

3.2 Methods

3.2.1 Molecular biology techniques

3.2.1.1 Cloning of shRNAs

shRNA sequences were taken from the RC consortium and syntesized by Thermo, and cloned into the lentiviral vector pLKO2mod-EGFP-WPRE (Kuhn et al. 2010). 1 μg of vector was digested with MluI and XmaI in a final volume of 20 μL, according to the manufacturer. The successful digestion was confirmed by agarose gel and the digested vector was purified from agarose. Annealling of shRNAs complementary sequences was performed at 95°C for 10 min. Ligation of the digested vector and annealed oligos was achieved overnight.

3.2.1.2 Agarose gel electrophoresis

For the separation of DNA fragments and for controlling the quality of DNA plasmids and RNA, agarose gels with different percentages of agarose were set up (1% and 2%-for fragments smaller that 500 bp-) in 1X TAE buffer. The agarose was boiled in 1X TAE buffer in the microwave until it was completely dissolved, the liquid solution was poured into chambers of different dimensions, and 0,2 ug/ml Ethiombromid were added into the liquid gel and thoroughly mixed. Combs with were inserted into the hot gel to create the wells. The 1Kb-DNA ladder was loaded as a marker. The electrophoresis was performed in 1XTAE buffer at 120-140 V.

3.2.1.3 Purification of DNA from agarose gel

After the electrophoresis, the agarose gel was placed on a UV-lamp and the wanted bands were identified and cut with a scalpel. The purification of plasmids, DNA fragments and PCR products from agarose slices was performed using the Nucleospin Extract Kit according to the instructions of the manufacturer. The eluted DNA was either immediately used for further processing or stored at -20°C.

3.2.1.4 Ligation

The insertion of DNA fragments into the wanted plasmid was performed using the T4-DNA-ligase. To increase the success of the reaction, different molar ratios between vector and insert were used. For the ligase reaction 1 µl T4 DNA ligase, 2 µl T4 DNA-Ligase-buffer and ratio of 1:1, 1:5, 1:10 between vector and insert were used. The reaction mixture was incubate either at RT for 3 hours, or at 16°C overnight.

3.2.1.5 Production of chemical competent bacteria E. coli DH5α

100 ml SOB-Medium was inoculated with E. Coli DH5α, and incubated overnight at 37°C at 200 upm. The following day the E.Coli colture was harvested. The culture was diluted with SOB medium until the OD600 reached 0.1, and incubated again at 37°C constantly monitoring the OD600 (every hour) for other 4-8 hours until the optical density reached 0.6. When the proper density was achieved, the colture was cooled down on ice for 10 min, centrifuged at 3500 rpm at 4°C for 20 min. The supernatant was discarded, the bacteria resuspended in TB medium and incubated on ice for another 10 min. after another centrifugation step at 3500 rpm 4°C for 20 min, the bacteria were resuspended in 20 ml TB medium with 7% (v/v) DMSO and incubated on ice for 10 min, and aliquots of 200 µl were frozen at -80°C.

3.2.1.6 Transformation

Chemical competent bacteria E. Coli DH5α were slowly thawed on ice. 100 µl bacteria were mixed with 20 µl of each ligation reations (1:1, 1:5, 1:10) and incubated on ice for 15 min. The transformation was performed by heat shock at 42°C for 2 min, the

reaction was cooled down for 2 min on ice and then 500 µl of LB medium were added on top of each reaction. The bacteria were allowed to recover for 30 min at 37°C in the shaking incubator.
The transformed bacteria were plated on LB agar plates with ampicillin (100 ug/ml), and incubated overnight at 37°C.

3.2.1.7 Preparation of DNA for screening of constructs – MINI preps

Single colonies of bacteria were picked up from the agar plates and incubated overnight in 5 ml LB medium with ampicillin (100 ug/ml) at 37°C in the shaking incubator at 300 rpm. The bacteria were centrifuged at 4°C at 4600 rpm for 15 min. the supernatant was discarded and the DNA was extracted using the Nucleospin plasmid kit following the instructions of the manufacturer.
The extracted DNA was digested with the proper restriction enzymes and the presence of the insert was proved running the digested reaction in the agarose gel. Positive clone were sent for sequencing, to verify the accuracy of the sequence.

3.2.1.8 Preparation of DNA for trasfection – MIDI preps

Bigger concentration of plasmids were generated retransforming the wanted plasmid into DH5alpha E. Coli. The bacteria were incubated overnight in 200 ml LB medium with ampicillin (100 ug/ml) at 37°C in the shaking incubator at 300 rpm. The bacteria were centrifuged at 4°C at 4600 rpm for 15 min. the supernatant was discarded and the DNA was extracted using the Nucleobond AX500 Kit following the instructions of the manufacturer.
The concentration of the extracted DNA was measured with the Nanodrop.

3.2.1.9 Sequencing

The sequencing reaction was performed from GATC Biotech. shRNA constructs cloned in the vector pLKO2mod-EGFP-WPRE were sequenced with the oligonucleotide 5'-CCTTCACCGAGGGCCTATTTCC-3'.

3.2.1.10 RNA isolation

In order to isolate the RNA from immortalised cells and neurons, the supernatant was removed and the cells were washed carefully with 1X PBS. The cells were then processed according to the RNeasy Mini Kit (Qiagen). To facilitate to homogenization, the QIAshredder spin colomn were used according to the instruction of the manufacturer. The quality of the extracted RNA was proved in the agarose gel, and the concentration was measured with the spectrophotometer. A pure RNA should have an A260/A280 of 1.8-2.0.

3.2.1.11 Reverse transcription of RNA

Purified RNA was reverse transcribed with the High Capacity Reverse Transcription kit (ABI) following the instructions of the manufacturer. Total RNA was reverse transcribed with random primers and the cDNA was used for detecting the expression of the wanted genes via qRT-PCR.

3.2.1.12 SYBR-Green qRT-PCR

The specific primers for qRT-PCR were designed in the following manner: the cDNA sequence of the target gene was downloaded from NCBI as RefSeq and pasted into Primer3Plus for the primer design. To avoid amplification of contaminating genomic DNA, the primers were designed as exon-introns panning, so that one half of the primer hybridizes to the 3' end of the exon and the other half to the 5' end of the following exon. The primers were tested using control murine or human cDNA in a standard curve dilution for efficiency and specificity. Different dilutions of cDNA amplified with the same primer pairs should give an efficiency of 1.8-2.2. The specificity was proved by the melting curve: a specific signal should show a sharp and clean pick for each amplified cDNA. The qPCR was performed using the qPCR machine "7500 fast real-time PCR" and the reaction was set up using the "power SYBR-Green PCR master Mix" according to the instruction of the producer. Each sample was measured in duplicate or triplicate in 96-well-plate for detecting the expression of the gene of interest. Constitutively expressed genes like beta Actin and GAPDH were used as references in the calculation of the final expression rate of the gene of interest.

The qPCR protocol was run as follows:
Step 1: 95°C, 10 min
Step 2: 95°C, 15 sec
Step 3: 60°C, 1 min
Step 4: repeat Step 2 to Step 3 40X
Step 5: melt curve 60°C-90°C with 0,75°C/min

3.2.2 Immortalized cell lines

3.2.2.1 Cell lines

Human Embryonic Kidney HEK293E (immortalized with Epstein-Barr nuclear antigen 1) were cultured in DMEM (Dulbecco´s modified eagles medium) with the addition of 10% FCS and 1% Penicillin/Streptomycin. HEK293E cells are easy to grow and trasfectable with plasmids and siRNA. Human Embryonic Kidney HEK293T (immortalized with SV40 large T-Antigen) were cultivated in DMEM with 10% FCS, 1% Penicillin/Streptomycin and 200 ug/ml G418. Human dopaminergic neuroblastoma cells SH-SY5Y cells were cultivated in DMEM-F12 (50% DMEM and 50% HAM´s F12) with the addition of 15% FCS, 1% Penicillin/Streptomycin, 2mM Glutamin and 1% NEAA (non essential aminoacids).

3.2.2.2 Cell culture

The regular passaging of cell lines was done under the sterile cell culture hood. The immortalized cells were first rinsed with 3 ml 1X PBS, incubated for few minutes with 1,5 ml of Trypsin-EDTA to detach them from the plastic dish or flask, harvested with DMEM-10%FCS and centrifuged for 5 min at 1000 g at room temperature. The pellet was resuspended in 5 ml/each trypsinized flasks or dishes and the cells were split in 1:3, 1:5 or 1:10 ratio dependently on the confluence of each cell line. Cultures were regularly expanded in either 10 cm dishes or T75 flasks. Cell viability and confluence were controlled under the light microscope.
HEK293E, HEK293T and SH-SY5Y cells were grown in the incubator at 37°C and 5% CO2.

3.2.2.3 Freezing and thawing cells

Stocks of each immortalized cell line were cryopreserved at -80°C or in liquid nitrogen. Cells were handled as for a regular passaging, the harvested pellet was resuspended in cold FCS with 10% DMSO and aliquots of 1 ml in 2 ml cryopreservation vials were made. Cells were incubated 10 minute on ice, and the transferred to cryoboxes for few days. Afterwards the vials were transferred into -80°C freezers and later to liquid nitrogen tanks.

For recultivation, cells were quickly transferred from liquid nitrogen tanks or -80°C to a 37°C water bath, until they were partially thawed, and taken up in the medium of the specific cell line. Cells were centrifuged at 1000 g for 5 min at room temperature, taken up again in fresh culture medium and plated in T75 flasks.

The proper recovery of the cell culture was checked under the light microscope the next day.

3 days later, the differentiation was checked under the light microscope, and cells were trypsinized and used for the experiment.

3.2.3 Transfection of immortalized cell lines

3.2.3.1 Poly-L-lysine coating

Prior transfection of immortalized cell lines, the surface of plates (96-well-plate, 24-well plate, 6-well-plate, 10 cm dishes) was coated with a sterile solution of Poly-L-lysin (1:1000 in sterile H20), and incubated under the sterile hood for at least 20 min. Subsequently the solution was washed away twice with 1X sterile PBS. The plates were then used for seeding the cells. This process ensures a better attachment of the cells to the surface.

3.2.3.2 Transient overexpression in HEK293E

For transient overexpression, the optimal number for an efficient transfection of HEK293E was tested. Cells were seeded in poly-L-lysine coated wells at the optimal density (circa $1*10^5$). 24- hours later, the transfection solution with Lipofectamine 2000 was set up: depending on the well size (see Table 12) the Lipofectamine was diluted in

OPTIMEM and incubated for 5 min at RT. In the meantime the DNA was as well diluted in OPTIMEM. The 2 solutions were combined and then incubated for other 20 minutes, to allow the liposome to entrap efficiently the DNA molecules.

Table 12: Lipofectamine 2000 transfection ratios.

Well size	DNA (µg)	LIPOFECTAMINE (µL)	Optimem (µL)
96-well	0.1	0.2-0.5	2x5 µL
24-well	0.5	1-2.5	2x25 µL
6-well	2.5	5-12.5	2x100 µL
10 cm dish	14-28	29-73	2x500 µL

The liposomal solution was dropped on the cells, and the plate was gently swirled prior the incubation at 37°C. The next day, the supernatant was changed. 2 days after the transfection, the supernatant was collected to be analysed for secreted and shed proteins, while the cells were harvested for the lysate preparation.

3.2.3.3 Transient siRNA knock-down in SH-SY5Y

siRNA based knockdown was used for screening the human kinome in SH-SY5Y cells. The siRNA kinase library was purchased by Dharmacon (Thermo Scientific) as sets of four pooled single siRNA for each of the 720 genes. The most interesting candidates were also studied performing the knock-down with different siRNA sequences, transfected in a single and a pooled manner, purchased from Qiagen.
The oligos were solubilized in 1X siRNA Buffer (Dharmacon) or RNAase free- ddH20 (QIAGEN) in stock of 1 or 10 uM, and then frozen at -20°C in aliquots. At the time of use, siRNA were first thawn at RT and then kept on ice. Reverse transfection with Lipofectamine RNAiMax (Invitrogen) was used to efficiently deliver siRNA oligos into the cell. The Lipofectamine RNAi MAX was previously incubated with OPTIMEM for 5 min at RT, and then added on top of the wanted amount of siRNA. The volume of LipofectamineRNAi Max, OPTIMEM and concentration of siRNA vary depending on the cell line, and on the size of the well, according to the instructions of the manufacturer. the mixture was incubated at RT for 20 min. In the meantime the cells were harvested, counted and diluted to the wanted concentration ($5*10^5$) in antibiotics free medium,

added to the mixture and plated out in the wanted well-size. siRNAs were used at concentrations of 5, 10, 15 nM: at these concentration siRNA off-target effects are minimized.

Table 13: RNAiMax transfection ratios.

Well size	siRNA nM	RNAiMax (µL)	Optimem (µL)
96-well	5-15	0.5	2x5 µL
24-well	5-15	1.5	2x25 µL
6-well	5-15	5	2x100 µL

The next day 1/3 of the antibiotics free medium was added to dilute the Lipofectamine to monimize toxic effects. The third day, the medium was replaced with fresh one. The fourth day the medium was collected for the analysis of secreted and shed proteins, and cells were harvested for lysis or processed for RNA extraction.

3.2.3.4 Toxicity assay Alamar Blue

Cell health was monitored with the Alamar Blue toxicity assay. Alamar blue cell viability reagent is based on the molecule resazurin, which detects the reducing power of living cells in a quantitative manner. Resazurin is a cell permeable compound, which is reduced to resorufin, changing the colour of the media. The Alamar Blue solution was added to the supernatant with a ration of 1/10, the cells were incubated for 1-4 hours at 37°C(the precise time was prior assessed with a standard curve specific for the cell line and the well size). The fluorescence was measured at 570 nm. Since resazurin is not toxic, supernatants and cells can be subsequently used for further read-outs.

3.2.4 Cortical and Hippocampal Embryonic Neurons

3.2.4.1 Dissociation of wild-type neurons

The primary neurons were extracted from E15-E16 wild-type (BL6 strain) mouse brains. The first day the wells were prepared for the neuronal culture, coating them with Poly-D-Lysine: 25 ug/ml of poly-D-lysine diluted in cell culture Tested Water (Gibco) was used for covering the surface of the well. The coating was left for 30 min under the UV-light,

or for 2-3 hours at 37°C in the cell culture incubator. Afterwards the wells were washed twice with cell culture tested water and finally with 1X PBS at pH 7.4 (Gibco). The plates were kept overnight in the incubator with 1X PBS.

The next day, the different media were prepared prior starting the explant of the neurons.

MEDIUM 1 (DIGESTION): 9,7 D-MEM Glutamax High Glucose, 0,01g L-Cystein, 200 U Papain, pH 7.4

MEDIUM 2 (DISSOCIATION): D-MEM Glutamax High Glucose with 10% FBS

MEDIUM 3 (PLATING): D-MEM Glutamax High Glucose with 10% FBS, 1% Pen/Strep

MEDIUM 4 (CULTURE): 9,6 ml Neurobasal, 100 µl Glutamin 50 mM, 100 µl Pen/Strep, 200 µl B27.

The different media were kept at 37°C in the water bath until they were used.

The pregnant mouse was sacrificed by cervical dislocation, the abdomen was sterilized with 80% EtOH and opened with a scissor. The embryos were transferred to a 10-cm dish in cold HBSS without antibiotics and decapitated. Brains were removed from the heads under a light microscope, the hemispheres were separated, the meninges were removed and the cortex was collected and placed in fresh cold HBSS without antibiotics.

The HBSS was changed with MEDIUM 1 and incubated at 37°C for 15-20 min (not more than 6-7 embryos/tube). Medium 1 was changed to 5 ml of Medium 2 for washing the tissues and then other 5 ml of medium 2 were added to the tissues for the dissociation process. Dissociation was performed with pipetting the neuronal tissue up and down with a 2 ml graduate sterile pipette. The dissociated neurons were collected apart from the debris in a new tube, which was spin down at 700-800 rpm for 5 min. Cells were counted with Trypan Blue, resuspended in medium 3 and plated at a density of $1,5*10^6$/well for a 6-well plate, $5*10^5$/well for a 24-well plate or $7,5*10^4$/well for a 96-well plate. The neurons were placed in the incubator at 37°C and left there for 2-3 hours. Afterwards the medium 3 was changed to medium 4 to keeps the cells in culture for several days. The medium was refreshed at need after few days, replacing only half of the volume to avoid stress to the neurons.

3.2.4.2 Transduction of embryonic cortical neurons with Lentivirus

Lentiviral particles were produced from HEK293T cells in 6-well plates or 10 cm dishes.

Transfection in 6-well-plates was performed as follow: 1,3 µg of transfer vector, 0,75 ug psPAX2 and 0,45 ug pcDNA3.1(-)-VSVG were mixed with 6,3 µl Lipofectamine 2000. Optimal density was calculated and cells were transfected in solution in 2 ml OPTIMEM. The following day, medium was changed to 2 ml of packing medium. After 24 hours, the packing medium containing the lentiviral particles was filtered with 0.45 µM sterile filters. Different dilutions of the medium are added to 60% confluent target cells. In this case the virus is not purified.
Transfection in 10 cm dishes was performed as follow: 30 µg of transfer vector, 20 µg psPAX2 and 10,8 µg pcDNA3.1(-)-VSVG were mixed with 136 µl Lipofectamine 2000. Optimal density was calculated and cells were transfected in solution in 9 ml OPTIMEM. The following day, medium was changed to 8.5 ml of packing medium. After 24 hours, the packing medium containing the lentiviral particles was filtered with 0.45 uM sterile filters. The conditioned medium was cleared through ultracetrifugation at 22000 rpm, 2 hours at 4°C. Afterwards the medium was discarded and the pellet containing the viruses was incubated with 250 µl of TBS-5-BSA buffer for 4 hours in the fridge. Viruses were carefully resuspended and spinned down at 3000 rpm at 4°C.
Aliquots were frozen at -80°C.

3.2.5 Protein analysis

3.2.5.1 Harvesting of supernatant

The conditioned medium was collected in Eppendorf tubes after 24 hours incubation, centrifuged at 13.000g for 1 min at RT and the supernatant was transferred to new Eppendorf tubes and either kept on ice for further use or frozen at -20°C for storage.

3.2.5.2 Cell lysis

For the investigation of intracellular proteins and membrane bound proteins, cells were washed once with cold 1X PBS and then incubated on ice with either 1% NP40 lysis buffer (STEN) or 1% Triton-X100 lysis buffer (STET) enriched with protein inhibitors mix for 15 min. The volume of the lysis buffer was adjusted depending on the well size: 200 uL/well were used for 24-well plate and 300 uL/well for a 6-well plate. After 15 min cells were resuspended in the buffer with the pipette, collected in Eppendorf tubes and

centrifuged at 13.000g for 5 min at 4°C. The supernatant was transferred to new Eppendorf tubes and kept on ice or frozen at -20°C.

3.2.5.3 Protein measurement

In general, the total protein concentration was measured using the BC Assay Solution A and B kit (Uptima). To determine the protein concentration, Solution A and Solution B were mixed in a 1:50 ratio. 10 µl of lysate from either immortalized cells or primary neurons are were pipetted in a 96-well-plate in duplicate and the mixture of solution A and B was added. The plate was incubated for 30 min at 37°C and the absorption was measured with ELISA reader at 562 nm according to the instruction of the manufacturer. As reference, a standard curve with predefined concentration of BSA was used.
Total protein concentration in cerebrospinal fluid and serum was assessed as follow: aliquots of 5 µL of CSF were used for determining the total protein concentration in the CSF, while serum was centrifuged at 3000xg for 10 min at 4°C, diluted 1:20 in PBS and then 5 µL were taken for BCA measurement. Each sample was measured in duplicate.

3.2.5.4 Immunoprecipitation

APPsα immunoprecipitation was performed using 5313 antibody, pre-bound to 25 µl of PAS beads. Samples were diluted in a final volume of 500 uL, PAS beads conjugated to the antibody were added and the mixture was incubated at 4°C for at least 2 hour on the inversion shaker. The immunoprecipitation was centrifuged at 8000 rpm for 1 min at 4°C. The supernatant was analysed in the immunoassay for depletion studies. The pellet was washed with 1 ml STEN-NaCl, STEN-SDS and STEN buffers. Immunoprecipitated proteins were dissociated from the antibody boiling the pellet with 2X Sample Buffer at 95°C for 5 min.

3.2.5.5 SDS-Polyacrylamide-gel electrophoresis

The protein concentration was assessed with the BCA measurement in the cell lysate and normalized in order to charge the same concentration for every sample. In preparation of the SDS-PAGE analysis, the lysate and supernatant samples were mixed with 4X SDS-

Sample Buffer and boiled for 5 min at 95°C. The samples in SDS-Sample buffer were either immediately used or frozen at -20°C.

SDS-PAGE (SDS- polyacrylamide gel electrophoresis) is the most widely used technique to separate proteins under denaturing conditions. Negatively charged proteins are separated according to their molecular weight. In this study the percentage of acrylamide in the SDS-PAGE varied according to the molecular weight of the proteins under investigation: 8% acrylamide gels were used to separate proteins between 50 kDa and 200 kDa, while 12% acrylamide gels were used for proteins between 10 kDa to 100 kDa. Polyacrilamide gels were poured in spaced glasses 1.5 mm thick, with 7.5 ml of separation gel and 2,5 ml of stacking gel (see composition in table 14), and allowed to polymerase.

Table 14: SDS-PAGE. Volume of reagents for 2 polyacrylamide gels.

Separation gel

Reagent	**8% Gel**	**12% Gel**
dH_20	8.8 ml	7.5 ml
40% Acrylamide-Bis	3.2 ml	4.5 ml
Low-Tris Buffer	4 ml	4 ml
10% APS	60 ul	60 ul
TEMED	30 ul	30 ul

Stacking gel

Reagent	**8% Gel**	**12% Gel**
dH_20	6.8 ml	6.8 ml
40% Acrylamide-Bis	1 ml	1 ml
Upper-Tris Buffer	2.5 ml	2.5 ml
10% APS	30 ul	30 ul
TEMED	30 ul	30 ul

Samples in Laemmli sample-buffer were loaded in the stacking gel and the gels were run with the Mini-Protean system of Biorad filled with Running Buffer was used for the electrophoresis in accordance to the instructions of the manufacturer. The molecular weight marker used the SeeBluePlus2 (Invitrogen).

3.2.5.6 Western blot

The proteins separated in the SDS-PAGE were transferred to a membrane for the detection of the protein of interest. The transfer of the proteins was performed with the Western Blot. A sandwich with a sponge, 2 whatman papers, the polyacrylamide gel, the membrane (PVDF), 2 whatman papers, a sponge was set up. The proteins are negatively charged, so they will move to the positive pole, from the polyacrylamide gel to the membrane. The Western blot was performed in Mini Protean Blotting System filled with Transfer Buffer. The transfer was done at 400 mV for 65 min. Afterwards the membrane was briefly washed in 1X PBS-Tween and blocked with 3% Milk in 1X PBS-Tween for 30 min on the horizontal shaker at RT. The membrane was then washed twice with 1X PBS-Tween for 5 min and incubated with the primary antibody against the protein of interest for either 1 hour at RT or overnight at 4°C on the shaker, depending on the antibody characteristics. The membrane was carefully washed 4 times with 1X PBS-Tween for 5 min/each washing step. The following incubation of the proper secondary antibody was done for 1 hour at RT on the shaker. Afterwards the membrane was again washed 4 times with 1X PBS Tween, 5 min/each step. The development of the Western Blot was done using either ECL or ECL plus according to the instructions of the manufacturer.

All primary antibodies were diluted in 10 ml 1X PBS Tween, 0,5% BSA, 0,05% w/v Sodium Azide at the concentration advised from the manufacturer. the secondary antibodies were also diluted in 10 ml 1X PBS Tween, 0,5% BSA, without Sodium Azide at a concentration of 1:10.000 or 1:5.000.

3.2.5.7 Detection and Quantification of ECL Signal

The signal of the peroxidase conjugated with the secondary antibody and excited with the ECL reagent was detected using the quantification machine Image System LAS-4000.

3.2.5.8 Immunoassays

APPsα (6E10) and APPsβ concentrations in CSF and serum were measured according to the manufacturers' instructions with the multiplex assay provided from Meso Scale Discovery (Gaithersburg, MD, USA). The assay uses the 6E10 antibody for detecting

human APPsα, binding the epitope at amino acids 3-8 of the Aβ sequence. APPsβ was detected in the same well with the ANGU antibody, raised against the peptide sequence of amino acids 591-596 of APP695 (β-secretase cleavage site). Briefly, 150 µL of blocking buffer was added into each well of a 96-well plate, incubated for 1 hour at room temperature (RT), washed three times with MSD washing buffer. Afterwards 25 µL of synthetic standards and samples were incubated for 1 hour at RT. Wells were washed three times. 25 µL of detection Sulfo-tag antibody P2-1 against the N-terminus of APP was added into each well and incubated for 1 hour at RT. After the last washing step, 150 µL of reading buffer was added to each well, incubated for 10 minutes at RT and the signal was measured using the Sector Imager 2400. Each sample was measured in duplicate.

Aβ40 concentration in cell culture supernatants was measured according to the manufacturers' instructions as above with the multiplex assay Aβ triplex provided from Meso Scale Discovery (Gaithersburg, MD, USA). Sulfo-Tag 6E10 antibody was used for detecting human Aβ40, whereas Sulfo-Tag 4G8 was employed for murine Aβ detection.

14D6 antibody was raised against amino acids 11-16 of the human Aβ sequence (Colombo et al., 2012), and it was biotinylated. APPsα measurements with new antibody 14D6 were done as above using the Meso Scale Discovery system and plates with the following changes: streptavidin coated plates from Meso Scale Discovery were blocked with 3% BSA and washed as above. 150 µL of biotinylated-14D6 antibody diluted 1:1000 in 1% BSA in washing buffer was added to the wells, incubated for 1 hour on the shaker and washed three times. 25 µL of either 1:2 diluted CSF or 1:2 diluted serum was added into the wells. In the meantime, 25 µL of 5313 antibody (1:1000) in 1% BSA solution were added to the wells and incubated for 2 hours. 5313 antibody was raised against amino acids 444-592 of APP sequence, provided by Christian Haass (Steiner et al., 1999). After three washing steps, 25 µL of MSD anti-rabbit-sulfo-tag antibody were added into each well and incubated for 1 hour. After the last washing step, 150 µL of reading buffer were added to each well, incubated for 10 minutes at RT and the signal was measured using the Sector Imager 2400. Each sample was measured in duplicate.

Aβ42, tau and phospho-tau181 levels were measured in the clinic as part of the routine diagnosis with standardized commercially available ELISA kits (Innotest, Innogenetics, Ghent, Belgium). Granulin levels were measured in the lab of Dr. Anja Capell.

3.2.6 Patients

All subjects included in the study were part of the sample collection from the Psychiatric Clinic, Ludwig-Maximilians University, Munich. The study was approved by the Ethics Committee of Munich University and was performed in accordance with the Declaration of Helsinki. All participants provided informed written consent. Patients with Alzheimer´s disease were recruited at the Memory Clinic of the Department of Psychiatry, University of Munich, Germany. Participants diagnosed with dementia associated with Alzheimer's disease fulfilled the criteria of probable Alzheimer´s disease, according to the NINCDS-ADRDA (McKhann et al., 1984). Cognitive testing by neuropsychological evaluation was performed in all patients according to Mini-Mental State Examination (MMSE) (Folstein et al., 1975), Consortium to Establish a Registry for Alzheimer's Disease (CERAD) battery (K.A. Welsh, 1994), Multiple Choice Vocabulary Test (MWT, Mehrfachwahl-Wortschatz-Test) (K.H. Schmidt, 1989) and a variant of the Trail Making Test (TMT) (K.H. Schmidt, 1989). Controls were recruited within the Munich hospital and did not have cognitive complaints and scored within 1 standard deviation from the age adjusted mean in all subtests of the CERAD cognitive battery. CSF and serum samples were collected from 26 controls and 27 Alzheimer's disease (AD) patients from both sexes. Samples were collected in 1.5 ml Eppendorf tubes, frozen in aliquots, and stored at -80°C. Aβ42, tau and ptau181 could be measured in 25 out of 26 controls, and in all 27 AD patients. APPsα and APPsβ could be measured in CSF of 23 out of 26 controls, and in 26 out of 27 AD patients. APPsα and APPsβ could be measured in serum of 24 out of 26 controls, and in 26 out of 27 AD patients. Age and MMSE could be evaluated in all 26 controls and all 27 AD patients.

3.2.6.1 Body fluid samples

CSF samples were thawn from -80°C at the time of measurement. CSF, plasma and serum samples were diluted 1:2, 1:4, 1:8 and 1:16 respectively in PBS with 1% BSA and PBS for test experiments. CSF was diluted 1:2 in PBS with 1% BSA and serum in PBS (1:2) for the measurement of APPsα and APPsβ in the small clinical study. Prior either ELISA or Western Blot analysis, serum samples were treated with 0.1% SDS and incubated for 10 minutes at 65°C, to mildly unfold the proteins.

3.2.7 Statistical analysis

Statistical anylsis of the screening was performed as follow: APPsα, APPsβ and Granulin levels were normalized to the mean of each screening plate. To assess the effect of the sigle kinase knock-down on APPsα, APPsβ and Granulin secretion, the mean of the two replicates was calculated. For each replicate, the correlation between two independent measurements of all three paramenters was analysed. The cut-offs for selecting the scoring hits were chosen arbitrarily (see Results section).

For experiments concerning the characterization of STK39 knock-down, two-tailed unpaired Student's t-test was performed and reached significance if $p< 0.05$.

In the biomarkers´study, statistical analysis was performed using GraphPad Prism 5 (GraphPad Inc., San Diego, CA, USA). The Mann Whitney U test (two-tailed p-value) and unpaired Student's t-test were used to test the statistical significance between the group of non-demented subjects and AD patients. Results are represented as medians and interquartile ranges. Differences between the two groups were considered significant if two-tailed $p<0.05$.
The receiver operating characteristic (ROC) curve was generated via analyses in R (R-project.org) with the help of Dr. Tobias Straub. The areas under the curve (AUC) were computed using the 'somers2' function in library 'Hmisc'.

4 RESULTS

4.1 A new sandwich immunoassay for detection of APPsα in cerebrospinal fluid and serum.

Biomarkers, as a helpful tool to diagnose Alzheimer disease, have been worldwide matter of deep investigation. In order to diagnose a particular disease state, it is important to find a molecule whose levels are easily detectable and significantly changed in healthy versus diseased conditions. Considering neuronal loss, extracellular amyloid plaques and intracellular neurofibrillar tangles as major pathological hallmarks of Alzheimer's disease, biomarkers linked to these neuropathological changes have been extensively investigated. Aβ42, tau and phosphorylated tau have been widely used to help the diagnosis of AD in association with cognitive test based on neuropsycological evaluation (Blennow et al., 2012). Subjects with higher level of tau and phospho-tau, and at the same time with lower levels of Aβ42 in comparison to controls are usually categorized as patients with probable dementia associated with Alzheimer´s disease.
Nevertheless these biomarkers do not allow a full and significant separation between the group of AD and non-demented subjects: for this reason, other proteins and peptides are investigated, to find meaningful changes that can be better correlated to the pathology.

4.1.1 New antibody for specific detection of APPsα

For example APPsα, the secreted fragment of APP produced from ADAM10 cleavage at the α-cleavage site, has been investigated as potential biomarker mainly in CSF and less in blood. Several studies have provided conflicting results: some studies reported an increase in APPsα levels, some other a reduction in AD patients in comparison to the controls (Fellgiebel et al., 2009; Gabelle et al., 2010; Lannfelt et al., 1995; Lewczuk et al., 2010; Olsson et al., 2003; Perneczky et al., 2011; Rosen et al., 2012; Selnes et al., 2010; Sennvik et al., 2000).
Most of these studies, regardless if they were performed in CSF or blood, used antibody 6E10 for detecting APPsα, which binds amino acids 1-16 (Covance) (Klyubin et al., 2005) of the Aβ sequence (Fig. 9A). For this reason, 6E10 antibody does not only detect APPsα, but also the alternative β-secretase cleavage product APPsβ´, probably influencing APPsα measurments.

In order to specifically detect APPsα, but not APPsβ´, the specific antibody 14D6 against the amino acids 11-16 of the human Aβ sequence was raised (Fig. 9A). To test the specificity of the antibody, HEK293 cells were transiently transfected with full-length APP wt, APPsβ, APPsβ´ , APPs-14, APPs-15, APPs-16 and the control plasmid (Linker). Lysates and media collected from the different transfections were tested with 14D6 antibody to detect specific recognition of APP soluble fragments. 14D6 recognizes specifically the soluble ectodomain of APP generated after the cleavage by α-secretase, and no cross reaction was detectable via immunoblot analysis for either APPsβ´ or APPsβ, as shown in Fig. 9B. As transfection control, intracellular APP was detected with the antibody 22C11.

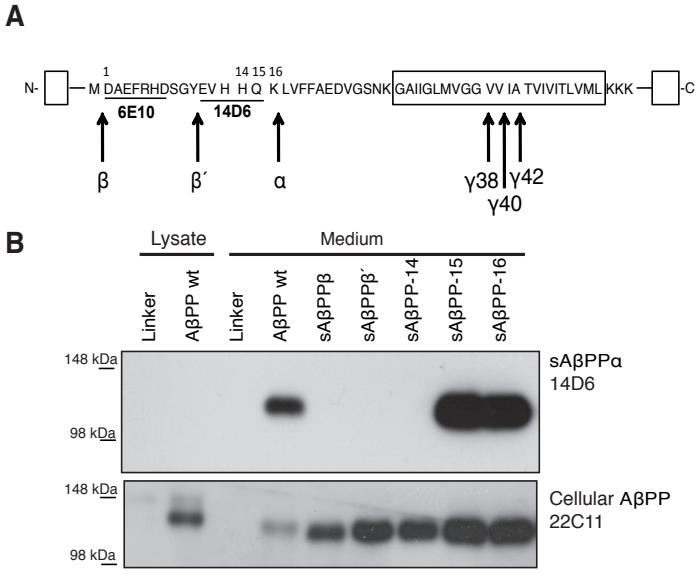

Fig. 9: Validation of the new 14D6 antibody. A) 14D6 and 6E10 epitopes are indicated on the schematic representation of APP. Amino acid numbers refer to Aβ sequence. Arrows correspond to α-, β-, β´-, γ38-, γ40-, γ42- cleavage sites. The box highlights the transmembrane domain. B) Immunoblot of supernatants and lysate of cells transfected with APP wild-type, APPsβ, APPs-14, APPs-15, APPs-16. 14D6 antibody specifically detects APPs-15 and APPs-16. Antibody 22C11 detects cellular APP (taken from Taverna et al. 2013 in press).

4.1.2 Validation of the new 14D6-immunoassay in supernatant from neuroblastoma cells

In order to selectively and efficiently capture APPsα in cell culture supernatant and body fluids, a sandwich immunoassay was generated. This assay is based on the trapping of the analyte by a first specific antibody, and the detection by a second antibody. This

method is very specific and sensitive for the analyte, because of the two recognition sites of the two antbodies. For this purpose, 14D6 antibody was biotinylated, to allow high affinity and specific binding to streptavidin coated plates. The biotin-streptavidin binding is widely used in sandwich immunoassay for the capability to give low background with high signal-to-noise ratio, and biotin is very unlikely to interfere with the epitope-antibody interaction.

To demonstrate the specificity of the new 14D6-immunoassay in detecting APPsα, either α-secretase ADAM10 or APP was knocked-down with siRNA pools in human neuroblastoma SH-SY5Y cells. The conditioned medium was collected and used for testing the specific detection of APPsα with 14D6-immunoassay. APPsα signal was reduced to almost 20% in the case of ADAM10 knock-down (Fig. 10). This result is in agreement with a previous work done in our lab using another antibody specific for APPsα (Kuhn et al., 2010). APP knock-down also reduced the APPsα signal to almost 5% (Fig. 10). This analysis could indeed validate the specificity of the new assay for detecting APPsα in supernatants from neuroblastoma cells.

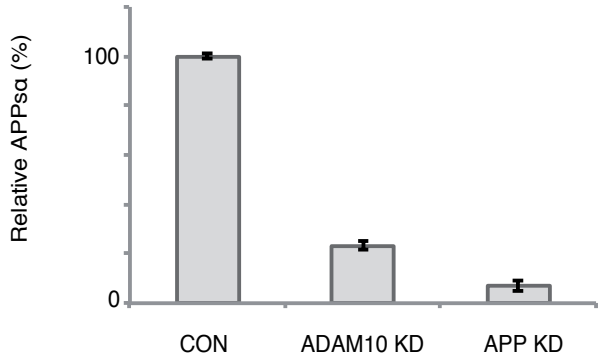

Fig. 10: Validation of 14D6 immunoassay with supernatants from neuroblastoma SH-SY5Y cells. APPsα signal decreased upon ADAM10 and APP knock-down. CON: scrambled siRNA; ADAM10 KD: ADAM10 knock-down with pooled siRNA; APP KD: amyloid precursor protein knock-down with pooled siRNA. Given are mean and standard errors relative to control of six independent experiments.

4.1.3 Validation of the new 14D6-immunoassay in human cerebrospinal fluid (CSF)

To test whether the 14D6-immunoassay was also suitable to detect APPsα in body fluids, namely CSF, plasma and serum, samples of human origin were collected and analysed. Serial dilutions of CSF were prepared and APPsα levels were analysed. In undiluted CSF

sample, APPsα concentration resulted in the range of 50 ng/ml (Fig. 11) being surprisingly not much different from the first 1:2 dilution. Further 1:2 dilutions (1:4, 1:8, 1:16 in Fig. 11) decreased the signal as expected. CSF APPsα concentration in other studies is described to have similar concentrations around 55 ng/ml, when using the 6E10 antibody in sandwich ELISA and Western Blot.

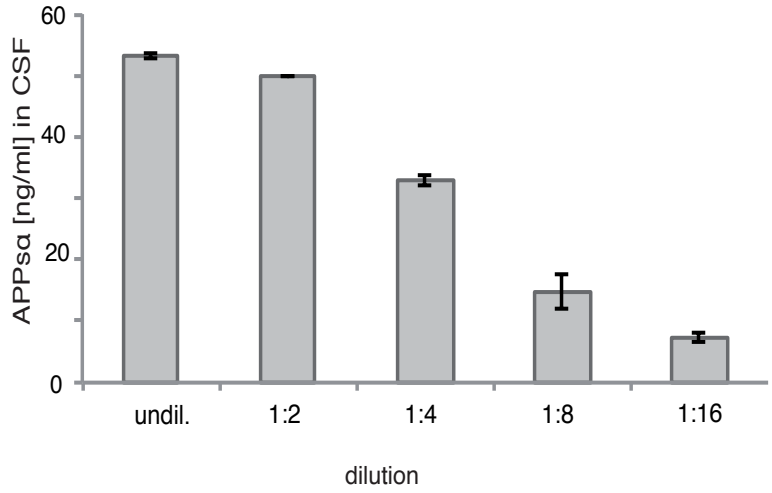

Fig. 11: 14D6-immunoassay in human cerebrospinal fluid (CSF). Undiluted APPsα, and serial dilutions of 1:2. 1:4, 1:8, 1:16 were analysed with 14D6-immunoassay. Given are mean and standard deviations of 2 technical replicates.

Presumably APPsα concentration in the undiluted CSF was higher than the detected amount, but probably some other proteins or components present in CSF interfered with the assay, thereby resulting in the discrepancy between expected and measured concentration.

To test this hypothesis, another CSF sample from a different subject was analyzed both by 14D6 assay and by immunoblot with the same 14D6 antibody. Also in this case, the concentration of APPsα given by the immunoassay was at about 35 ng/ml, and did not change in the expected ratio when it was diluted 1:2 (Fig. 12B). The measured concentration in the immunoblot for the 1:2 dilution was similar to the immunoassay, but not for the undiluted sample (Fig. 12A), as assessed by densitometric comparison with known concentrations of APPsα standards purchased by Meso Scale Discovery. This is a demonstration that in the undiluted CSF there are indeed some components interfering with the detection of APPsα signal. Therefore, latter measurements of APPsα in CSF were always performed using 1:2 dilution of CSF samples.

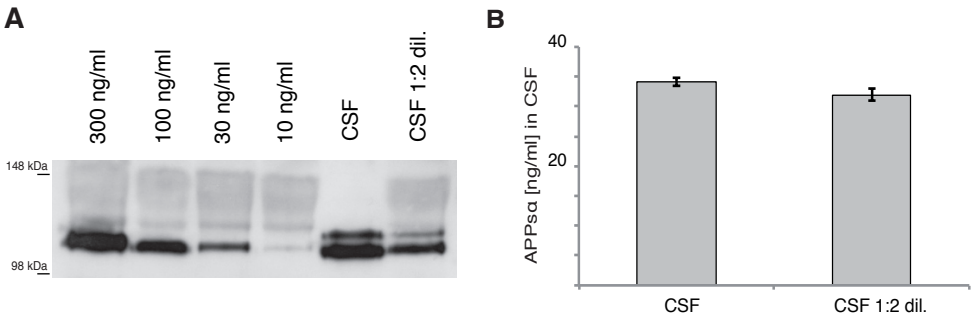

Fig. 12: Analysis of undiluted and diluted CSF. A) 14D6 immunoblot for detecting APPsα in CSF. Same volumes of APPsα standards with known concentrations were loaded as reference. The same CSF sample was loaded undiluted and diluted in 1% BSA solution. B) 14D6 immunoassay of the same sample analysed in A).

To further confirm the identity of the signal in 14D6-immunoassay, APPsα was immunoprecipitated in a CSF sample using antibody 5313. This antibody binds to the middle region of the APP ectodomain, specifically to amino acids 444-592 (Steiner et al., 1999). The CSF APPsα-depleted was analyzed in the 14D6 assay, and APPsα signal was reduced by 88% compared to the control (no IP), confirming once more the specificity of the signal. As further controls, immunoprecipitations without antibody and with antibody against E-cadherin were carried out in parallel. Both controls did not decrease the APPsα signal compared to the control (no IP) (Fig. 13).

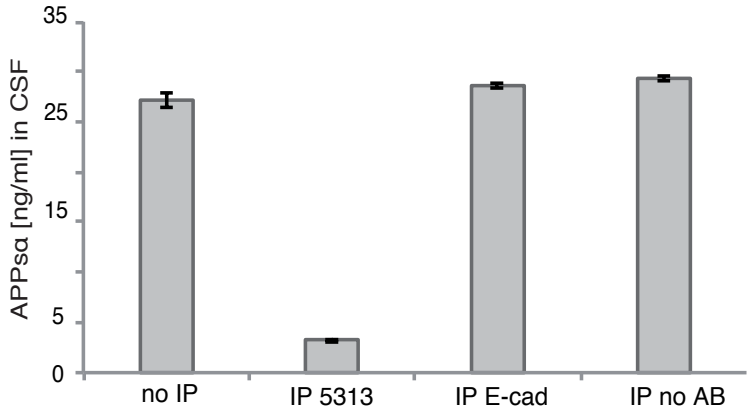

Fig. 13: Immunoprecipitation of APPsα in human CSF. APPsα was depleted in CSF with 5313, control antibody (E-Cadh) and without antibody (no AB) as further control. No IP: no immunoprecipitation; IP 5313: APPsα was depleted from CSF with polyclonal 5313 antibody; IP E-cad, IP no AB: immunoprecipitation with a control antibody against

E-Cadherin or without antibody (IP no AB; beads only). The signal detected with 14D6 immunoassay decreased only when APPsα was depleted. Given are mean and standard deviations of 2 technical replicates.

Taken together, these results show that the signal measured with 14D6-immunoassay is specific for soluble α-cleaved APP in human CSF.

4.1.4 Validation of the new 14D6-immunoassay in human serum

Recently, the search of peripheral biomarkers as a measure of AD pathology has become of primary importance. Currently, the best diagnostic methods used for the determination of AD are expensive techniques, such as MRI and PET, or invasive procedure like lumbar puncture for the collection of CSF. Both those methods are certainly not suitable for massive monitoring of the progression of the disease in the population. Therefore, other peripheral biomarkers, which can reflect the intracerebral pathology, are under investigation. In particular, since CSF is daily assimilated into the blood, plasma and serum are valuable source of AD biomarkers. In addition, blood is normally a low-cost, easily accessible and collectable body fluid.

For this reason, serum and plasma samples were collected and analyzed with the 14D6-immonoassay. The concentration of APPsα in the plasma was ca. 6 ng/ml, much lower in comparison to the CSF levels and close to the detection limit of the immunoassay. Moreover, APPsα signal was totally abolished in further 1:2 dilutions. Thus, plasma was not included in the study (Fig. 14A).

In opposition to the plasma, APPsα seems to be more abundant in the serum, giving a concentration of around 25 ng/ml. Further dilutions performed as expected, decreasing APPsα concentration in 1:2 ratio (Fig. 14B).

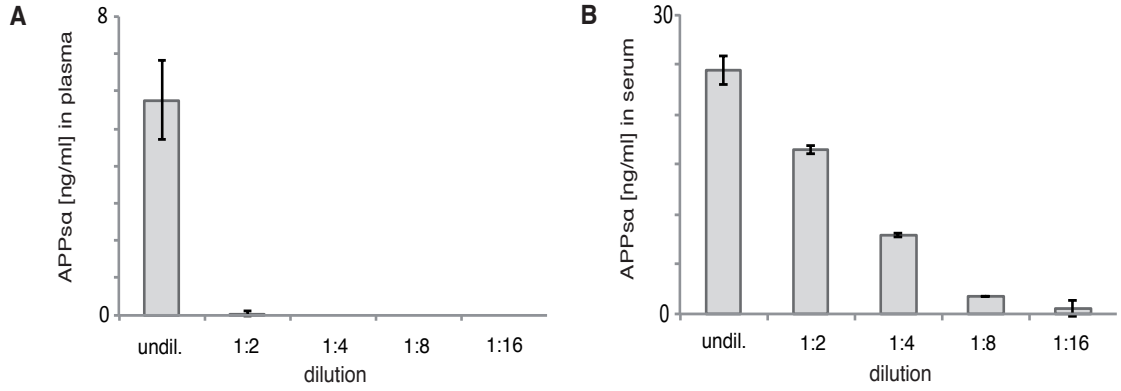

Fig. 14: Validation of 14D6-immunoassay in human plasma and serum. A) APPsα was detected in undiluted plasma, and in serial dilutions 1:2, 1: 4, 1: 8, 1: 16. APPsα concentration was close to the detection limit. B) APPsα was detected in undiluted serum, and in serial dilutions 1:2, 1: 4, 1: 8, 1: 16. APPsα concentration decreased accordingly to the dilutions. Dilutions were made in PBS. Given are mean and standard deviations of 2 technical replicates.

Similar to specificity tests performed for CSF, sera from 3 different subjects were analyzed by immunoblot. The serum is very rich in its total protein content, especially in albumin and globulins. Thus, it was necessary to immunoprecipitate APPsα from the 3 samples with antibody 5313, in order to enrich APPsα content in the samples, and to allow APPsα detection via Western blot using 14D6 antibody (Fig.15A). Immunoprecipitated APPsα from serum has the same molecular weight as the recombinant APPsα-751. Due to alternative splicing, APP in the serum is APP751, whereas the main APP isoform in CSF is APP695 (Tanaka et al., 1988).

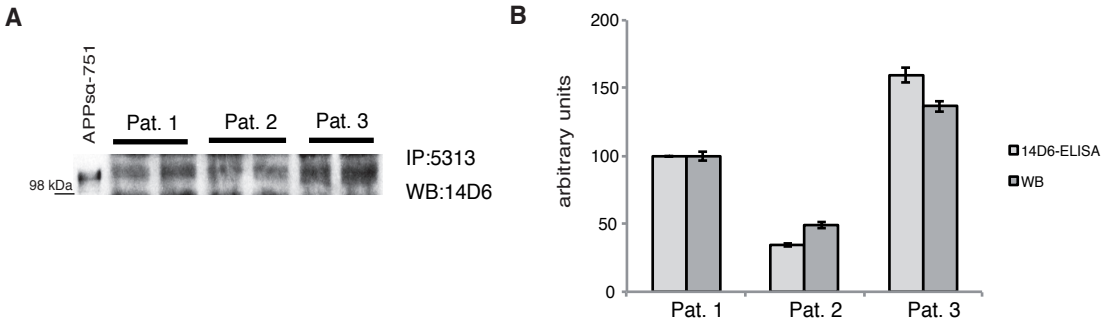

Fig. 15: Analysis APPsα detection in sera from 3 different patients. A) To detect APPsα with 14D6 immunblot in serum, it was necessary to perform the immunoprecipitation with 5313 antibody. Recombinant APPsα751 was used as control. The most abundant isoform in the serum is APP751. B) Comparison between densiometric quantification of APPsα in A) and APPsα detected in sera from the same patients with 14D6 immunoassay. Relative differences in APPsα levels were similar for

both assays. APPsα751: soluble APPsα from 751 isoform; Pat: patient. Given are mean and standard deviations of 2 technical replicates.

The same samples were also analysed by 14D6-immunoassay: the densitometric analysis of the detected bands in the Western Blot and the normalized APPsα concentrations measured with the immunoassay revealed that the differences in APPsα levels in 3 different samples were comparable, validating the specificity of the assay in the serum (Fig. 15B).

Taken together, these experiments demonstrate that 14D6-immunoassay is suitable for the detection of APPsα in CSF and serum from human origins, and it can be used for analysis of larger groups of individuals.

4.1.5 Aβ42, tau and p181-tau measurements in CSF of controls and AD patients

To test whether APPsα measured with 14D6-immunoassay could have valuable diagnostic performance in the analysis of patients with Alzheimer´s disease, CSF and serum of 53 subjects were collected. 27 of these subjects were diagnosed with Alzheimer´s disease and 26 were age-matched non-demented controls. Patients with Alzheimer´s disease were chosen after evaluation of the mini-mental state (MMSE) (see Table 15), and the diagnosis was supported by the panel of neurochemical tests regularly performed in the clinics to categorize patients with dementia. The neurochemical tests are based on the levels of Aβ42, tau and phosho-tau181.

In the subjects selected for this study, tau and phospho-tau were significantly increased in AD patients, whereas Aβ42 levels were lower, but could not reach statistical significance with the Mann Withney test (see Table 15 and Fig. 16). The results from these biomarkers were as expected (Blennow et al., 2012). Aβ42, tau and ptau181 could be measured in 25 out of 26 controls, and in all 27 AD patients (Table 15).

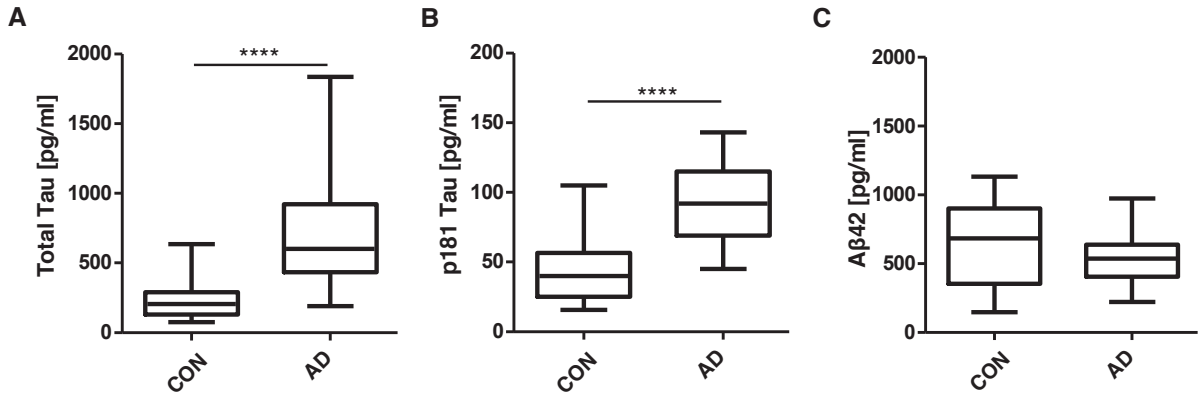

Fig. 16: Measurement of Aβ42, total tau and p181-tau in CSF with Innotest (Innogenetics, Ghent, Belgium) in patients with AD and controls. A) Total tau levels. The Mann Whitney U test showed significant differences between controls (CON) ad Alzheimer´s disease patients (AD): p<0,0001****. B) p181-tau levels. The Mann Whitney U test showed significant differences between controls (CON) ad Alzheimer´s disease patients (AD): p<0,0001****. C) Aβ42 levels. p= n. s. CON, n=25; AD, n=27; confidence intervals =95%. The boxplots show the maximum, the minium, the upper and the lower quartile, and the median. These measurements were performed in the Psychiatric Clinic, LMU, Munich.

4.1.6 APPsα measurement in CSF of controls and AD patients

Next, 14D6-immunoassay was used to measure APPsα concentration in CSF samples. This measurement was performed in parallel with the commercially available APPsα/APPsβ immunoassay from Meso Scale Discovery. This APPsα-assay uses antibody 6E10 as capture antibody, which detects both APPsα and APPsβ´products of APP shedding. CSF samples from 23 out of 26 controls and in 26 out of 27 AD patients could be included in the analysis. The concentration range of APPsα in CSF varied between about 25 ng/ml to 100 ng/ml in both 14D6- and 6E10-immonoassay, considering all samples from both groups. Afterwards APPsα measurements were normalized to the corresponding protein concentration of each sample. A statistically significant increase in APPsα concentration was detected in AD patients compared to controls when the α-cleaved peptide was measured with 14D6-immunoassay, whereas APPsα measurement performed using 6E10-assay showed only a trend to increase, which did not reach significance. APPsβ levels did not change in AD patients when compared to the controls group of subjects (Fig. 17).

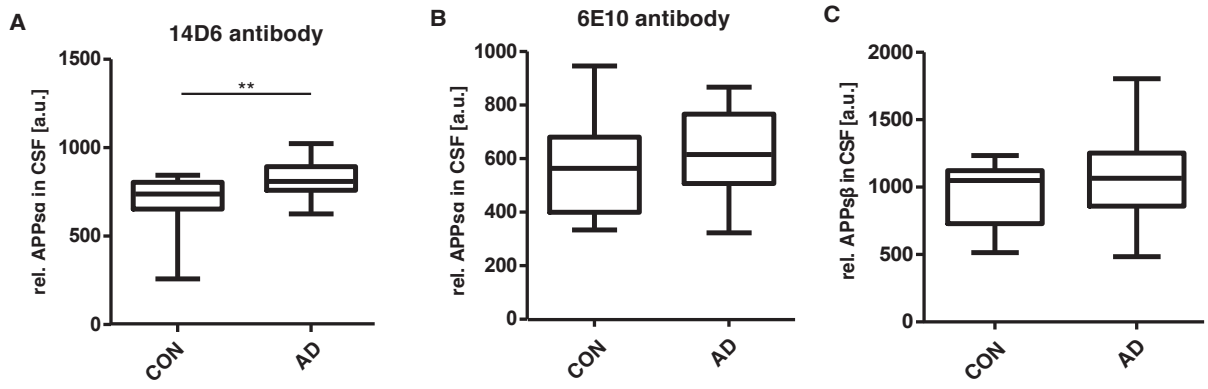

Fig. 17: Measurement of APPsα in CSF from AD patients and controls with 14D6- and MSD multiplex immunoassays. A) 14D6 immunoassay. APPsα concentrations were normalized to the intensity of total protein concentration (a.u.: aribitrary units). The Mann Whitney U test showed significant differences between controls (CON) and Alzheimer´s disease patients (AD): p<0,005**. B) 6E10 Multiplex MDS immunoassay. APPsα concentrations were normalized to the intensity of total protein concentration (a.u.: aribitrary units). p= n. s. C) APPsβ Multiplex MSD immunoassay. APPsβ concentrations were normalized to the intensity of total protein concentration (a.u.: arbitrary units). p= n. s. The boxplots show the maximum, the minium, the upper and the lower quartile, and the median. Con, n=23; AD=26, confidence intervals =95%.

4.1.7 APPsα measurement in serum of controls and AD patients

Considering the importance of peripheral biomarkers for Alzheimer´s disease, 14D6-immunoassay was also employed to measure APPsα concentration in serum samples. The analysis was performed in parallel with the commercially available Multiplex immunoassay from Meso Scale Discovery, which uses the 6E10 antibody. Serum samples from 24 out of 26 controls, and in 26 out of 27 AD patients could be included in the final analysis. The concentration range of APPsα in sera varied between about 5 ng/ml to 70 ng/ml for 14D6-immunoassay, and between 1 ng/ml and 20 ng/ml in the case of 6E10-immonoassay. APPsα measurements were normalized to the corresponding protein concentration of each sample. APPsα concentration in sera from AD patients was significantly lower compared to controls when measured with both 14D6-immunoassay and 6E10-assay. In the case of 6E10 assay, the significance was higher compared to the 14D6-immunoassay. Additionally, APPsβ levels decreased in sera from AD patients when compared to the control group of subjects (Fig. 18).

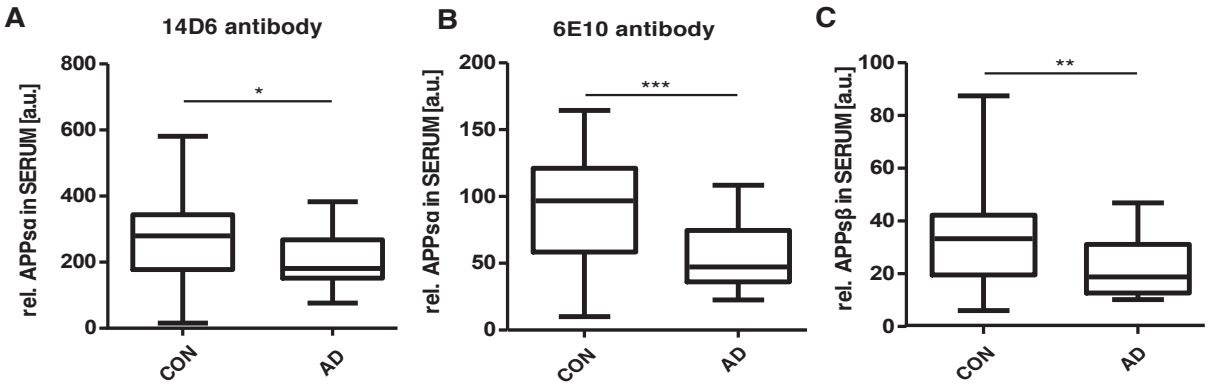

Fig. 18: Measurement of APPsα in sera from AD patients and controls with 14D6- and MSD multiplex immunoassays. A) 14D6 immunoassay. APPsα concentrations were normalized to the intensity of total protein concentration (a.u.: aribitrary units). The Mann Whitney U test showed significant differences between controls (CON) ad Alzheimer´s disease patients (AD): $p<0,05$ **. B) 6E10 Multiplex MDS immunoassay. APPsα concentrations were normalized to the intensity of total protein concentration (a.u.: aribitrary units). The Mann Whitney U test showed significant differences between controls (CON) ad Alzheimer´s disease patients (AD): $p<0,001$ ***. C) APPsβ Multiplex MSD immunoassay. APPsβ concentrations were normalized to the intensity of total protein concentration (a.u.: arbitrary units). The Mann Whitney U test showed significant differences between controls (CON) ad Alzheimer´s disease patients (AD): $p<0,01$ **. The boxplots show the maximum, the minium, the upper and the lower quartile, and the median. Con, n=24; AD=26, confidence intervals =95%.

Table 15: Absolute values of the studied parameters in controls and AD patients.

	CON	**AD**
APPsα 14D6	70033.4 ± 13290.3	78477.0 ± 9162.4
CSF (pg/ml)	n=23	n=26
APPsα 6E10	55326.8 ± 15901.2	59507.2 ± 13848.5
CSF (pg/ml)	n=23	n=26
APPsβ 6E10	93769.3 ± 22365.1	103021.3 ± 27160.8
CSF (pg/ml)	n=23	n=26
APPsα 14D6	34575.9 ± 15108.9	31498.9 ± 11106.9
SERUM (pg/ml)	n=24	n=26
APPsα 6E10	11868.7 ± 5137.0	8281.4 ± 3239.6
SERUM (pg/ml)	n=24	n=26
APPsβ 6E10	4305.0 ± 2162.9	3306.6 ± 1478.9
SERUM (pg/ml)	n=24	n=26
Aβ42 (pg/ml)	613.9 ± 303.5	529.4 ± 188.6
	n=25	n=27
Age (years)	60.9 ± 9.5	67.3 ± 8.6
	n=26	n=27
Total tau (pg/ml)	233.9 ± 139.9	715.8 ± 408.4
	n=25	n=27

p181 tau (pg/ml)	43.5 ± 23.4 n=25	92.0 ± 29.7 n=27
MMSE	28.92 ± 1.06 n=26	23.81 ± 4.28 n=27

Abbreviations: CON: control individuals; AD: Alzheimer´s disease patients; CSF, cerebrospinal fluid; APPsα, soluble amyloid precursor protein α; APPsβ, soluble amyloid precursor protein β; Aβ42, amyloid β 1-42; MMSE, Mini Mental State Examination; n, number of subjects. Given are mean and standard deviations of the mean.

4.1.8 ROC analysis

The Receiver Operating Characteristic (ROC) is a tool broadly used to evaluate the performance of a diagnostic test. The ROC graphic gives information about the sensitivity and specificity of a test, represented by plotting the true positive rate in function of the false positive rate for different cut-off points of the parameter of interest. With this analysis, it is possible to judge the accuracy of a test in distinguishing between the group of the diseased subjects and non-diseased cases.

To evaluate specificity and sensitivity of the new 14D6 immunoassay, ROC analysis was performed on APPsα concentrations obtained from control and AD patient samples and compared to the 6E10 measurements. Normalized APPsα measurements of CSF showed ROC AUCs (area under the curve) of 0.75 (14D6) and 0.61 (6E10), proving the better performance of the 14D6 assay when compared to the 6E10 assay. In contrast, in the serum, the AUC of 6E10 assay at 0.79 scored better than the 14D6 immunoassay with AUC being 0.68 (Fig. 19).

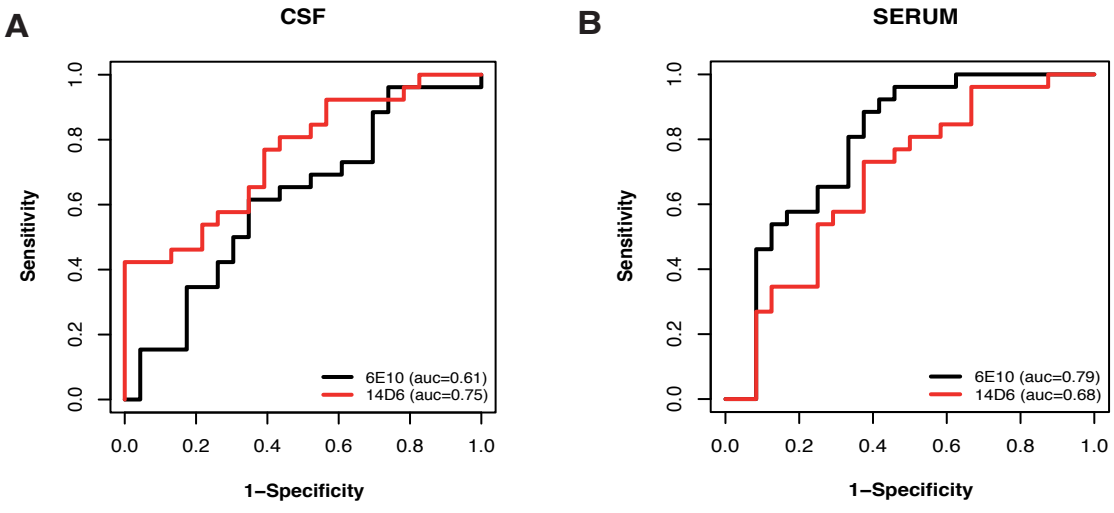

Fig. 19: Receiver-operating characteristic (ROC) curves for APPsα measurement in CSF and serum. The performance of 14D6-immunoassay in detecting APPsα is

compared with 6E10 assay. A) ROC curve for CSF APPsα. The area under the curve (AUC) for 14D6-antibody (0.75) indicates a better performance of the assay when compared to the AUC of 6E10 (0.61). B) ROC curves for serum APPsα. The 6E10 AUC (0.79) indicates a better ferformance of the assay when compared to 14D6-AUC (0.68).

4.1.9 Conclusion 1

In conclusion, a new ELISA-like immunoassay was developed for specific APPsα detection in cell culture media, human cerebrospinal fluid and serum. The assay is sensitive and specifically detects APPsα-cleaved APP, but not APPsβ´, such as other commercially available antibodies. The assay was tested in a small clinical study including Alzheimer´s disease patients and non-demented controls. A significant increase in CSF APPsα was measured in AD patients in comparison to controls, whereas a significant decrease in serum APPsα was assessed. This assay needs to be further tested in larger cohort of patients to obtain more value as potential biomarker for AD, and as putative clinical monitor for disease-modifing drugs in clinical trials.

4.2 Screening of kinases as modulators of APP-shedding and Aβ generation

4.2.1 Kinase screening set-up

APP is phosphorylated at three main sites, Thr668, Tyr 682 and Tyr687, in its cytoplasmatic tail. The main studied phosphorylation site is Thr668, which is phosphorylated by cdk5/cdc2, GSK3 and JNK (mainly in stress conditions) and it is associated to conformational changes of the cytoplasmatic region of APP. These changes may function as molecular switch, as regulation of protein-protein interaction, signaling and intracellular traffick of APP and CTFs. Therefore, kinases are involved not only in APP phosphorylation, but also in controlling APP shedding and Aβ generation.
At the moment, few kinases have been deeply characterized for their role in APP shedding and Aβ generation. Moreover, kinases may be putative drug targets, with the aim of reducing Aβ levels and tau pathology in Alzheimer´s disease.
To identify new kinases involved in APP shedding in endogenous conditions, a siRNA based screening of the human kinome in neuroblastoma SH-SY5Y cells was established. The neuroblastoma SH-SY5Y cells are commonly used as an in vitro model for neurobiology, because they provide an unlimited supply of cells of human origin with neuronal properties, and they are efficiently trasfectable with siRNA. In this study, SH-SY5Y cells expressed endogenous APP, in order to minimize the artificial environment of the experimental setting. In order to evaluate the effect of the knock-down of each kinase on APP shedding, the commercially available Multiplex ELISA-like immunoassay APPsα/APPsβ from Meso Scale Discovery was used. This assay is based on multi-array technology: in a single well of a 96-well plate, 2 different antibodies against APPsα (6E10) and APPsβ (ANGU) are spot, resulting in the simultaneous detection of both soluble APP. The signal is measured by electrochemiluminescence, which is very sensitive and allows detection of endogenous APPs.

For a successful performance of a screening, it is important to set up a robust and specific assay for the biological process being investigated. The first step in designing a successful screen is to carefully choose positive and negative controls, in order to validate the read-out assay and to evaluate the strength of the phenotype observed.
First of all, negative controls were evaluated. The non targeting siRNA controls, called CON siRNA, is a mixture of four different sequences of oligos with no complementarity

to any known transcript. It is a measure of the baseline changes on total gene expression caused by siRNA delivery in the cell system. Therefore, the siRNA transfection with this control has to give a very similar effect (no effect) to the so called "water control", named "non transfected" (Fig. 20). This non-transfected negative control is represented by seeded cells that are treated with no transfection reagent nor siRNAs. It is used to define whether the non-targeting siRNA treatment affects assay results. Indeed, the effect of non-targeting siRNA transfection on APPsα/APPsβ shedding measured with MSD-Multiplex Immunoassay was similar to the treatment of the "water control", excluding prominent off-target effects due to the siRNA treatment itself in the screening set up. The CON transfection was subsequently used as a reference for the whole screening and further validation.

In endogenous conditions APP shedding is constitutively carried out by the α-secretase ADAM10, responsible for the initiation of the non-amyloidogenic pathway, and β-secretase BACE1. Cleavage by BACE1 initiates the amyloidogenic pathway.

To evaluate the knock-down efficiency and the specificity of the signal of APPsα/APPsβ multiplex immunoassay, the secreateses ADAM10 and BACE1 were knocked-down with pools of 4 different siRNA oligos per gene (Dharmacon), and the secretion of soluble APPs was analyzed. Upon ADAM10 knock-down, APPsα shedding showed a decrease of about 30% compared to the non-tageting siRNA pool used as control, whereas APPsβ levels increased to about 140%. As BACE1 was knocked-down, APPsβ levels decreased to about 5%, together with a very slight decrease in APPsα shedding. In fact, ADAM10 knock-down lowers consistently APPsα shedding, whereas BACE1 knock-down decreased dramatically APPsβ processing, as expected (Colombo et al., 2012; Jorissen et al., 2010; Kuhn et al., 2010). These results confirm on the one hand the sensitivity of the multiplex immunoassay to detect endogenous levels of APP shedding products in SH-SY5Y cells, and on the other hand the read out for the process of interest is validated (Fig. 20).

To complete the set of the screening controls for APP shedding, APP itself was knocked down. This control serves as a measure about knock-down efficiency, to set the background noise of the read-out assay, and to further validate the screening set-up. Upon APP knock-down, the signal of both APPsα/APPsβ was close to detection limit (Fig. 20).

The expected results on APP shedding achieved by ADAM10, BACE1 and APP knock-down validated the screening set-up. These controls, together with the water and the non-targeting controls were included as quality controls in each plate of the final screening.

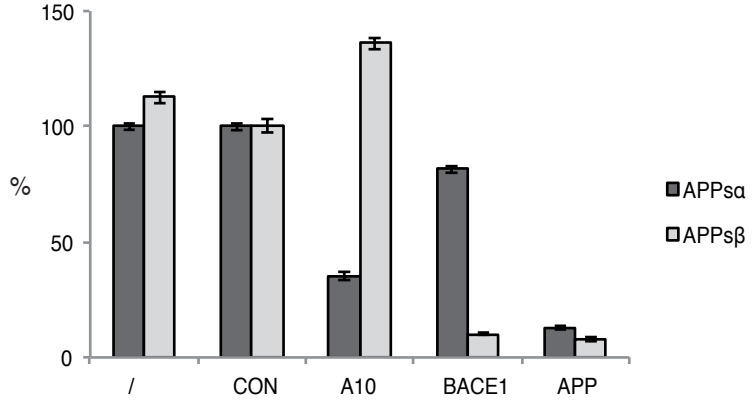

Fig. 20: Controls for the kinase screening from Dharmacon. /: negative control with no transfection reagents nor siRNA; CON: mixture of 4 non-targeting siRNA; A10: α-secretase ADAM10 knock-down with pooled siRNA. BACE1: β-secretase knock-down with pooled siRNA; APP: APP knock-down with pooled siRNA. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively. Given are standard errors of at least 6 independent experiments.

4.2.2 Kinase screening for APP shedding

RNA interference was used to selectively knock-down the human kinome in neuroblastoma cells SH-SY5Y, which expressed APP at endogenous levels. The kinase library from Dharmacon (Thermo Fisher) includes siRNA pools against 720 known and predicted kinases. The human kinome comprises 518 kinases, but the library includes also predicted kinases or kinases found only at transcriptional level. In case of multiple splicing isoforms of a specific gene, variants-specific siRNA were provided. The silencing of each kinase was achieved by reverse siRNA transfection using pooled siRNA (4 independent sequences targeting different regions of the same transcript). Variations in APP shedding were measured by Multiplex APPsα/APPsβ assay.

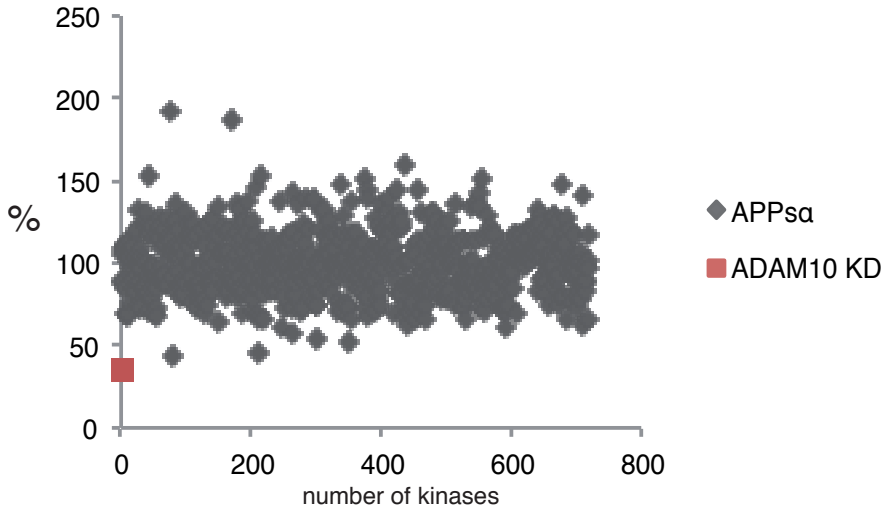

Fig 21: Screening of the human kinome for APPsα modulators. Red spot: APPsα level measured with Multiplex APPsα/APPsβ Immunoassay upon ADAM10 knock-down. Grey spots: APPsα levels measured with Multiplex APPsα/APPsβ Immunoassay upon knock-down of each single kinase of the human kinome (720 transcripts). Down-regulation was performed with 10 nM siGenome pools from Dharmacon in 2 independent experiments. APPsα: soluble amyloid precursor protein α; ADAM10 KD: ADAM10 knock-down.

ADAM10 is the constitutive α-secretase of APP. ADAM10 knock-down showed a decrease in APPsα-shedding down to 40%. Therefore, as ADAM10 is the responsible secretase for the direct APPsα cleavage, the knock-down of any kinase was not expected to decrease APPsα shedding more than ADAM10 knock-down. In fact, the knock-down of none of the 720 kinases lead to a decrease of APPsα shedding more than ADAM10 knock-down. This control was important in one hand to validate the experimental performance of each screening plate, and in the other hand to set the strength of the observed phenotype (Fig. 21).

Apparently, as shown in Fig. 21, the knock-down of many kinases could impair APPsα shedding at different degrees, with some of them showing stronger phenotypes in comparison to others. Upon systematic knock-down of 720 kinases, APPsα levels either decreased to around 50% in comparison to the control, or increased to 2-fold. APPsα values were normalized to the mean of each plate, and the mean of 2 independent experiments was calculated.

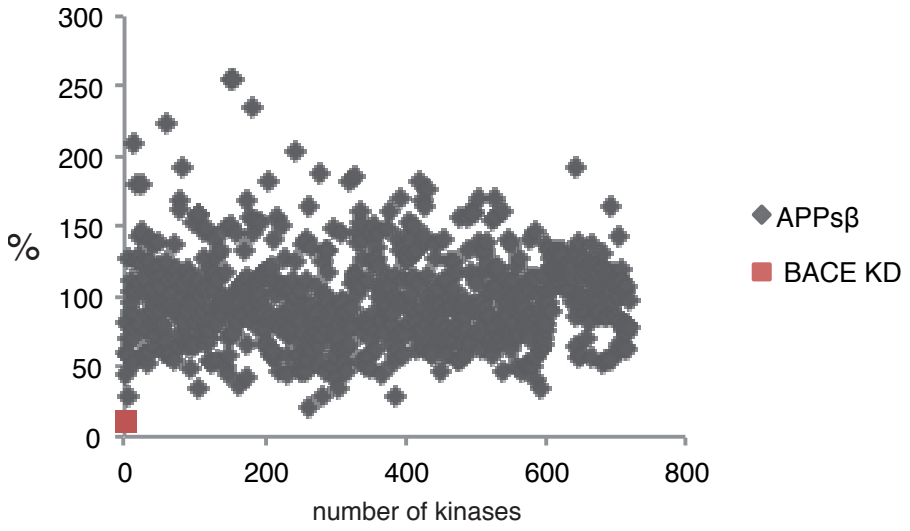

Fig. 22: Screening of the human kinome for APPsβ modulators. Red spot: APPsβ level measured with Multiplex APPsα/APPsβ Immunoassay upon BACE1 knock-down. Grey spots: APPsβ levels measured with Multiplex APPsα/APPsβ Immunoassay upon knock-down of each single kinase of the human kinome (720 transcripts). Down-regulation was performed with 10 nM siGenome pools from Dharmacon in 2 independent experiments. APPsβ: soluble amyloid precursor protein β; BACE KD: BACE knock-down.

At the same time, effects produced upon knock-down of the human kinome on APPsβ shedding were evaluated. BACE1 is the β-secretase responsible of β-cleavage of APP, and its knock-down resulted in a decrease of APPsβ shedding down to almost 10% (Fig. 22). As for APPsα, this treshould was considered to be the maximum effect expected on APPsβ shedding upon knock down of any kinase. Interestingly, knock-down of the human kinome resulted in broader impairment of APPsβ shedding in comparison to APPsα, leading to the observation that kinases are largely involved in controlling APPsβ shedding mechanisms. Upon systematic knock-down of 720 kinases, APPsβ levels either decreased to around 20% in comparison to the control, or increased to 2.6-fold. APPsβ values were normalized to the mean of each plate, and the mean of 2 independent experiments was calculated (Fig. 22).

As protein-secretion control, granulin levels were measured upon knock-down of each kinase with a specific granulin immunoassay. Granulin is a secreted protein with multiple biological roles. In this context, it was used as a marker for controlling the integrity of the secretion machine in the cells treated with siRNA, and to evaluate whether the knock-down of a specific kinase affected the overall secretion mechanism. Knock-down of the human kinome broadly affected granulin secretion, leading to either a decrease to ca. 50%, or an increase of almost 2-fold of granulin levels. For each kinase, granulin levels

were compared to the effects on APPsα/APPsβ shedding. As control for this read-out, knock-down of granulin was performed, leading to a decrease in the secretion to almost 20% (Fig. 23).

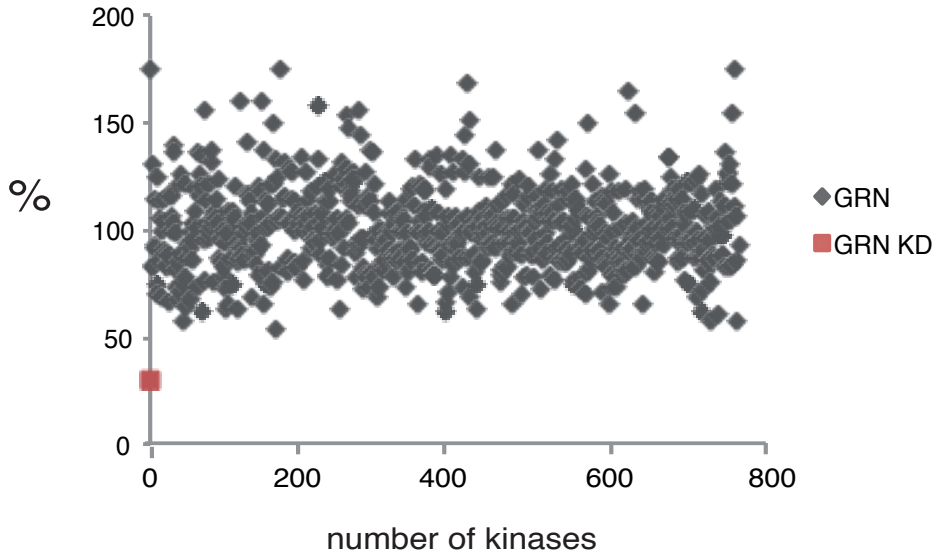

Fig. 23: Screening of the human kinome for modulators of granulin secretion. Red spot: Granulin measured with Granulin-Immunoassay upon Granulin knock-down. Grey spots: Granulin levels measured with Granulin Immunoassay upon knock-down of each sigle kinase of the human kinome (720 transcripts). Down-regulation was performed with 10 nM siGenome pools from Dharmacon in 2 independent experiments. GRN: granulin; GRN KD: granulin knock-down.

In order to control cytotoxicity of siRNA mediated knock-down, the toxicity assay Alamar Blue was employed. This assay measures the health status of the used cell line, through the metabolic activity of the respiratory chain. Viable cells continously reduce resazurin to resorufin, a cell permeable compound that, when reduced, produces red fluorescence. In the screening, 16 genes out of 720 displayed more than 20% decrease in the Alamar Blue assay. These genes were excluded from the hit selection analysis.

As further validation of the siRNA screening, kinases already known to be involved in APP processing were analysed, namely GSK3 and cdc2 as homologue of Cdk5 in cancer cells. The knock-down of GSK-3α and cdc2 led to a decrease in APPsβ levels only. These data could confirm the robustness of the approach in the identification of relevant kinases as APP shedding modulators (Fig. 24).

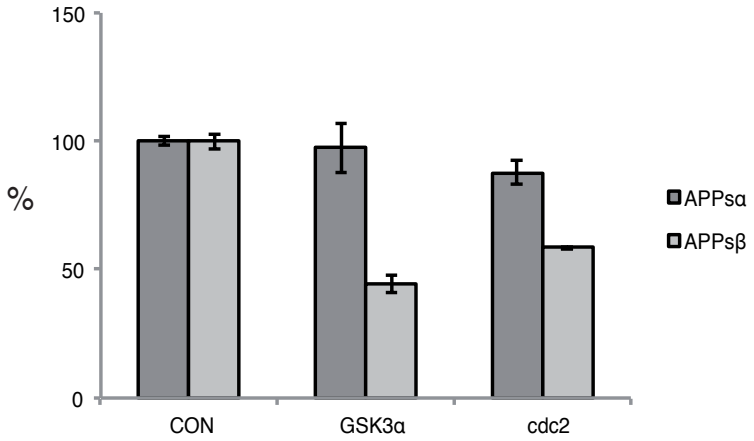

Fig. 24: Positive controls from the kinase screening. CON: non-targeting siRNA; GSK3α: Glycogen synthase kinase 3-α; cdc2: cyclin-dependent kinase 1. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively. Down-regulation was performed with 10 nM siGenome pools from Dharmacon in 2 indipendent experiments.

4.2.3 Primary validation of best scoring hits with Dharmacon siRNA pools

56 hits were selected as modulators of APP shedding. The cut-off values were assigned arbitrarily. In case of APPsα, the knock-down of 4 genes out of 720 determined an increase higher than 150% in APPsα shedding in comparison to the control (non targeting siRNA), whereas a decrease in APPsα shedding of less than 65% was detectable upon down regulation of only 1 gene (Fig. 25).

Since effects of kinases´ knock-down was stronger for APPsβ shedding, candidates impairing the amyloidogenic pathway were chosen with different cut-off values. The knock-down of 4 kinases led to an increase of APPsβ more than 190%, whereas 20 genes were selected for decreasing APPsβ shedding more than 45% (Fig. 25).

A group of hits were found to be involved in the modulation of both amyloidogenic and non- amyloidogenic pathways: down regulation of 6 genes led to an increase in both APPsα and APPsβ shedding, 20 genes led to the decrease in the levels of both solubles, and 1 of them was associated to an increase in α- and a decrease in β-shedding.

None of these 56 candidates showed changes neither in granulin levels nor in Alamar Blue viability assay.

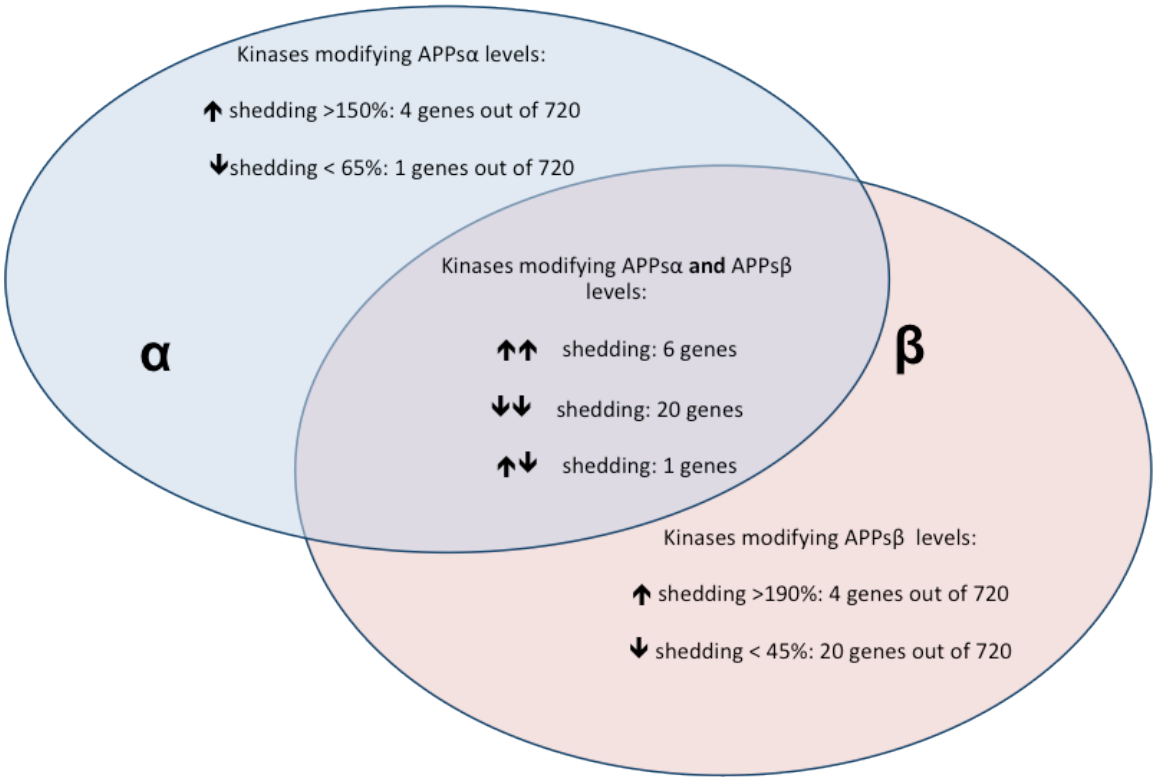

Fig. 25: 56 best scoring hits from the primary screening selected with given cut-off criteria. The blue set summarizes the number of hits where the knock-down modulated only APPsα shedding. the red set sums up hits where the knock-down modulated only APPsβ shedding, and the overlap contains hits where the knock-down modulated the shedding of both APPsα and APPsβ, not included in the other two sets. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively.

To minimize the presence of false-positive hits, these 56 candidates were re-screened in triplicate with lower concentration of siRNA pools in SH-SY5Y cells. The effects on APPsα/APPsβ shedding could be specifically reproduced for 23 out of 56 genes using 5 nM siRNA pools for reverse trasfection. The hits were categorized in 6 groups, depending on the effects of the kinase knock-down on APPsα/APPsβ shedding. Granulin secretion was measured as control. The down regulation of 8 out of 23 genes showed an increase in different degrees in both APPsα and APPsβ shedding, together with no change in granulin levels; 10 genes were correlated to decreased levels in both solubles. In addition, one gene was confirmed to increase APPsα and in the meantime to decrease APPsβ. Moreover, one gene was responsible for a decrease in only APPsα, with no change in APPsβ; another one led to an increase in APPsβ only, whereas two genes mildly lowered APPsβ levels only (Fig. 26).

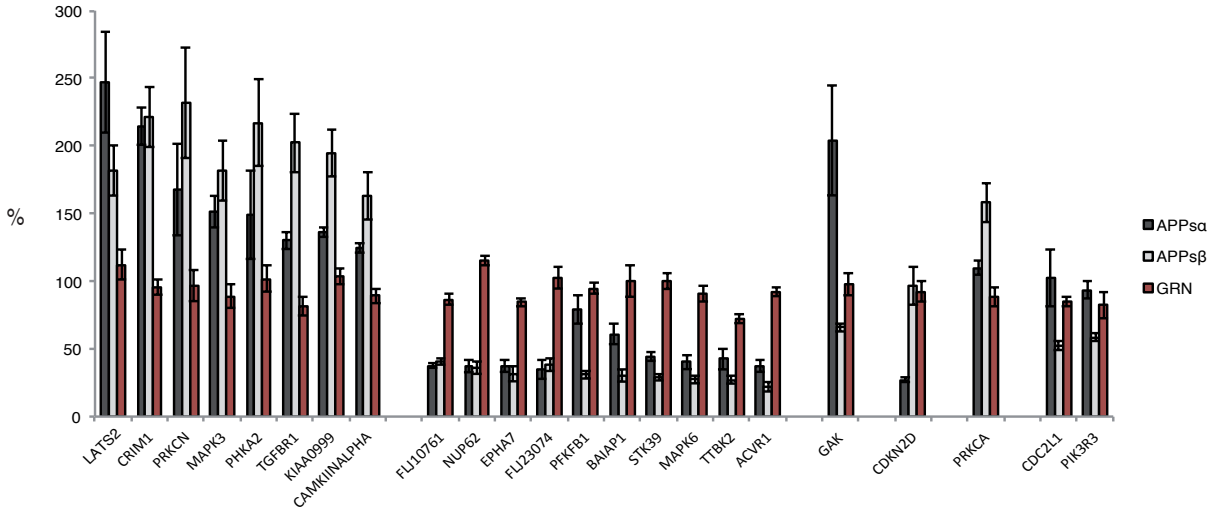

Fig. 26: Validation of best-scoring hits with 5 nM siGenome siRNA pools from Dharmacon. The effect on APP shedding was reproducible for 23 out of 56 genes. Genes were divided into effect-related groups. Granulin was measured as secretion marker. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively. GRN: Granulin. Given are standard errors of 3 independent experiments.

4.2.4 Secondary validation of best scoring hits with Qiagen siRNA pools

Afterwards, to rule out off-target effects, these hits were screened using siRNA pools from Qiagen. These siRNA pools consist in a mixture of 4 oligos for each gene, which bind to different regions of the target transcript, initiating the RNAi mediated down-regulation.

First of all, Qiagen controls for APP shedding were tested via reverse transfection in SH-SY5Y cells expressing endogenous APP at the concentration of 15 nM/pool. The effects were compared to the validated controls from Dharmacon. The strong decrease in APPsα shedding upon ADAM10 knock-down achieved with siRNA pools from both companies was associated to slightly different effects on APPsβ shedding, where its down regulation led to increased APPsβ levels using Dharmacon oligos. Upon BACE1 knock-down, the expected decrease in APPsβ shedding was observed with siRNA pools from both companies, whereas an increase in APPsα was detected only in case of down regulation with Qiagen siRNAs. A recent study from Colombo et al. showed that in differentiated SH-SY5Y cells α-and β-cleavage did not compete for APP shedding. Thus, the increase in APPsβ shedding upon ADAM10 knock-down with Dharmacon oligos and in APPsα

upon BACE1 knock-down with Qiagen siRNAs is likely to be an RNAi mediated off-target effect (Fig. 27).

APP knock down led to the depletion of both APP shedding products with siRNA pools from either Dharmacon or Qiagen.

The effect of negative control CNAS (non targeting siRNA pools from Qiagen) was comparable with the C2P (non targeting siRNA pools from Dharmacon), resulting to no change in APPsα and APPsβ levels. Thus, treatment with non-targeting siRNA did not impair the physiological enviroment concerning APP shedding (Fig. 27).

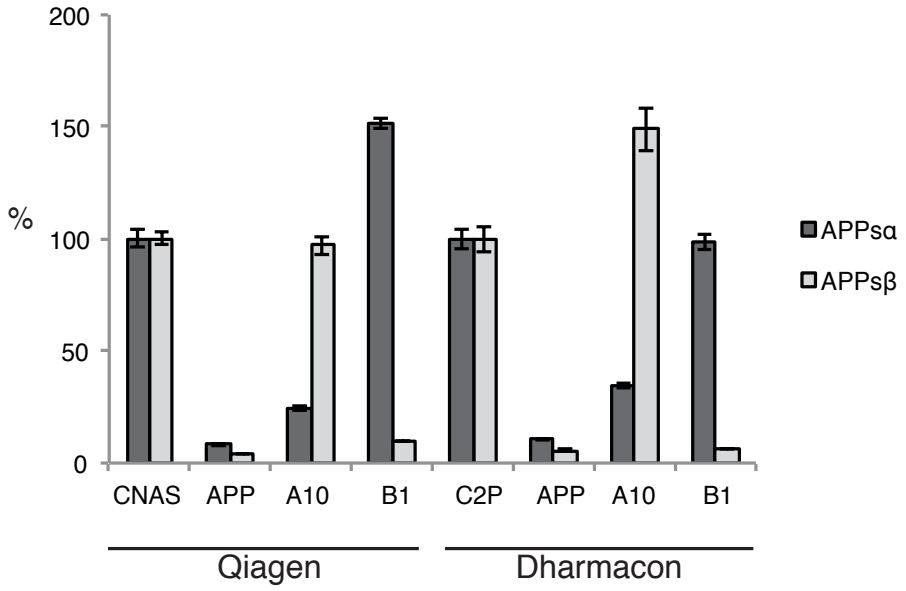

Fig. 27: Comparison between controls for the hits validation from Qiagen and Dharmacon. CNAS and C2P: mixture of 4 non-targeting siRNA; A10: α-secretase ADAM10 knock-down with pooled siRNA. B1: β-secretase BACE1 knock-down with pooled siRNA; APP: APP knock-down with pooled siRNA. Given are standard errors of at least 6 indipendent experiments. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively.

The validated controls from Qiagen were then used in the further confirmation of selected hits with Qiagen siRNA pools. The 23 selected genes were knocked down with 15 nM Qiagen siRNA pools in SH-SY5Y cells, and the effect on APPs levels was measured with Multiplex APPsα/APPsβ immunoassay. The resulting phenotypes were compared with the variations measured in the re-screen with 5 nM siRNA pools from Dharmacon.

The effects of 9 out of 23 genes were confirmed with siRNA pools from both companies: PRKCN, STK39, MAPK6, TTBK2, GAK, CDC2L1, PIK3R3, EPHA7, PFKFB1 (Fig.

28). Considering the low expression rate in SH-SY5Y cells indicated in "The human protein atlas" (www.proteinatlas.org) of EPHA7 and PFKFB1, off-target effects could have provoked the seen phenotype. For this reason, these genes were excluded from further characterization.

PRKCN knock-down showed an increase in both APP soluble levels in comparison to the control, whereas STK39, MAPK6, TTBK2 down regulation was confirmed to decrease both APPsα and APPsβ. Concerning GAK, CDC2L1 and PIK3R3, the knock-down led to decreased APPsβ levels. This effect was confirmed with siRNA pools from both companies, while APPsα levels varied depending on the siRNA used, showing that probably α-secretase pathway is more susceptible to off-target effects.

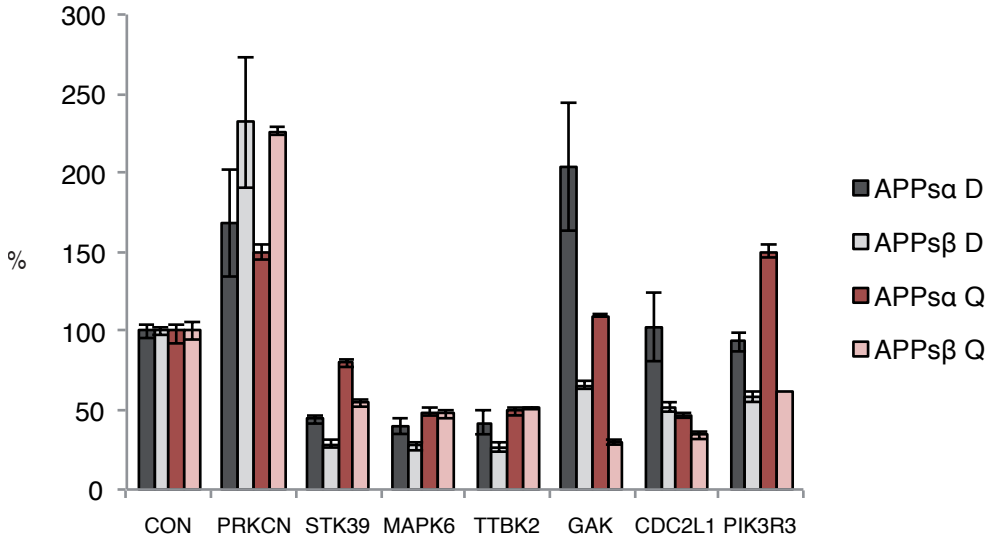

Fig. 28: Effects on APP shedding for the 7 validated kinases. Down-regulation was performed with Qiagen (Q) and Dharmacon (D) siRNA pools. APPsα, APPsβ: soluble APP cleaved by α- and β-secretase respectively. Given are standard errors of 3 independent experiments.

4.2.5 Effects on Aβ generation and APP mRNA upon knock-down of the validated hits

To better understand the impact of the knock-down of 7 validated kinases on APP metabolism, Aβ40 secretion was measured using Mutiplex Aβ Immunoassay from Meso Scale Discovery. This assay is sensitive enough to detect endogenously generated Aβ40 from SH-SH5Y cells. In the opposite, the concentration of Aβ38 and Aβ42 was too close to the lower detection limit, and therefore not included in the analysis. The knock-down

of all selected kinases but PRKCN caused a decrease in Aβ40 secretion, which is in correlation with the observed decrease in APPsβ (16).

To investigate whether the concomitant variation of APP soluble levels were caused by an upstream regulation on APP mRNA levels, qRT-PCR was performed. APP mRNA strongly increased only in case of PRKCN knock-down (Table 16) in correlation with the observed increase in both solubles (Fig. 28). The variation on APP shedding products for the other genes could not be explained by consistent changes in APP mRNA levels, leading to the conclusion that other mechanisms may be responsible for these phenotypes. Finally, the knock-down efficiency was proved for each studied gene (16).

Table 16: Aβ40 and APP mRNA levels upon knock-down of the 7 validated hits normalised to control.

Name	Aβ40		APPmRNA		KD efficiency	
	% to CON	S.E.M	% to CON	S.D.	% to CON	S.D.
PRKCN	121,3	14,0	162,0	0,0	30,6	4,8
STK39	53,3	3,1	71,6	4,4	22,5	1,2
MAPK6	52,8	0,6	89,6	11,0	25,4	4,0
TTBK2	64,0	1,8	103,1	12,9	47,0	9,9
GAK	64,9	3,3	91,4	6,7	33,8	5,6
CDC2L1	44,5	1,0	75,1	0,6	11,0	7,1
PIK3R3	49,1	4,4	104,1	16,6	27,9	12,5

These effects are correlated to knock-down efficiency. Given are standard errors (S.E.M.) or standard deviations (S.D.).

4.2.6 Validation of STK39 in mouse embryonic neurons with shRNAs

In Alzheimer´s disease pathology, an important role is given to a misregulation of Aβ production, which triggers plaque deposition and compromises synaptic functions and cognitive performance in humans. The amyloid precursor protein APP is highly expressed in neurons at an endogenous level. Within the group of the newly identified kinases in SH-SY5Y neuroblastoma cells, four of them show high expression levels in the mouse brain, according the Allen Brain Atlas (www.brain-map.org) and BioGPS (biogps.org). These kinases are MAPK6, TTBK2, STK39 and GAK.

Therefore, in order to investigate whether these newly identified kinases had a role in APP metabolism and in particular in Aβ generation in primary cortical neurons, a screening with lentivirus carrying shRNA sequences against these candidates was performed. shRNA sequences for knocking-down the selected kinases were chosen from

the RNAi consortium (www.broadinstitute.org) and cloned into pLKO-lentiviral vector (Kuhn, Wang et al. 2010). The viruses were tested for infectivity, knock-down efficiency and effect on Aβ40 secretion in murine E15/E16 isolated neurons. The best correlation between changes in Aβ secretion levels and knock-down efficiency was achieved for STK39 (Fig. 29) and shRNA "KD3" and "KD6" were selected and used for further detailed experiments. STK39 is highly expressed in neurons and has an important role in the phosphorylation of NMDA receptors at postsynaptic level (Ben-Ari, 2002).

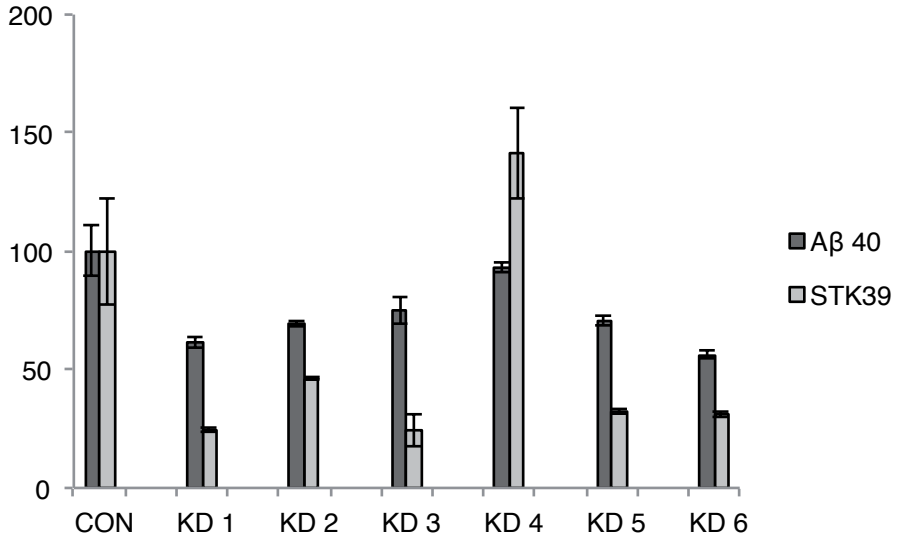

Fig. 29: Effects of STK39 knock-down in primary neurons on Aβ shedding. Down-regulation was performed with different shRNA sequences, cloned into the pLKO lentiviral vector. STK39 knock-down efficiency (KD) was assessed via qRT-PCR, and Aβ levels were measured via MSD-Multiplex ELISA. Aβ: amyloid β peptide. Given are standard errors of 3 independent experiments.

The effect of STK39 knock-down with the 2 selected shRNA (further called shRNA1, shRNA2) on APP shedding in primary cortical neurons was analysed. Both shRNA sequences targeted the coding region of STK39 transcript, but different exons. shRNA1 binds to exon 2, while shRNA2 targets exon 14. They have been selected for being very efficient in knocking-down STK39, without impairing the neuronal viability. In knock-down experiments, the down-regulation of a gene with at least 2 different shRNA sequences targeting the same transcripts minimize the risk of off-target effects in the studied pathway. The scrambled shRNA sequence was used as control, and the transduction with this virus did not show any adverse effect on the APP processing if compared to the non-trasfected (/) neurons (Fig. 30A). Therefore, unspecific effect due to viral trasfection and off-target effects caused by the scrambled sequence could be ruled

out. The supernatant was harvested 3 days after the viral infection, to allow an effective down-regulation of the kinase, and analysed for APPsα and APPsβ secretion via Western Blot, as well as Aβ40 generation using the Multiplex Aβ Immunoassay. The neurons were collected for the analysis of lysate, in order to address effects on full length APP, BACE1 and ADAM10 protein levels, and to confirm STK39 knock-down. Calnexin was used as loading control. As shown in Fig. 30, APPsα levels did not significantly change upon STK39 knock-down with both shRNA, while ADAM10 levels mildly decreased in comparison to control. Significant changes were instead detected for APPsβ, BACE1 levels and Aβ40, where STK39 knock-down resulted in lower the protein level of all these three parameters (Fig. 30 A,B,C). The decrease was stronger in case of the first shRNA, and milder for the second shRNA. This result is probably caused by the slightly more efficient knock-down achieved by the first sequence of shRNA.

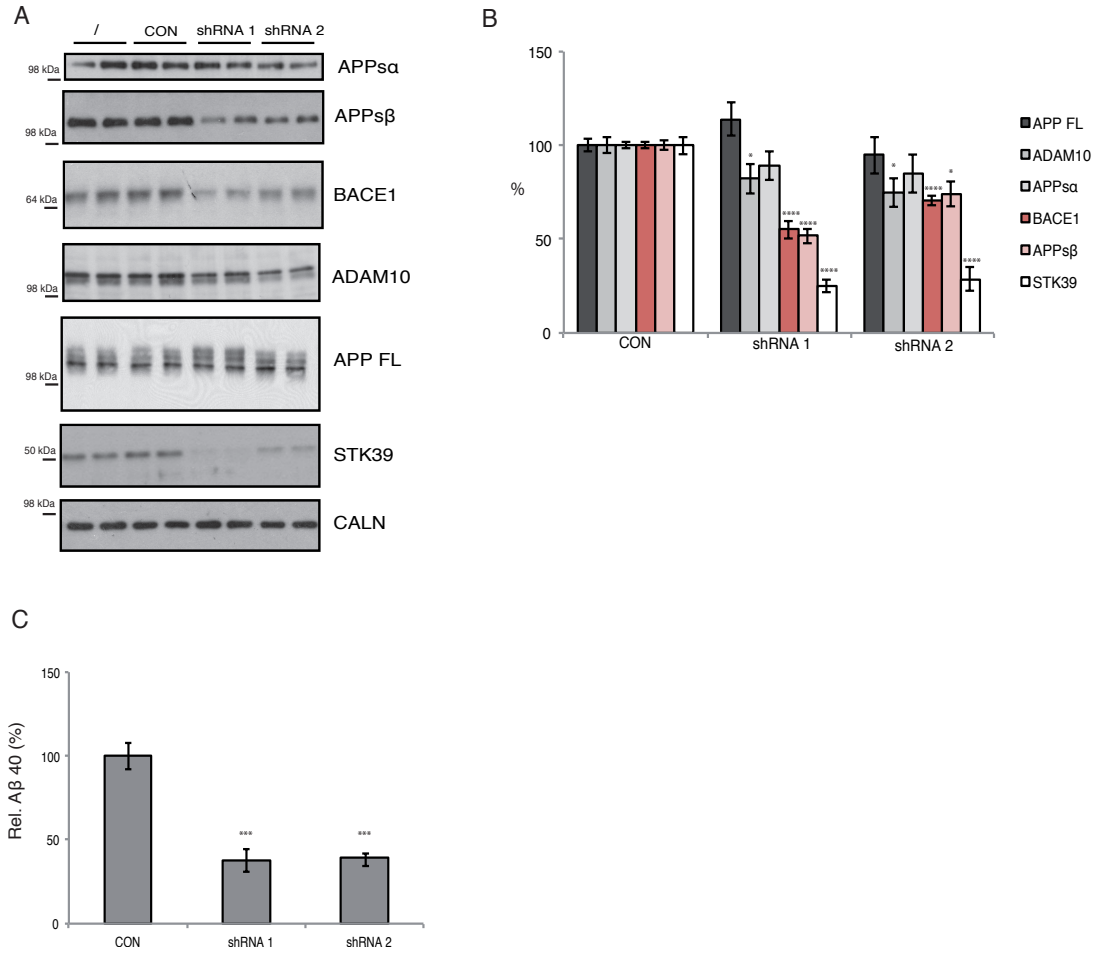

Fig. 30: STK39 knock-down with shRNAs in primary cortical neurons. A) Western Blot analysis of APPsα, APPsβ, BACE1, ADAM10, APP full length (FL), STK39 and Calnexin (Caln) protein levels upon STK39 knock-down with 2 different shRNA

(shRNA1, shRNA2). B) Quantification of A). C) Aβ40 levels measured with Multiplex Aβ Triplex Immunoassay (MSD) upon STK39 knock-down with 2 different shRNA (shRNA1, shRNA2). Given are standard errors of at least 6 indipendent experiments.

Apparently, decreased BACE1 protein levels upon STK39 knock-down subsequently impaired the β-secretase cleavage of APP, resulting in decreased in APPsβ and Aβ production.

In order to evaluate whether the decrease in BACE1 protein level had in impact also in other known BACE1 substrates, the effects of STK39 knock-down were assessed also for APLP1. This protein is reported to be a substrate very efficiently cleaved by BACE1 (Kuhn et al., 2012). STK39 knock-down resulted in a significant and reproducible decrease in APLP1 β-cleavage, whereas no significant change was detected in intracellular APLP1 of embryonic neurons (Fig. 31).

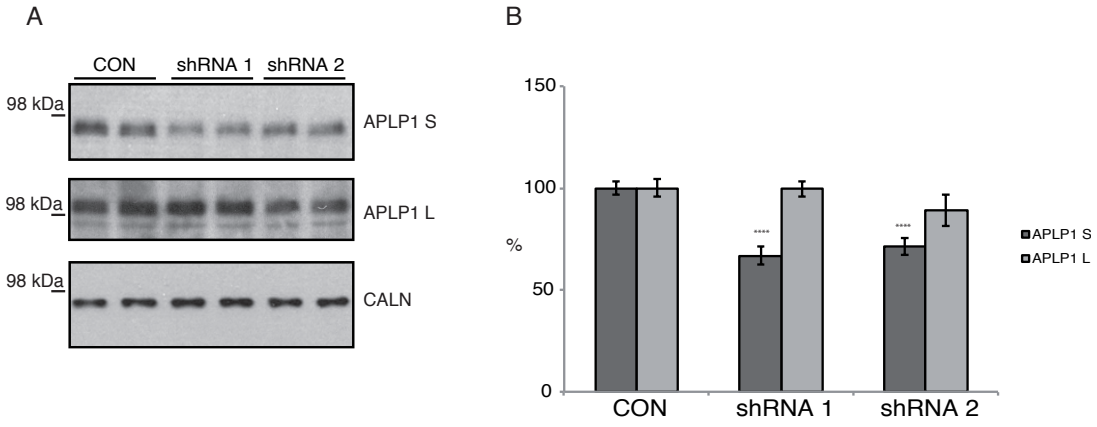

Fig. 31: STK39 knock-down with shRNAs in primary cortical neurons. A) Western Blot analysis of APLP1 protein levels upon STK39 knock-down with 2 different shRNA (shRNA 1, shRNA 2). B) Quantification of A). Given are standard errors of at least 6 independent experiments.

4.2.7 Validation of STK39 in SH-SY5Y cells with single siRNAs

Next, single siRNA oligos with different sequences from the previous pooled siRNA from Qiagen and Dharmacon were employed to reconfirm the phenotype observed in SH-SY5Y cells upon STK39 knock-down. The 2 siRNA sequences showed a decent knock down efficiency, decreasing STK39 protein levels to less than 50% (Fig. 33).

APPsα, APPsβ, and Aβ levels were measured in supernatants from transfected SH-SY5Y cells with the Multiplex APPsα/APPsβ immunoassay. Upon STK39 siRNA knock-down, the shedding of APP soluble forms significantly decreased as well as Aβ secretion (Fig. 32) According to previous experiments with SH-SY5Y cells, where STK39 was knock-down with siRNA pools, not only levels of APPsβ and Aβ40, but also APPsα decreased, probably due to either a major contribution of the α-secretase pathway in SH-SY5Y cells in comparison to neurons towards APP processing, or the susceptibility of α-secretase pathaway to off-target effects.

In the cell lysate, BACE1 levels significantly decreased upon knock-down with the second but not with the first siRNA. Intracellular APP slightly accumulated when STK39 was down-regulated with the first siRNA (Fig. 33). This effect was not seen so far, being probably a specific off-target effect of that siRNA sequence. Levels of mature and immature ADAM10 did not change in comparison to the control (Fig. 33).

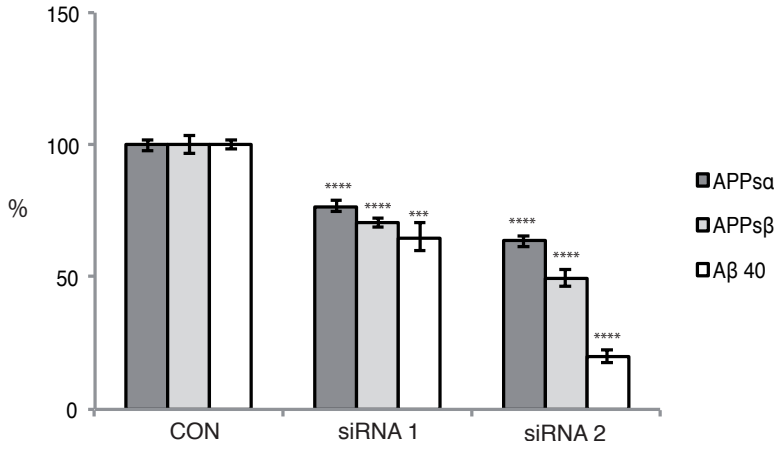

Fig. 32: STK39 knock-down with single siRNA from Qiagen. APPsα, APPsβ and Aβ40 levels were measured with respectively Multiplex APPsα/APPsβ Immunoassay and Multiplex Aβ Immunoassay (MSD). APPsα: soluble amyloid precursor protein α, APPsβ: soluble amyloid protein β; Aβ: amyloid β peptide. Given are standard errors of at least 6 independent experiments.

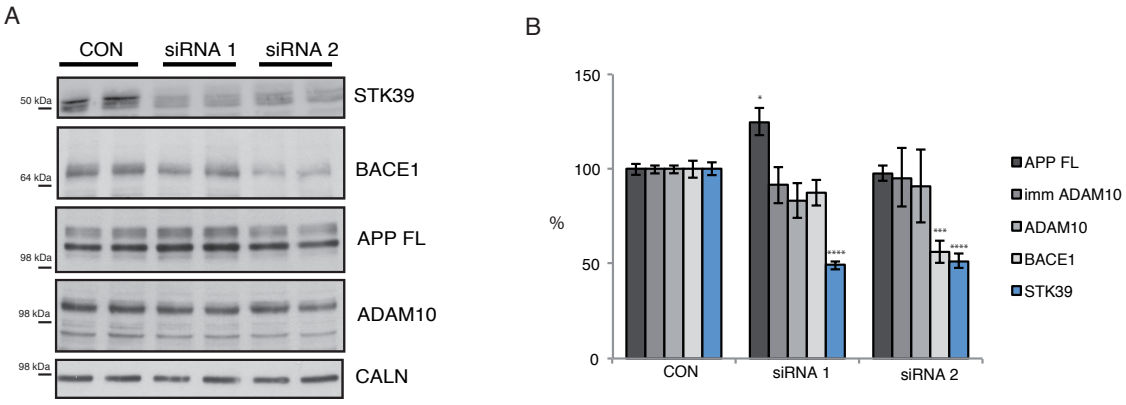

Fig. 33: STK39 knock-down with single siRNA from Qiagen. A) STK39, APP full length (FL), BACE1, ADAM10 and Calnexin (Caln) protein levels were analysed via Western Blot. B) Quantification of A). Given are standard errors of at least 6 independent experiments.

4.2.8 Conclusion 2

In conclusion, the human kinome was successfully screened using siRNA high-throughput screening technique, leading to the identification of new kinases as modulators of APP shedding and Aβ generation. Major efforts were dedicated to re-screen the most interesting candidates with different validation approaches in order to minimize false-positive hits and off-target effects.

Finally, STK39 kinase was identified as new modulator of APPsβ, Aβ and BACE1 protein level.

A detailed analysis is required to disclose the specific mechanism concerning how partial depletion of STK39 affects APPsβ, Aβ and BACE1 protein levels, and whether its trascription, translation or degradation results in lowered levels of the protein. Moreover, further investigation is needed to unveil how STK39 is involved in APP phosphorylation and in particular how it regulates the amyloidogenic pathway.

In conclusion, inhibition of STK39 could be a potential new drug target in AD.

5 DISCUSSION

In the first part of this thesis, a new sandwich immunoassay for detection of APPsα in CSF and serum was established.
In the second part of this work, a screening of the human kinome was performed to find new modulators of APP shedding and Aβ secretion. The data described in the results of this thesis will be discussed and compared with the scientific literature in the following section.

5.1 Biomarkers in Alzheimer´s disease

Alzheimer´s disease is the most common form of dementia worldwide in people 65 years of age or older. The pathological hallmarks associated with Alzheimer´s disease are the extracellular presence of β-amyloid plaques, intracellular tau tangles, neuronal loss and consequent cognitive decline (Ballard et al., 2011).
Several studies have proven the sensitivity and specificity of the combination between ELISA-like assay measurements of Aβ42, tau and phospho-tau for the diagnosis of AD (Hansson et al., 2006; Maddalena et al., 2003; Mattsson et al., 2010), together with the neuropsycological testing from the Mini Mental State battery, the NINCDS/ADRDA criteria and imaging techniques. These measurements are nowadays used to discriminate between nondemented aged subjects and AD patients with a sensitivity and specificity of above 80% (Blennow, 2004).
Nevertheless, the diagnostic performance of CSF biomarkers in distinguishing AD from other types of dementia is not yet optimal for several reasons. For example, for many years the high percentage of clinical misdiagnosis in samples used for research on AD-specific biomarkers had a negative impact in the output of these studies (Formichi et al., 2006). Specifically, AD pathology overlaps with other kinds of dementia, namely the Lewy body dementia and cerebral amyloid angiopathy or vascular dementia (Jellinger and Attems, 2008; Schneider and Bennett, 2010). Moreover, a consistent percentage of the elderly population have β- amyloid plaques and tagles without showing any symptom related to dementia (Snowdon, 1997). These reasons and the fact that the precise pathophysiological mechanisms at the basis of AD are still unknown generate basic problems such as finding a biomarker that is 100% reliable for the diagnosis of AD.

Moreover, since the pathological process of Alzheimer´s disease begins many years before the clinical manifestation, a big challenge in AD research is development of early predictive biomarkers to develop disease-modifying drugs, in order to prevent the progression of the prodromal state of dementia.

Therefore, in the first part of this thesis I focused my efforts on finding a new approach for possibly increasing the sensitivity and specificity of the AD diagnosis, at early stages of the disease and preferably in human samples that can be collected in a noninvasive manner, such as for example blood.

Aside of Aβ42, other biomarkers related to the metabolic processing of APP such as APPsα and APPsβ, which are secreted into the extracellular space in the brain, have been matter of investigation in patients with Alzheimer´s disease. Intriguingly, CSF levels of both APPsα and APPsβ in MCI and AD patients have been found to be either unaltered or moderately increased (Olsson et al., 2003; Zetterberg et al., 2008; Lewczuck et al., 2010; Rosen et al., 2012; Gabelle et al., 2000). In particular APPsα is considered an interesting biomarker for following new treatment studies. An increase in α-cleavage processing of APP is believed to shift the amyloidogenic pathway to the non-amyloidogenic pathway (Armstrong et al., 2005; Tippmann et al., 2009).

APPsα levels have been mostly investigated in CSF and little in blood. In particular, research studies reported conflicting results concerning changes in the levels of this fragment, finding an increase or no change between controls and AD patients. Moreover, a study (Wu et al., 2012) recently reported and increase in APPsα in plasma from AD patients, while another one (Colciaghi et al., 2004), described a reduction of APPsα and ADAM10 activity in platelets from AD patients. The discrepancy in these studies may be the result of the antibodies used for the detection of APPsα.

5.2 The new 14D6-immunoassay for specific detection of APPsα in cell culture, CSF and serum

Generally, most of the studies detected APPsα with antibody 6E10, which does not exclusively detect the product of α-secretase cleavage, but also the β-secretase alternative cleavage at β´ position (see Fig. 9A,B). This proteolytic activity of BACE1 results in the secretion of APPsβ´ fragment, which is thought to contribute to almost 25% of the total APP shedding (Zhou et al., 2011). Moreover, BACE1 activity and expression is found to increase in some patients with AD (Hebert et al., 2008). To overcome this problem, the

new antibody 14D6, which specifically detects APPsα, but not APPsβ´, was generated in our lab (Fig. 9A,B). This antibody was used for establishing a new sandwich immunoassay, which was proved to specifically detect the α-secretase product of APP both in CSF and serum (Fig. 12,13,14,15). Subsequently, 14D6 immunoassay was tested in a group of AD patients versus non-demented controls, in order to identify changes in the level of APPsα in CSF and serum. The new assay demonstrated higher sensitivity in CSF (Fig. 17, 19) in the detection of APPsα in comparison to the commercially available 6E10 immunoassay. In particular, CSF levels of APPsα were significantly higher in AD patients in comparison to the controls (Fig. 17), but the overlap between the two groups did not allow a full separation of the healthy and diseased condition, as it is reported in other studies using the 6E10 antibody (Gabelle et al., 2010; Lewczuk et al., 2010; Perneczky et al., 2011; Selnes et al., 2010). For this reason, APPsα measurement alone may not reach the value of a single biomarker for the diagnosis of Alzheimer´s disease. However, since other studies showed either no change or even a decrease in APPsα levels in CSF from AD patients (Fellgiebel et al., 2009; Lannfelt et al., 1995; Sennvik et al., 2000), the general conclusion of the overall picture is that there is no dramatic change in APPsα in CSF of AD patients. Therefore, a very sensitive method such as the 14D6-immunoassay is needed to pick fluctuation of this APP metabolic product. This concept can be also translated further to better understand the molecular basis of the onset of AD. In fact, it was believed that enhanced β-secretase cleavage of APP is coupled to decreased processing of APP through the α-secretase pathway. Recently, it has been shown that reduced α-secretase activity does not automatically result in an increased β-secretase cleavage, in cell culture experiments and murine embryonic neurons (Colombo et al., 2012; Jorissen et al., 2010).

The Mini Mental State of the majority of AD patients included into the study is above 23, targeting the patients´group to the very mild demented subjects. The new 14D6 assay significantly detects an increase in APPsα levels in AD patients, even if they are affected from mild AD dementia. For this reason, the new assay may be further tested as an additional tool for both pre-diagnosis of Alzheimer´s disease and also for monitoring effects on APP processing of treatments with disease-modyfing drugs in CSF and serum. Additionally, new drugs are typically tested in cell culture models, to monitor effects of new compounds in vitro, preferentially under endogenous APP expression to prevent artificial regulation of APP trafficking and metabolism. 14D6 assay can also be useful in this context, being sensitive enough to specifically detect endogenous APPsα levels in

cell culture models (Fig. 10). For example, acitretin is an approved drug currently in clinical trials for treatment of psoriasis, and it has recently been discovered to be an activator of ADAM10 expression, thereby increasing APPsα cleavage. Through stimulation of ADAM10 promoter activity, this drug promotes an increase of the mature form of ADAM10, enhancing the non-amyloidogenic pathway in cell culture and APP transgenic mice (Tippmann et al., 2009). Other AD modifying drugs acting as inhibitors of β-secretase activity can be tested in the same manner, suppressing BACE1 expression (i.e. PPARγ agonists) or inhibiting its proteolytic activity (i.e. MK-8931 from Merk, being actually in phase III clinical trial or LY2811376 from Ely Lilly) (May et al., 2011; Stamford and Strickland, 2013). In humans, BACE1 inhibition results not only in reduced Aβ and APPsβ generation, but also in a shift from the amyloidogenic to the non-amyloidogenic pathway, increasing two- to three-fold the levels of APPsα shedding (May et al., 2011; Wu et al., 2012). In this case, the compensatory mechanisms at the basis of the enhanced APPsα shedding may be used as indirect read-out for the inhibitory treatment of BACE1. Moreover, being serum an easily accessible body fluid, the new 14D6-immunoassay is also suitable for monitoring response to treatment in a less-invasive and broader manner.

Interestingly, 14D6-immunoassay performed better than the commercially available 6E10 antibody in detecting changes of APPsα levels in AD patients in the CSF. For this reason, this antibody should be preferred on the other one for future α-secretase processing of APP in clinical studies.

In contrast, the new 14D6 immunoassay did not show a better performance in serum in comparison to the 6E10, but it allows significant and specific detection of APPsα also in this blood fraction. Probably, as β-secretase activity is very low in blood, the interference of APPsβ´ while measuring APPsα concentration with the 6E10 antibody is minimal (Decourt and Sabbagh, 2011; Vassar et al., 1999). Therefore, the 14D6 immunoassay is not preferable over the 6E10 assay for detecting APPsα in serum. APPsα levels were found to significantly decrease in serum from patients with AD in comparison to non-demented controls with both assays. This finding is consistent with low levels of ADAM10 in platelets in AD patients reported by another research group (Colciaghi et al., 2004). As in the case of the CSF, contradictory results concerning APPsα levels in blood fraction were reported. In fact, a recent study reported mildly increased levels of APPsα in the plasma of AD patients (Wu et al., 2012). This difference can be caused by the use of different sources of blood biomarkers.

In conclusion, a new immunoassay for the specific detection of APPsα in cell culture experiments, CSF and serum has been established and tested in a small clinical cohort of Alzheimer´s disease patients with very mild dementia. Further confirmation of the value of this assay will be provided with larger number of patients, ideally sub-divided into differential stages of AD. Ideally, this assay could also be used as a tool to monitor the effect of new drugs on α-secretase stimulation or BACE1 inhibition, in cell culture experiments under endogenous APP expression levels and later on in clinical trials.

5.3 Screening of the human kinome in SHSY5Y cells

RNA interference has become a broadly used technique to study gene function and to identify new components on already known pathways. The power of this technique is attributable to the efficient silencing of single genes (and sometimes even specific isoforms) using the physiological mechanism to repress gene expression in the cellular system. Nowadays, RNA interference is used for high-throughput screenings in different cell lines and for various purposes. For this reason, this tool was used in this thesis to better understand how APP shedding can be modulated by changes in the kinase activity. So far, only a small number of kinases have been studied in the context of Alzheimer´s disease.

First of all, the activity of many kinases is reported to increase in Alzheimer´s disease brain. For example, increased phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) have been described in AD, in association with amyloid plaques pathology and tau tangles (Hensley et al., 1999). Likewise, the activity of cdk5 was reported to be higher in post mortem AD brains (Lee et al., 1999), and its protein levels are elevated in brains from mild cognitive impairment (MCI) patients (Sultana and Butterfield, 2007). Moreover, the expression level of casein kinase 1 (CK1) has been shown to be up-regulated in brains of AD patients (Yasojima et al., 2000). Another very well studied kinase in Alzheimer´s disease context, GSK3, is up-regulated at different levels: GSK3 expression was increased in the hippocampal area of AD patients, as well as an increased activity of GSK3 was found in the frontal cortex of AD patients, due to hyperphosphorylation of Tyr216 (Leroy et al., 2007).

Second, kinases can have a role in the onset of Alzheimer´s disease interfering with different pathways: they can control APP processing, tau phosphorylation, neurotoxicity and neuroinflammation. In this thesis, we decided to focus on finding new kinases that

modulate APP processing. Kinases are described to play a role in the phosphorylation of the cytoplasmic tail of APP at position 688, and at the YENPTY motif, which represents not only a very important internalisation motif in the APP sequence, but also a regulated docking site for several interaction proteins involved in APP trafficking and cellular signaling (da Cruz e Silva and da Cruz e Silva, 2003). The main phosphorylated amino acid within APP cytoplasmic sequence is the Thr668 (numbering from the APP695 isoform). Cdk5 and GSK-3β constitutively phosphorylate APP at this residue in neurons, whereas JNK phosphorylates it only under stress conditions (Kimberly et al., 2005; Liu et al., 2003; Taru et al., 2002).

The goal of screening the human kinome was to find new modulators of the amyloidogenic and non-amyloidogenic pathways, which are at the basis of Aβ generation and dysregulation of APP metabolism. The complete human kinome was published in 2002 by Manning and colleagues (Manning et al., 2002), and it described 518 protein kinases. For our screening, the kinase library from Dharmacon (Thermo Scientific) was used. The library is composed of siRNA pools, which can efficiently knock down single genes. The siRNA-library is designed to target 720 known and putative kinases. In order to develop a robust and specific assay for the screening, several parameters were evaluated and tested. First of all, the neuroblastoma cell line SH-SY5Y was chosen for screening the human kinome in a 96-well-plate format. Since the final aim of the project was to find modulators of APP shedding in the neuronal context and to be able to translate the study into Alzheimer´s disease molecular background, this cell model was preferred because they express neuronal features. Additionally, this cell line is particularly suitable for reverse transfection, which delivers siRNAs into the cytoplasm of the cell with the help of transfection reagents. To specifically identify changes in APP solubles levels, namely APPsα and APPsβ, the Multiplex APPsα/APPsβ Immunoassay from Meso Scale Discovery was employed. This assay detects APP solubles simultaneously and in a very sensitive manner in the same well of a 96-well plate. This immunoassay is sensitive enough to detect the endogenous secretion of the soluble APP fragments from SH-SY5Y cells. This was particulary remarkable, because it allowed to screen in minimal artificial conditions. In opposite, other studies were able to perform screening for APP processing only upon APP overexpression (Bali et al., 2012; Majercak et al., 2006). To control for general toxic effects of siRNA treatment, the toxicity assay Alamar Blue was used. It has been reported that high concentration of siRNA can increase the rate of toxicity (Birmingham et al., 2006). To lower the risk to induce

toxicity, a concentration of 10 nM/siRNA pool was used. Each siRNA transfection was performed in duplicate, to increase the confidence of finding positive hits and to avoid high false negative rate (Boutros and Ahringer, 2008).

Positive and negative controls were selected for testing the specificity of the read-out, the efficiency of the reverse transfection, and the expected strength of changes in the biological process of interest. Knock-down of the secretases primarly involved in APP shedding, namely the α-secretase ADAM10 and the β-secretase BACE1, together with the knock-down of their substrate APP, was performed. ADAM10 knock-down showed a decrease in APPsα shedding, whereas BACE1 down-regulation resulted in lower levels of APPsβ secretion, confirming the specificity of the signal detected with the Multiplex immunoassay. Additionally, the signal of both APP fragments was depleted upon APP knockdown. The non-targeting siRNA pool did not display any effect on APP shedding nor major toxicity in comparison to the control with non-treated cells, further confirming the reliability of the experimental set up (Fig. 20, 27) (Boutros and Ahringer, 2008).

The screening of 720 kinases revealed diverse effects on α- and β- secretase pathways: the knock-down of the human kinome seems to have a major role in modulating APPsβ shedding, whereas the down-regulation of the kinases had a minor role in the regulation of APPsα shedding, because the number of hits identified for APPsβ was higher in comparison to APPsα (Fig. 21, 22, 25). None of the genes was found to decrease APPsβ more than BACE1 KD (Fig. 22). Interestingly, the down-regulation of the human kinome had a broad effect also in the secretion of granulin, which was chosen as a marker for controlling the general secretion machinery of the cell (Fig. 23).

Afterwards, the data from the primary screening were normalized to allow comparison and combination of data from various plates (Birmingham et al., 2009) and the cut-offs for APPsα-and APPsβ were set: 4 out of 720 genes increase APPsα levels above 150 and 1 of them decreased APPsα shedding below 65%; the knock-down of 4 out of 720 genes resulted in an increase of APPsβ levels above 190%, and for 20 of them a decreased below 45% was detected. Down regulation of 6 out of 720 genes increase the shedding of both fragments, whereas 20 of them displayed a role in decreasing their cleavage. The knock down of 1 gene of the whole kinase library had a differential effect on APPsα and APPsβ shedding. Since the down regulation of the human kinome had different effects towards the amyloidogenic and non-amyloidogenic pathway, it was not possible to set a common cut-off strategy. None of the included genes displayed a toxicity of more than 20% in comparison to the control. In total, the screening of the human kinome for new

modulators of APP shedding revealed 56 genes capable of modifying the shedding of APPsα and/or APPsβ, without affecting cell viability.

As further validation of the siRNA screening, kinases already known to be involved in APP processing were analysed, namely GSK3 and cdc2 as homologue of cdk5 in cancer cells. Knock-down of GSK-3α and cdc2 led to a decrease in APPsβ levels only. These data could confirm the robustness of the approach in the identification of relevant kinases as APP shedding modulators.

5.4 Validation of best-scoring hits from the kinase screening

As described in the introduction, siRNA technique has major advantages to study the physiological function of specific genes within a pathway. Nevertheless, a successful validation strategy is necessary to avoid the high rate of false positive hits due to off-target effects (Sigoillot and King, 2011). Off target effects may be non-sequence and sequence specific. Non-sequence specific off-target effects can arise either when siRNA/shRNA treatment disturbs the endogenous RNAi machine, which is involved in the maintenance of the cell homeostasis by an accurate regulation of gene translation, or when the treatment induces stress-related response in the cell (Persengiev et al., 2004; Rao et al., 2009a; Semizarov et al., 2003) (Khan et al., 2009). The sequence specific off-target effects originate when a siRNA sequence is responsible for the down-regulation of others than the target mRNA (Jackson and Linsley, 2010; Scacheri et al., 2004). siRNA, shRNA and miRNA may target several transcripts with a non-specific basepair match and downregulate the expression of unwanted genes. The probability of unspecific sequence match is higher if the concentration of siRNA is elevated (Sigoillot and King, 2011).

Here, the strategy adopted to minimize the risk of sequence-related off target effects was to use several siRNA oligos targeting the same transcript at different regions for each gene, and correlate phenotype and knock-down efficiency (Mohr et al., 2010; Sigoillot and King, 2011). In particular, only hits showing the same phenotype upon transfection of siRNA pools in SH-SY5Y cells with different sequences were considered as validated, namely PRKCN, STK39, MAPK6, TTBK2, GAK, CDC2L1, PIK3R3. Low concentration of siRNA pools implicates even lower concentration of single siRNA oligos, leading to lower probability of inducing sequence-related off-target effect. (Rao et

al., 2009a) Afterwards, small concentrations of single siRNA sequences were correlated to the knock-down efficiency concerning STK39.

Moreover, shRNAs are believed to promote less off-target effects than siRNA sequences, maily because they are endogenously transcribed and processed in a very low but constant manner (Klinghoffer et al., 2010; Rao et al., 2009a). In fact, the plasmid containing the shRNA is immediately transferred into the nucleus by the lentivirus, where shRNA molecules are synthesized in a hairpin like stem-loop, which is processed into individual shRNAs with 2 nucleotides at the 3´overhang. After several modifications, mature shRNAs are finally transported into the cytoplasm where they are processed by the RISC complex into shorter molecules of anti-sense oligos (Rao et al., 2009a). shRNA mediated knock down allows to achive higher level of repression efficiency, as the plasmid is constantly transcribed into the nucleus via RNA polymerase II, and it has a longer life in comparison to synthetic molecules of siRNA. For this reason, phenotypes of neuronal expressed genes were validated also using shRNA sequences. The selection of a neuronal cell line, namely SH-SY5Y cells, facilitates the research of neuronal expressed kinases within the group of the 7 genes validated with 2 sequence-indipendent pools of siRNA. In order to investigate the expression profile of the validated genes in murine brain and neurons, educated search in literature, BioGPS and The Brain Atlas was performed. Finally, 4 out of 9 genes were consistently described to be highly expressed in neurons: MAPK6, TTBK2, STK39, GAK. The shRNA mediated-knock down screening performed with lentiviruses in murine embryonic neurons revealed that STK39 exhibited the strongest correlation between Aβ phenotype and knock-down efficiency. The correlation between knock-down efficiency and phenotype is one of the validation strategies to confirm the consistency of the phenotype. In contrast, together with sequence specific off-target effects, shRNA can also stimulate the immune response of the cell against the virus used for the delivery of the plasmid. Moreover, a higher viral titer could cause toxicity or unspecific modulation of APP processing. These effects were excluded through the transduction with a plasmid containing a scrabled shRNA sequence and the not –treated control. When two or more sequence-independent shRNA diplay a strong correlation between knock-down and phenotype, the probability that the selected hit is a false positive is lower.

5.5 STK39 knock-down lowers APPsβ, Aβ40 and BACE1 protein levels in primary neurons and neuroblastoma cells

STK39 knock-down with 2 sequence-independent shRNAs reduced endogenous APPsβ shedding, Aβ40 secretion and lowered BACE1 protein level, without dramatically affecting APPsα and ADAM10 levels (Fig. 30). In contrast, STK39 siRNA mediated knock-down with both pools or single siRNA oligos at different concentrations (Fig. 26, 28, 31) showed a decrease in APPsα shedding, as well as lower levels of APPsβ and Aβ40. This discrepancy between the effect of STK39 knock-down in neuroblastoma cells and neurons concerning APPsα cleavage can be interpreted from 2 different sides: it can be caused in one hand by siRNA mediated off-target effects, and in the second hand because of the different expression profile of ADAM10 and BACE1 in tumor cells and neurons. In the first case, miRNA off-target effects produced from siRNA oligos has been recently decribed in details. In a study from 2003 (Jackson et al., 2003) a series of siRNAs were specifically designed to target MAPK14 and IGF1R. The authors evaluated the effects of each single anti-sense sequence through transcriptional profiling analysis, and they could correlate different patterns of effects on transcription to each siRNA. The expression of other genes than the target of siRNA sequence was modulated in comparison to the control, also at very small concentration of siRNA (4 nM). Another similar study drawing the same conclusions was performed knocking down 12 genes with different siRNA, but with higher siRNA concentration (Birmingham et al., 2006). Concerning the different expression pattern of ADAM10 and BACE1 in different cell lines, BACE1 expression is high in neurons but not in cells from peripheral tissue (Vassar et al., 1999), whereas ADAM10 expression is prominent in SH-SY5Y cells. STK39 knock-down showed a highly significant reduction in APPsα shedding only in SH-SY5Y cells, independently upon down regulation with 2 single siRNA oligos or two different pools (Fig. 26, 28, 32). In neurons, STK39 knock-down lowered APPsβ, Aβ and BACE1 levels, without having a major effect on APPsα processing. A recent study investigated the contribution of α- and β-secretases in APP shedding in murine embryonic neurons: coupling between the activity of ADAM10 and BACE1 for APP cleavage was demonstrated (Colombo et al., 2012). Inhibition of β-secretase activity resulted in increased ADAM10 cleavage of APP, in contrast to the observed effect of STK39 knock-down in this work. However, APP clevage is very much dependent on the availability of the substrate and on the compartimentalization of mature APP. It is reported that only a little amount of mature APP reaches the plasma membrane, where α-

secretase cleavage occurs, whereas the majority of APP is localized into the Golgi apparatus and the trans-Golgi network, where it is mostly cleaved by BACE1 (Haass et al., 2012). Probably, upon STK39 knock-down, trafficking of either APP or BACE1 is modified, and thereby α-secretase cleavage of APP does not increase.

Moreover, APP intracellular domain can be phosphorylated at different residues, modulating APP processing through interaction with other proteins as well as CTFs localization. The further deals with APP internalization motif, YENPTY, which is implicated in APP internalization and regulation of Aβ production. A number of cytosolic adaptor proteins have been described to bind APP internalization motif, including Fe65, Fe65L1 and 2, Mint1 (X11α), 2 and 3, Dab1, sortin nexin 17, and c-Jun amino-terminal kinase-interacting protein family members (Haass et al., 2012). The sequence of these APP binding proteins presents phosphotyrosine-binding domains. For example, lower Aβ levels and amyloid plaque deposition were detected when Mint 1, Mint 2 or Fe65 were overexpressed in APP transgenic mice. In particular Mint proteins bind also ADP-ribosylation factors, which are involved in APP vesicular trafficking (Haass et al., 2012). The latter involves phosphorylation of specific residues at CTFs level, which define the destiny of CTFs processing, such as α- rather than β-cleavage, and APP subcellular localization. In particular, APP phosphorylation at either Tyr682 or Tyr687 can drive APP and/or the CTFs to the amyloidogenic or the non-amyloidogenic pathway. For example, a recent study described phosphorylation at Tyr687 as important for α- and γ-secretase processing, leading to an increase in α-CTF generation (Takahashi et al., 2008). In contrast, phosphorylation at Thr668 seems instead to enhance APP processing by β-secretase, increasing Aβ secretion (Lee et al., 2003). Moreover, it is not yet clear how phosphorylation at Thr668 influences Fe65 binding to APP. In some studies, Fe65 is reported to interact with the internalization motif YENPTY of APP and to regulate APP processing, decreasing APP endocytosis and Golgi and endosomal localisation APP (Nakaya et al., 2008; Nakaya and Suzuki, 2006; Santiard-Baron et al., 2005). In opposite, others describe that APP phosphorylation at residue Thr-668 introduces a conformational change in APP structure, so that binding of Fe65 is prevented, increasing the availability of APP for β and γ-secretase cleavage (Nakaya et al., 2008; Nakaya and Suzuki, 2006). Additionally, phosphorylation at Tyr682 plays an important role in commiting APP to β-secretase processing by increasing the production of βCTF. This mechanism seems to be mediated by Shc/Grb2/APP interaction (Russo et al., 2002). Therefore, downregulation of the newly identified kinase STK39 may inhibit

the phosphorylation of APP residues important for the commitment to the β-secretase cleavage, namely Tyr682 and Thr668, and thereby resulting in decrease APPsβ and Aβ40 secretion. Alternatively, MAPK are described to be activated by Shc/Grb2/APP complex (Russo et al., 2002; Russo et al., 2005). Partial inhibition of MAPK signaling upon STK39 knock-down may potentially lead to a downregulation of BACE1 transcription levels, resulting in less protein levels. For example, increased mRNA levels of BACE1 have been correlated with overexpression of p25, which is the activator of cdk5 in mice. The p25/cdk5 complex phosphorylates the transcription factor STAT3, which binds to BACE1 promoter, increasing BACE1 mRNA levels (Wen et al., 2008). Two independent studies showed that STK39 overexpression in vitro resulted in specific activation of p38-MAPK, but not JNK or ERK (Johnston et al., 2000; Yan et al., 2009). STK39 mRNA is also up-regulated in inflammatory conditions such as under stimulation of cancer cells with interferon and TNF-α ((Yan et al., 2007)). This finding further underlines that a down regulation of STK39 may be beneficial in neurodegenerative diseases that have inflammatory features like Alzheimer´s disease.

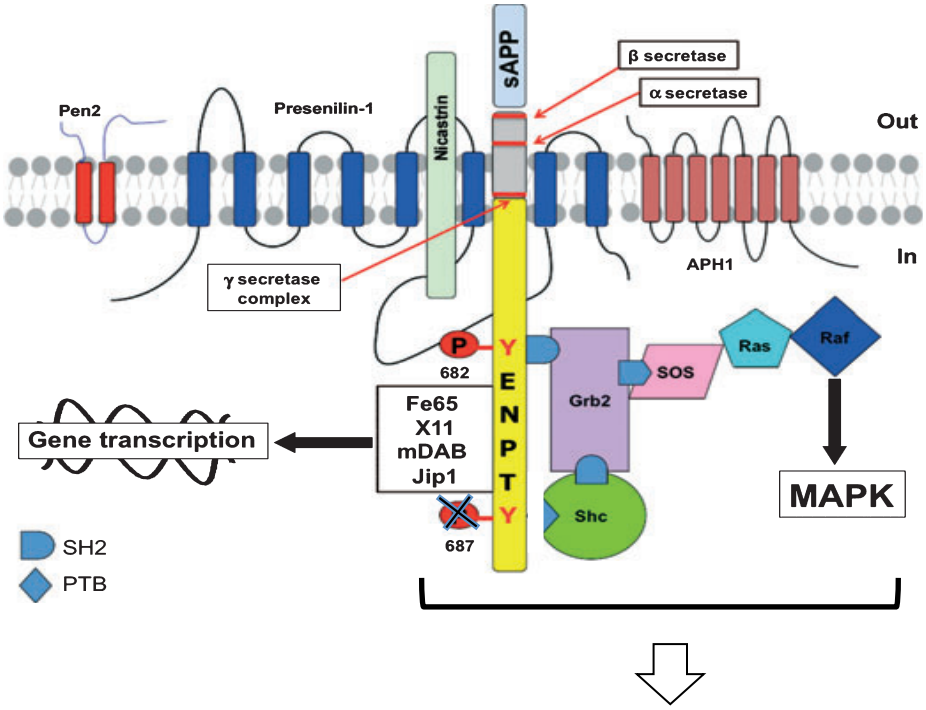

Fig. 34: Schematic representation of APP intracellular domain and kinases interactions with APP phosphorylation sites. YENPTY is the interaction motif, adaptor proteins are named in the white box. Tyr682 phosphorylation can favour the binding of Shc and/or Grb2 adaptor proteins, leading to the activation of MAPK pathway,

and promoting the commitment of βCTFs to amyloidogenic pathway (modified from Schettini et al., 2010).

Interestingly, whereas αCTFs are reported to be not phosphorylated in AD brains, βCTFs are instead phosphorylated (Russo et al., 2002; Russo et al., 2001). In particular, APP phosphorylation at Thr668 is up-regulated in hippocampi of AD patients, exhibiting colocalization with enlarged endosomes, where BACE1 is mostly active. Moreover, this specific APP phosphorylation has been found within dystrophic neurites around β-amyloid plaques, and in association with phosphorylated tau. These findings have been confirmed also in cortical neurons, where Thr668-phosphorylated APP colocalised with Rab5-positive endocytic compartments (Lee et al., 2003).
Apparently, STK39 knock-down lowers the levels of BACE1 protein in neurons and in SH-SY5Y cells. As well as its probable role in BACE transcription, STK39 can also influence the degradation of BACE1, thereby controlling the intracellular protein levels. The C-terminus of BACE1 is characterized by a di-leucine motif DISLL (residues 496-500), which is necessary to target the protein to the lysosomal pathway, where it undergoes degradation (Koh et al., 2005). Interestingly, the activity of the di-leucin motif LL is regulated by the phosphorylation of the adjacent serine residue S498, which controls BACE recycling from early endosomes to the TGN and the cell surface (von Arnim et al., 2004). STK39 is a serine/threonine kinase, and therefore a direct role in controlling BACE degradation can also be invisaged.

5.6 STK39 is linked to neurological diseases

STK39 is a serine/threonine kinase, member of the STE20 family of mitogen-activated protein kinase-like protein kinases (Gagnon and Delpire, 2012). STK39 is widely expressed, with higher expression levels in brain, whereas its close homolog OSR1 is barely expressed (Geng et al., 2009). STK39 and OSR1 display 89% identity in their catalytic domain, located at the N-terminus of the sequence. Their regulatory domain is located to the C-terminus, where With-No-Lysine kinases (WNKs) are found to interact. These kinases directly interact with cation chlorid cotransporters, such as NKCC1 and NKCC2, modulating their activity (Gagnon and Delpire, 2012). The cation chlorid cotransporters are involved in the intracellular accumulation of chloride in cortical and dorsal root ganglial neurons. Dysregulation of the sodium and potassium gradients may impair the functionality of synapses (Ben-Ari, 2002).

In fact, neurological disorders have been linked with mutations in STK39 gene. A genetic study involving 334 families found three single nucleotide polymorphisms (SNPs) in STK39 associated to autism (Ramoz et al., 2008). Another genome-wide association study demonstrated the correlation between higher risk to develop Parkinson disease and a SNP in STK39, which increased the activity of the kinase (Nalls et al., 2011). Additionally, an increase in protein expression of STK39 homologue OSR1 and WNK3 were associated to schizophrenia (Arion and Lewis, 2011). These data provide futher evidence that STK39 downregulation could possibly be beneficial for a number of neurological diseases. Importantly, STK39 knock-out mice do not show any major adverse neurological phenotype (Geng et al., 2010). The development of STK39 inhibitors is currently under debate (Glover and O'Shaughnessy K, 2011).

6 OUTLOOK

In this thesis, the new 14D6-immunoassay for specific detection of APPsα in cell culture media, CSF and serum has been established as a tool to better separate a small group of AD patients from non-demented subjects. To increase the value of the new immunoassay and the statistical significance of the study, CSF and serum samples from a larger group of diseased and healthy subjects need to be collected. Since the analysis of combined biomarkers (Aβ, tau and p-tau) showed a better diagnostic performance for Alzheimer disease than any biomarker alone, it would be interesting to analyse a larger number of human samples in a multivariate analysis. This approach would allow to get more precise information about the temporal dynamics correlating AD symptoms and biochemical changes in the brain. Moreover, the new 14D6-immunoassay could offer a valid method to monitor the effect of disease-modifying drugs in clinical trials, where higher concentrations of APPsα might indicate successful inhibition of disease progression.

In the second part of this thesis, the kinase STK39 was newly found to be involved in APP shedding via RNAi high-throughput screening of the human kinome. In neuroblastoma cells and embryonic neurons STK39 knock-down showed reduced Aβ generation, as well as decreased APPsβ and BACE1 protein levels. Since APP intracellular domain can be phosphorylated at Thr668, commiting βCTF to undergo preferentially β-secretase cleavage, it remains still to investigate whether upon STK39 knock-down Thr668 is less phosphorylated, thereby diminuishing the products of β-secretase cleavage, namely APPsβ and Aβ. This inhibition of APP phosphorylation at Thr668 may also result in an impaired binding of specific APP binding partners, such as Shc and Grb2. The APP/Shc/Grb2 complex is reported to activate MAPK signalling pathway. On one hand, the reduction of BACE1 protein level upon STK39 knock-down may be caused by a dysregulation of MAPK cascade, resulting in a partial transcriptional inhibition of BACE1 mRNA, and thereby in lower protein level. On the other hand, STK39 may also influence BACE1 degradation by controlling the transport to the lysosome. Imaging experiments could track the destiny of BACE1 protein upon STK39 down-regulation.

Additionally, more experiments are needed to map the downstream events upon STK39 knock-down involving the MAPK pathway. Therefore, a deep investigation of the

phosphorylation pattern in the whole cellular system and more precisely of the MAPK pathway could be of great interest to shed light on the mechanism.

7 SUMMARY

Alzheimer´s disease (AD) is the most common form of dementia in the elderly worldwide. β-amyloid depositions and neurofibrillary tangles are the most common histopathological hallmarks of AD. The amyloid precursor protein (APP) contains the Aβ sequence, the highly aggregating toxic peptide found in β-amyloid plaques, which is generated through the sequential cleavage of APP by β- and γ-secretases. A third protease with α-secretase activity cleaves APP within the Aβ peptide, preventing Aβ production.

This thesis successfully addressed two major topics in Alzheimer´s disease:

- Investigation of products of APP metabolism as new potential biomarkers in AD.

Cerebrospinal fluid biomarkers such as Aβ42, tau and phospho-tau are used as biomarkers for helping AD diagnosis. Since the specificity of these biomarkers is far below 100%, especially for precise individual diagnosis, other products of APP metabolism have been proposed as putative biomarkers, such as APPsα. A number of studies investigated APPsα levels in CSF and plasma of AD patients versus non-demented controls, with poor results. In this thesis, a new antibody for detecting APPsα in a more specific manner was used to establish an APPsα-specific immunoassay. The immunoassay was tested for specificity in cell culture media, CSF and serum. Furthermore, its performance was assessed by analysing APPsα levels in a group of AD patients versus non-demented controls. A moderate, but significant increase in APPsα levels in CSF was detected in AD patients versus controls, whereas APPsα levels were significantly decreased in serum. The new 14D6 immunoassay performed better than the commercially available one for APPsα detection. For this reason, the new 14D6-immunoassay has wide applications for specific APPsα measurement in cell culture media, CSF and serum.

- RNAi- screening of the human kinome to identify new modulators of APP shedding.

Detection of the soluble APP is not only important for diagnostic purposes, but also for better understanding how APP processing is regulated. In particular, dysregulation of kinases can impair the physiological process of APP shedding. Hyperphosphorylated tau and impaired APP phosphorylation are key mechanisms for the onset of tau tagles and amyloid plaques. So far, only few kinases have been extensively studied for their role in Alzheimer´s disease. In the second part of this thesis, an RNAi high-throughput screening of the human kinome was performed to identify new kinases involved in the modulation of APP shedding and consequently in Aβ generation. The screening was performed in neuroblastoma cells expressing endogenous APP, in order to select for kinases implicated in the physiological processing of APP. Several candidates were identified and validated in neuroblastoma cells to exclude false-positive hits due to RNAi-mediated off-target effects. The effect of the downregulation of neuronally expressed genes was further assessed in murine embryonic neurons expressing endogenous APP. Finally, the kinase STK39 was identified as new modulator of BACE1 protein level. STK39 knock-down showed reduced Aβ generation, as well as decreased BACE1 and APPsβ levels in neuroblastoma cells and embryonic neurons.
Therefore, inhibition of STK39 could represent a new strategy for AD therapy.

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