Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Medicine
Department of Human Genetics
Laboratory of Neuronal Differentiation

Validation in mammalian systems of γ-secretase activity modulators identified in a *Drosophila* genetic screen

Vasiliki MICHAKI

Doctoral thesis in Biomedical Sciences

Leuven, 2010

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For my parents
Μαμά μου, μπαμπά μου, για εσάς

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Φυσικά, θα ήθελα να ευχαριστήσω την οικογένειά μου για όλη τους την στήριξη, ακόμα και από μακριά. Τα λόγια είναι φτωχά για να περιγράψουν πόσο σημαντική είναι η «αόρατη» βοήθειά τους και πόσο τους αγαπώ.
Μαμά, μπαμπά, αδερφάκια μου, σας ευχαριστώ.
Και τέλος, τον αγαπημένο μου Παναγιώτη. Για όλα.

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Men ought to know that from the brain and from the brain only arise our pleasures, joys, laughter, and jests as well as our sorrows, pains, grieves and tears. ... It is the same thing which makes us mad or delirious, inspires us with dread and fear, whether by night or by day, brings us sleeplessness, inopportune mistakes, aimless anxieties, absent-mindedness and acts that are contrary to habit...

Hippocrates

Οι άνθρωποι πρέπει να ξέρουν πως από τον εγκέφαλο και μόνο από τον εγκέφαλο πηγάζουν οι ηδονές μας, οι χαρές μας, το γέλιο μας και το κέφι, όπως και οι λύπες μας, οι πόνοι μας, οι θρήνοι μας και τα δάκρυά μας. ... Είναι το ίδιο πράγμα που μας τρελαίνει ή μας φέρνει παραλήρημα, μας προκαλεί φόβο και τρόμο, είτε τη μέρα είτε τη νύχτα, μας φέρνει αϋπνία, άτυχα λάθη, άσκοπα άγχη, αφηρημάδα και πράξεις αντίθετες από τις συνηθισμένες...

Ιπποκράτης

Section 1

Summary (English & Dutch)

1. Summary

One of the most devastating neurodegenerative diseases in the elderly population is Alzheimer's disease, which causes a progressive cognitive decline and extensive neuronal loss. A major pathological feature of the disease is the presence of amyloid plaques, observed extracellularly in brains of post-mortem patients (Forstl *et al.,* 1999, Selkoe 2001, Cummings *et al.,* 2007). The Amyloid (A) β peptide, a major constituent of these plaques, is generated by the cleavage of the Amyloid Precursor Protein (APP) by γ-secretase (De Strooper *et al.,* 2000). Consensus exists that the presence of excess of this peptide at synaptic and perisynaptic places is a major factor in the appearance of the disease. Hence, a race has begun to identify the regulatory mechanisms that govern Aβ generation by γ-secretase. Yet, the fact that numerous proteins are processed by γ-secretase made necessary the detection of substrate specific mechanisms. Because not all different substrates could be analyzed, the priority for this project was put in determining regulatory molecules of γ-secretase cleavage on APP, independent from the cleavage of the Notch receptor. Notch signaling, which is exerted upon γ-secretase proteolysis, controls important biological events (Struhl *et al.,* 2000, Louvi and Artavanis-Tsakonas, 2006) and poses, therefore, a major obstacle for a therapeutic approach based on γ-secretase inhibition. Even though the knowledge surrounding γ-secretase is increasing, the potential for activity regulation has not been thoroughly investigated. In an effort to shed some light on that, we performed a genetic screen in a *Drosophila melanogaster* model for APP cleavage. Subsequently, positive hits were tested in a Notch signaling *Drosophila* model. This strategy allowed us to identify eight suppressors and eight enhancers of APP cleavage. Without underestimating the value of the suppressors, we decided for this project to focus on the enhancers, reasoning that in a putative therapeutic scenario the most commonplace way to follow is the blockage of a molecule. Once we determined that five of them, *Abca2*, *Atp5g2*, *Habp4*, *Ddx5* and *Pairbp1*, are significantly expressed in the mouse brain we continued with the validation in a human cell line. Knockdown of all five genes led to a significant decrease in Aβ secretion. When a cell-free assay was compared to a cell-based assay for γ-secretase activity it was observed that only in the latter the enzymatic activity was disturbed. This suggests that the effect is not directly on general γ-secretase activity. A series of experiments in the cell line also suggested that β- and α-secretase are not affected, the maturation of APP is occurring normally, and there is not retention of Aβ intracellularly. As it was expected from our data showing that γ-secretase activity is not the direct target, we were also able to show that the levels of the enzyme

components are not altered. Importantly, knockdown of two of the genes, *Abca2* and *Habp4*, did not interfere with Notch Intracellular Domain (NICD) generation significantly, while lentiviral-based reduction of these genes in the more relevant system of primary hippocampal neurons could again reduce rat Aβ secretion. Altogether, our results suggest that these genes most probably act on the substrate-enzyme interaction level and provide evidence that γ-secretase activity on APP and Notch can be differentially regulated.

1. Samenvatting

De ziekte van Alzheimer, een van de meest voorkomende neurodegeneratieve aandoeiningen bij ouderen, leidt tot uitgebreid verlies van neuronen en bijgevolg een progressieve cognitieve achteruitgang. Een pathologisch kenmerk van de ziekte is de aanwezigheid van extracellulaire amyloide plaques in de hersenen van post-mortem patienten. De belangrijkste component van deze plaques is het Amyloid (A)β peptide dat ontstaat bij de klieving van het amyloid precursor proteine door γ-secretase. Het is algemeen aanvaard dat een overmaat van dit peptide op synaptische en presynaptische plaatsen een voorname factor is in het ontstaan van deze ziekte. De identificatie van de regulatiemechanismen die leiden tot Aβ vorming door γ-secretase wordt bemoeilijkt door het feit dat verschillende eiwitten gekliefd wordt door het enzymcomplex. Dit noodzaakt de zoektocht naar eventuele substraatspecifieke mechanismen. Vermits niet alle verschillende substraten geanalyseerd kunnen worden, werd in dit project de focus gelegd op de regulatie van γ-secretase klieveing van APP onafhankelijk van de klieving van de Notch receptor. Signaaltransductie via Notch, na γ-secretase gemedieerde proteolyse, controleert belangrijke biologische processen en is daarom een belangrijk obstakel voor een therapie gebaseerd op inhibite van γ-secretase. Ondanks een toenemende kennis van γ-secretase is de regulatie van de activiteit weinig bestudeerd. Om hier meer inzicht in te krijgen hebben we een genetische screen uitgevoerd in een *D. melanogaster* model van APP klieving. De mutanten met effect in dit model werden nadien getest in een Notch signalisatie model. Op deze wijze zijn we erin geslaagd om acht onderdrukkers en acht activatoren van APP klieving te identificeren. Hoewel ook de onderdukkers waardevol zouden kunnen zijn hebben we besloten om ons te richten op de activatoren vermits, in geval van een therapeutische toepassing, het blokkeren van een molecule de meest voorkomende aanpak is. Na bevestiging van expressie van vijf van deze eiwitten, *Abca2*, *Atp5g2*, *Habp4*, *Ddx5* en *Pairbp1,* in de hersenen van muizen zijn we verder gegaan met validatie in humane cellen. Uitschakeling van alle vijf genen leidde tot een significante daling van Aβ secretie. Bij het vergelijken van de γ-secretase activiteit in een cell-vrije versus een cell-gebaseerde assay merkten we op dat enkel in deze laatste de enzymatische activiteit verstoord wordt. Dit toont aan dat de γ-secretase activiteit niet rechtstreekt beinvloed wordt. Bijkomende experimenten in de cellijn wijzen erop dat β- en α-secretase niet aangetast zijn, dat de maturatie van APP normaal gebeurt en dat er geen intracellulaire retentie is van Aβ. Zoals verwacht uit het feit dat γ-secretase activiteit niet

rechtstreeks beinvloed is konden we aantonen dat ook de stabiliteit van het enzym componenten niet veranderd is. Opmerkelijk was dat uitschakeling van twee van de genen, *Abca2* en *Habp4,* geen significante invloed had op de vorming van Notch Intracellular Domain (NICD). Anderzijds reduceerde lentiviraal gebaseerde vermindering van deze genen in primaire hippocampale neuronen, een relevanter model, toch rat Aβ secretie. Samengevat suggereren onze resultaten dat deze genen waarschijnlijk betrokken zijn bij de interactie tussen het substraat en het enzyme en het bevestigt dat γ-secretase activiteit op APP en Notch verschillend gereguleerd kunnen worden.

Section 2

Introduction

2. Introduction

2.1 Alzheimer's disease: Neuropathological and clinical characteristics

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and the most common cause of dementia in the modern society. Currently there is no disease-modifying cure available, and the treatment is solely symptomatic. Age is considered to be the major risk factor for AD and millions suffer from AD worldwide and with continuous rise in life expectancy the number of AD patients is set to increase rapidly in coming years (Sisodia, 1999, Ferri *et al.,* 2005). Clinically, AD is characterized by progressive loss of memory associated with cognitive deficits extending to language skills, decision-making ability, movement and recognition (Borson *et al.,* 1997, Förstl *et al.,* 1999, Arnaiz *et al.,* 2003). The histopathological features of the disease were first described in 1906 by the psychiatrist Alois Alzheimer (Figure 1) (Alzheimer A, 1907) who autopsied his patient Auguste D. and connected the presented dementia with the neuropathological lesions of her brain.



Figure 1. German neuropathologist and psychiatrist Alois Alzheimer was the first to describe the histopathological features of the presenile dementia, later termed Alzheimer's disease.

The "miliary bodies" and the "dense bundles of fibrils" that Alzheimer observed at the time were extensively analysed many years later and are currently the hallmarks of the brains of Alzheimer's disease patients, namely the amyloid (senile) plaques and the neurofibrillary tangles (Selkoe, 2000).

The amyloid plaques are extracellular spherical lesions of the brain. They are mostly made up from the hydrophobic 4kDa amyloid-beta peptide (Aβ), but degenerating neurites, activated microglia and reactive astrocytes are also present inside and around the plaque. The intraneuronal fibrillary tangles consist of paired-helical filaments of abnormal hyperphosphorylated insoluble tau, which is a microtubule-associated protein (Figure 2) (Selkoe, 2000).

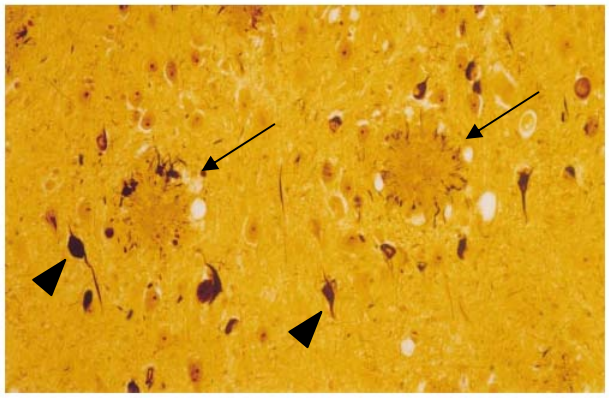


Figure 2. Brain section from an Alzheimer's patient demonstrating two senile plaques (arrows) containing spherical deposits of extracellular amyloid that are surrounded by dystrophic neurites. Neurons (arrowheads) that contain darkly stained neurofibrillary tangles can also be seen (from Selkoe, 2000).

Although these two features can occur separately in the diseased brain, many studies during the years have placed the formation of the tangles in a pathway that starts with the accumulation of Aβ peptide and leads through a series of events to AD pathology. This pathway comprises the amyloid cascade hypothesis, which although controversial, still is widely accepted to be correct. In particular, what the amyloid hypothesis proposes is that the initial accumulation of Aβ is the first step towards AD and that the rest of the symptoms, like microglial activation, synaptic injury and tangle formation, are a consequence of this event (Figure 3) (Hardy and Selkoe, 2002). Even though in the past the amyloid plaques were held responsible for the subsequent events leading to the disease, a lot of studies have provided good evidence that the toxicity is a feature of the Aβ oligomers before they reach the fibrillar state, which can cause synaptic defects. A recent study in fact proposed that the impairment of the synaptic plasticity produced by Aβ oligomers is mediated by binding of the oligomers to the cellular prion protein (Lauren *et al.,* 2009). Therefore, while in numerous occasions tangles are evident at sites without direct correlation with Aβ cumuli this can be due to the

toxicity of oligomeric soluble Aβ, well before its accumulation (Klein, 2002, Walsh *et al.*, 2002, Walsh *et al.*, 2005, Lesne *et al.*, 2006, Townsend *et al.*, 2006, Shankar *et al.*, 2007, Walsh and Selkoe, 2007).

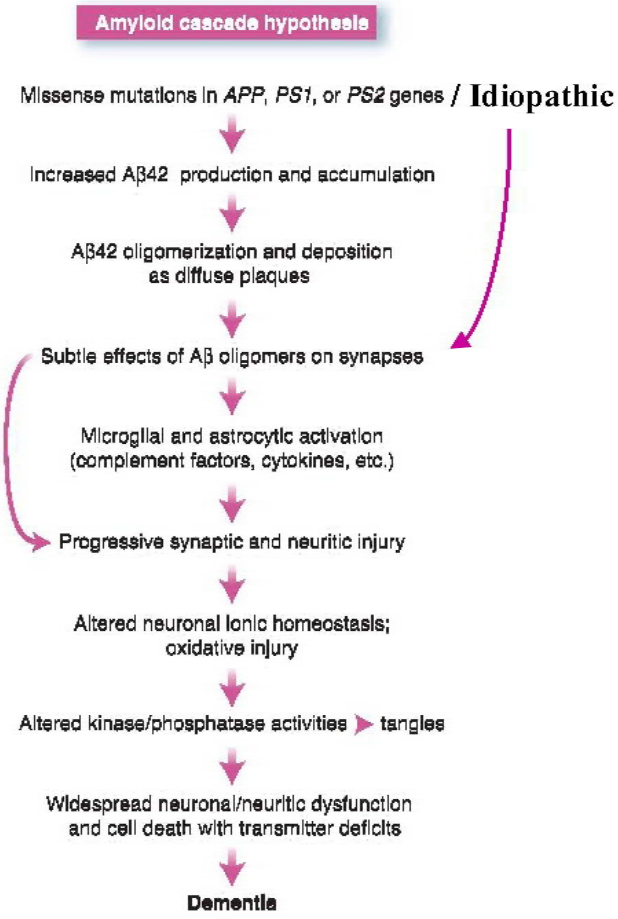


Figure 3. Sequence of events according to the amyloid cascade hypothesis. Several recent studies have shown that Aβ oligomers can impair long-term potentiation and lead to synaptic injury (curved arrow, Walsh *et al.*, 2002, Lesne *et al.*, 2006), apart from activating microglia and astrocytes (adapted from Hardy and Selkoe, 2002).

Aβ is produced also in healthy individuals, as studies of the cerebrospinal fluid have shown (Ida *et al.*, 1996, Vigo-Pelfrey *et al.*, 1993). Most of the peptide produced in physiological conditions is Aβ40; however, in AD there is a shift towards the production of a longer isoform, Aβ42 that is the major Aβ species deposited in the plaques, although this peptide represents only 5–10% of all Aβ produced. Aβ40 and Aβ42 peptides show significant differences in the biophysical properties; due to a greater hydrophobicity Aβ42 is prone to

aggregate faster and was described as a 'nucleation' factor, which notably accelerates the aggregation of Aβ *in vitro* (Barrow *et al.,* 1991, Jarrett *et al.,* 1993).

2.2 APP: structure and proteolytical processing

APP (Glenner *et al.,* 1984, Masters *et al.,* 1985) is a type-I transmembrane protein with a still unclear physiological role. It is synthesized as an immature protein and undergoes several post-translational modifications, like glycosylation and phosphorylation (Tomita *et al.,* 1998, Takahashi *et al.,* 2008). APP can be processed in two different pathways, the amyloidogenic and the non-amyloidogenic (Selkoe, 2001). In the amyloidogenic, sequential processing of APP by β- and γ-secretase in the secretory and endocytic pathway results in generation of Aβ (Figure 4) (Mills *et al.,* 1999, Walter *et al.,* 2001, De Strooper *et al.,* 2000). In particular, APP is cleaved at the N-terminus of the Aβ domain by β-secretase resulting in the secretion of soluble APP (APPsβ). The 99-amino acid long APP C-terminal fragment (APP-CTFβ) is tethered within the membrane bilayer and is substrate to a multimeric protein complex called γ-secretase, which cleaves CTFβ within the transmembrane domain leading to generation of Aβ. Alternatively, APP can be processed in a non-amyloidogenic fashion. Initial cleavage by the α-secretase within the Aβ domain precludes formation of Aβ peptides and results in the formation of soluble APPα (APPsα) and 83-amino acid long CTFα. CTFα is also cleaved by γ-secretase and generates p3, which is not known to aggregate like the amyloid peptide. Because of the differential actions of α- and β-secretase, the non-amyloidogenic and the amyloidogenic processing pathways of APP are mutually exclusive.

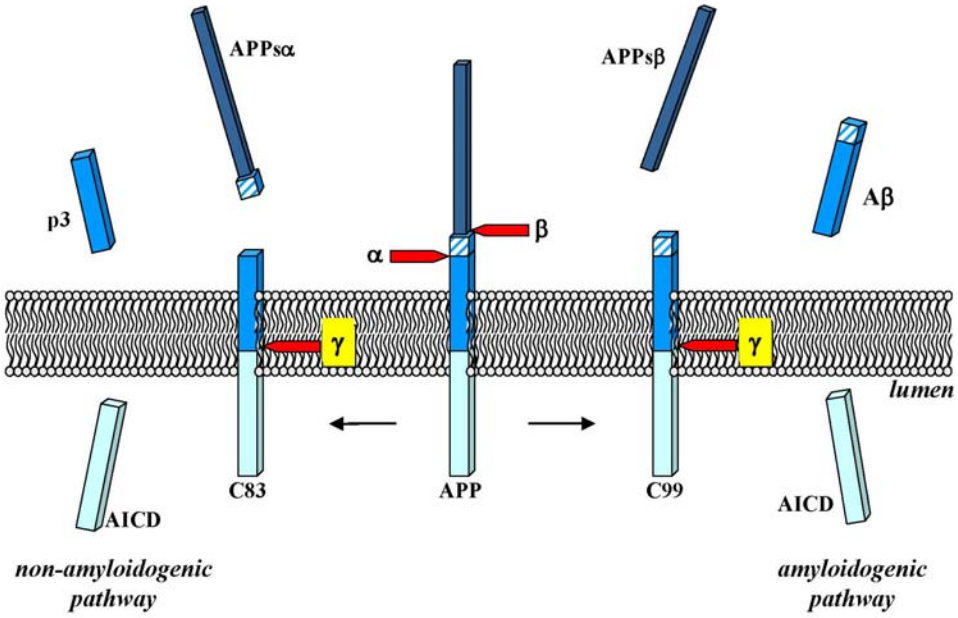


Figure 4. Schematic depiction of the two different proteolytic pathways of APP. In the amyloidogenic pathway (right part) APP is cleaved by β- and γ-secretase and APPsβ and Aβ is released. On the other hand, in the non-amyloidogenic pathway (left side) α-secretase is performing the first cut and γ-secretase the second and APPsα and the non-amyloidogenic p3 peptide is released. AICD is generated in both pathways.

Three related metalloproteases of the ADAM (a disintegrin and metalloprotease) family exert α-secretase activity, ADAM10 (Lammich *et al.,* 1999), ADAM17/TACE (tumor necrosis factor-a converting enzyme) (Buxbaum *et al.,* 1998), or ADAM9 (Koike *et al.,* 1999).

Four independent approaches led to the identification of β-secretase or BACE1 (β-site APP-cleaving enzyme1) (Hussain *et al.,* 1999, Sinha *et al.,* 1999, Vassar *et al.,* 1999, Yan *et al.,* 1999). BACE1 is a type 1 transmembrane protein and belongs to the pepsin family of aspartyl proteases. At the plasma membrane it has been shown to localize in lipid rafts, where it cleaves APP, but also to be endocytosed and cleave APP in the pH optimal endosomes (Haass, 2004). The biological role of this enzyme is not completely understood yet, although other substrates, like P-selectin glycoprotein ligand-1, ST6GalI (Lichtenthaler *et al.,* 2003, Woodard-Grice *et al.,* 2008), and NRG1 (Willem *et al.,* 2006) have been identified.

Even though APP has been extensively studied and plenty of light has been shed on its structure, proteolysis and involvement in disease, its physiological biological function is yet to be determined (Thinakaran and Koo, 2008). Because of its structure and similarities to other receptors the possibility that it acts as one has been suggested. However, the pursuit of ligands has not been fruitful (De Strooper and Annaert, 2000). Interestingly, a physiological

function has been appointed to the APP intracellular domain (AICD). It has been shown that AICD can bind to the histone deacetyltransferase TIP60 and the nuclear adaptor protein Fe65 and form a complex that can regulate gene transcription. This event has been proposed to maintain calcium levels in the endoplasmic reticulum and suppress retinoid-dependent gene expression (Fortini, 2002). Nevertheless, it needs to be noted that the signaling role of AICD have been recently contradicted, and it is not determined if *in vivo* this signaling is present (Waldron *et al.,* 2008, Ma *et al.,* 2008). In addition to these functions, experiments in cells and in brain have given evidence that APP might have growth factor properties (Saitoh *et al.,* 1989, Meziane *et al.,* 1998). Furthermore, genetic studies in mice lacking or under-expressing APP have led to the conclusions that APP plays a role in axon guidance, dendritic arborization and neuronal survival. It is noteworthy, however, that APP knockout mice have a mild neurological phenotype (Muller *et al.,* 1994, Magara *et al.,* 1999, De Strooper and Annaert, 2000). In line with its putative role in neurite outgrowth, APP has been shown to colocalize with integrins on axons and contain a motif that promotes cell adhesion (Yamazaki *et al.,* 1997).

In summary, even if the exact function of APP is unclear, still these potential roles should be taken into consideration and strategies involving the decrease of APP in humans need to be examined with caution.

2.3 The γ-secretase complex

γ-secretase is a multimeric membrane bound protein complex that catalyses regulated intramembranous proteolysis (RIP) of APP-CTFs generated by either β-secretase or α-secretase to liberate Aβ and p3, respectively (Wolfe *et al.,* 2001). Four essential components of γ-secretase have been identified by now, namely Presenilin1 or 2 (Levy-Lahad *et al.,* 1995, Sherrington *et al.,* 1995), Nicastrin (Edbauer *et al.,* 2002, Kopan *et al.,* 2002, Lai, 2002), PEN-2 (presenilin enhancer protein 2) (Francis *et al.,* 2002, Steiner *et al.,* 2002, Luo *et al.,* 2003), and APH-1 (anterior pharynx defective 1) (Francis *et al.,* 2002, Goutte *et al.,* 2002, Lee *et al.,* 2002, Luo *et al.,* 2003) (Figure 5). These proteins form a series of high molecular weight complexes and cooperatively regulate each other's expression and maturation (Kimberly *et al.,* 2003, Takasugi *et al.,* 2003, Edbauer *et al.,* 2003), while they have been shown to be necessary and sufficient to reconstitute the enzymatic activity even in yeast that lacks endogenous γ-secretase (Edbauer *et al.,* 2003, Fraering *et al.,* 2004).

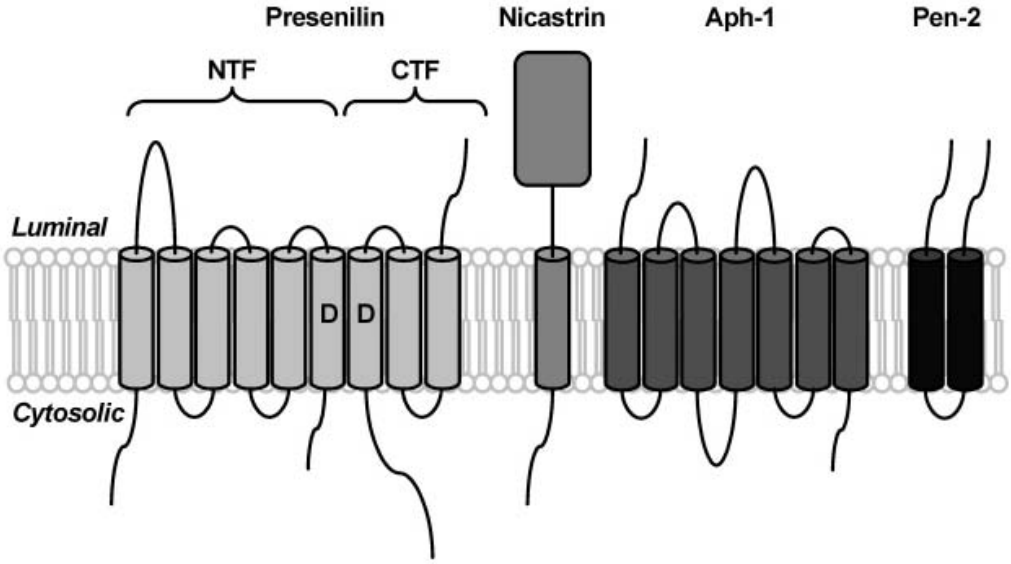


Figure 5. The four components of γ-secretase complex. Presenilin with nine transmebrane domains (TMDs), Nicastrin with one TMD, Aph-1 with 7 TMDs and Pen-2 with 2 TMDs. The generated complex possesses in total 19 TMDs, which explains its high hydrophobicity (image from Wolfe, 2006).

Presenilin 1 (PS1) and Presenilin 2 (PS2) are the mammalian prototype members of a rapidly growing protein family with highly conserved GXGD motif at the catalytic site (Haass *et al.,* 2002). Structurally they are polytopic transmembrane proteins, with a nine-transmembrane domain topology that are cleaved endoproteolytically shortly after biosynthesis (Thinakaran *et al.,* 1996, Podlisny *et al.,* 1997, Spasic *et al.,* 2006) to generate the N-terminal fragment (NTF) and the C-terminal fragment (CTF), which remain bound to each other.

In 1995, mutations in the presenilins were linked with early-onset familial Alzheimer's disease (Levy-Lahad *et al.,* 1995, Rogaev *et al.,* 1995, Sherrington *et al.,* 1995). The AD-associated mutations were determined to increase the ratio of Aβ42 to Aβ40 (Aβ42/Aβ40) in mice and humans (Citron *et al.,* 1992, Borchelt *et al.,* 1996, Scheuner *et al.,* 1996), indicating that presenilins might modify the way in which γ-secretase cuts APP. To day there are more than hundred autosomal dominant mutations in PS connected to early onset AD. Studies that were showing that PS1 knockout decreases Aβ (De Strooper *et al.,* 1998) and that when both PS1 and PS2 were removed then Aβ production was abolished (Herreman *et al.,* 2000, Zhang *et al.,* 2000) proved that PS is the catalytic core of γ-secretase. Further proof of that was the presence of two conserved aspartate residues in the transmembrane domains 6 and 7 of PS, which form the catalytic core of the enzyme (Wolfe *et al.,* 1999). 3D structure of γ-secretase

complex, determined by electron microscopy (Lazarov *et al.,* 2006), revealed a large cylindrical interior chamber, which is postulated to provide a hydrophilic milieu necessary to accomplish peptide bond hydrolysis. The contribution of TMD6 and TMD7 to such water-containing cavity was verified by cystein scanning mutagenesis (Tolia *et al.,* 2006).

Nicastrin is a type I transmembrane glycoprotein that has been suggested to act as a gatekeeper of the γ-secretase complex. The extracellular domain of nicastrin binds specifically to the amino terminal residue of membrane bound protein fragments generated by sheddases and is then able to position the bound substrates so as to facilitate their cleavage by the γ-secretase complex (Shah *et al.,* 2005, De Strooper *et al.,* 2005). As expected nicastrin knockout mice exhibit a very similar phenotype as that of Notch and presenilin knockout mice (Li *et al.,* 2003). Francis *et al.* (2002) identified two presenilin enhancers in C. elegans namely APH1 and PEN2. Both are multipass polytopic membrane proteins. APH1 stabilizes the presenilin holoprotein in the complex, whereas PEN2 is required for endoproteolytic processing of presenilin conferring, thus, γ-secretase activity to the complex (Takasugi *et al.,* 2003).

A series of studies proved that APP proteolysis is more complex than initially described. Thus, APP can also be cleaved by γ-secretase at ε- and ζ-site. ε-cleavage of APP-CTF after Aβ49 position liberates AICD. ζ-cleavage of APP-CTF after Aβ46 position by γ-secretase has also been reported. Recently it was shown that γ-cleavage is dependent on prior ζ -cleavage which takes place after ε-cleavage (Qi-Takahara *et al.,* 2005, Wolfe *et al.,* 2007). Evidence indicates that γ-secretase cuts APP initially at the ε-site and then progressively removes C-terminal residues until the γ-cleavage site has been reached (Kakuda *et al.,* 2006).

As aforementioned, γ-secretase is responsible for the intramembrane cleavage of several type I transmembrane proteins. Therefore, in addition to APP, several other γ-secretase substrates have been identified with a variety of functions. Examples include the Notch receptor, the Lipoprotein-Related Protein (LRP), the members of the Cadherin family, APP-like-Proteins (APLP) 1/2, Erb4, CD44, Nectin-1, Voltage-gated Sodium Channel b2-subunit and MHC class I protein HLA-A2 (Kim *et al.,* 2002, Lammich *et al.,* 2002, Lee *et al.,* 2002, Jung *et al.,* 2003, Marambaud *et. al.,* 2003, Gowrishankar *et al.,* 2004, Kopan *et al.,* 2004, Kim *et al.,* 2005, Maretzky *et al.,* 2005, Carey *et al.,* 2006). This has led to the suggestion that the γ-secretase complex might act as a proteasome of the membrane by degrading and clearing the membrane from prior cleaved proteins (Kopan *et al.,* 2004).

2.4 Notch signaling

Prominent among the substrates of γ-secretase and center of numerous studies is the Notch receptor (Louvi and Artavanis-Tsakonas, 2006). Notch signaling controls embryonic cell-fate decisions in variety of cell lineages (Struhl and Adachi, 2000) and Notch1 knockout in mice is embryonic lethal. Furthermore, Notch signaling is also necessary during the adult stage controlling cell differentiation processes like axon guidance, neurite outgrowth and arborization (Wittenburg *et al.,* 2000, Figueroa *et al.,* 2002, Selkoe and Kopan, 2003) and in hematopoiesis (Schroeder and Just, 2000).

Most signaling pathways function via the transmission of the signal from the plasma membrane to the nucleus, with the participation of intermediate proteins. However, the Notch signaling relies on the proteolytical generation of Notch intracellular domain (NICD) by γ-secretase. NICD is then binding directly to the transcription factors of the CSL family (C promoter binding factor/Suppressor of Hairless/Lag-1) and modulate target genes (Fortini, 2002).

Apart from the pivotal role of Notch signaling during development, the Notch pathway is also functional and important in the adult brain, where it is still expressed even at a low level (Berezovska *et al.,* 1998). Studies in *Drosophila* using Notch mutants and RNA interference to disturb the pathway have shown that Notch is involved in the formation of long-term memories (Presente *et al.,* 2004, Ge *et al.,* 2004). The same was proposed after analyzing mice heterozygous for a Notch mutation and observing that their spatial learning capacity was decreased (Costa *et al.,* 2003). In line with that, mice that express only 50% of the normal Notch mRNA levels and have less protein in the hippocampus do not exhibit long-term potentiation, suggesting that Notch signaling is implicated in synaptic plasticity (Wang *et al.,* 2004). Additionally, experiments using primary postmitotic neurons have given evidence that Notch signaling can be activated in the mature neurons and that it affects neurite maintanance (Berezovska *et al.,* 1999). Moreover, Notch signaling has been shown to regulate adult neurogenesis, by maintaining the neural stem cell population (Hitoshi *et al.,* 2009, Alexson *et al.,* 2006, Androutsellis-Theotokis *et al.,* 2006), and to keep the forebrain ependymal cells quiescent until they need to respond to brain injury (Carlen *et al.,* 2009).

2.5 APP and Notch transport and proteolysis

The transport of proteins upon synthesis is an important biological event in order to achieve post-translational modifications, function and residence in the correct compartment. For APP the transport from the endoplasmic reticulum (ER) to the plasma membrane and the subsequent endocytosis/recycling/degradation are imperative for its maturation and proteolysis. Briefly, the trafficking of newly synthesized APP through the constitutive secretory pathway begins with its transport from the ER to the Golgi membranes and from there, vesicles directly bud and fuse with the plasma membrane. Importantly, the maturation of APP occurs during the passage from the ER, and includes modifications such as glycosylation and phosphorylation. Once the mature APP reaches the plasma membrane, it gets quickly endocytosed and is either recycled back to the surface or degraded in lysosomes (Figure 6) (Thinakaran and Koo, 2008). Specifically in neurons, the transport of APP happens anterogradely along the axons of the peripheral or central nervous system, where also the proteolysis takes place, however the exact place where Aβ is produced is not yet clear (Buxbaum *et al.,* 1998).

As aforementioned, there are three different enzymes involved in the processing of APP. It is characteristic that not only these three enzymes constitute two separate pathways for APP cleavage but also that their site of action can differ, giving an additional importance to the trafficking of APP. So, while α-secretase acts at the plasma membrane, β-secretase can act at the plasma membrane, late Golgi and endosomes upon APP endocytosis (Haass, 2004). For γ-secretase the situation is more complicated because activity has been shown in multiple sites, namely the ER, late Golgi, endosomes and a small activity also at the plasma membrane (Greenfield *et al.,* 1999, Chyung *et al.,* 2005, Vetrivel and Thinakaran, 2006).

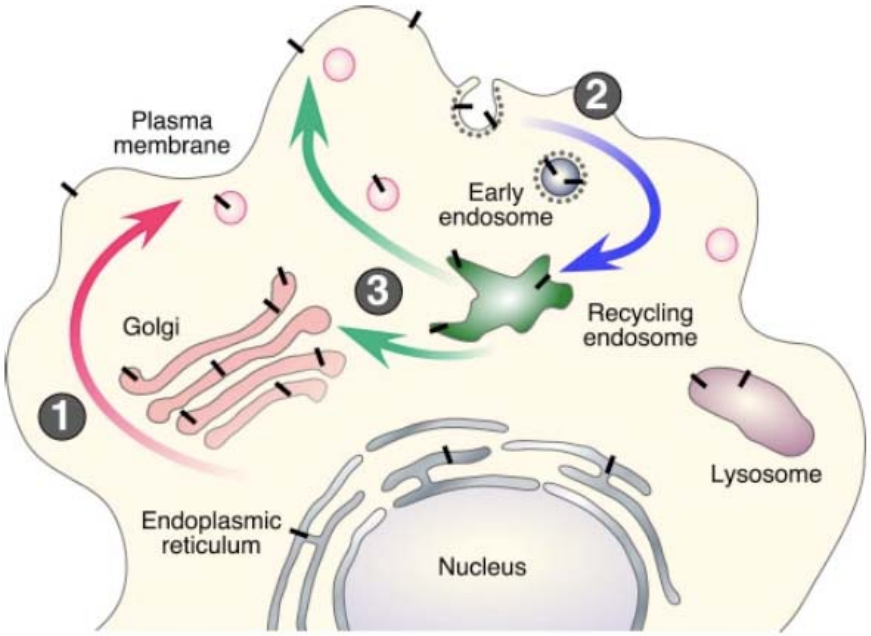


Figure 6. New immature APP molecules (black bars) undergo several post-translational modifications while passing through the ER towards the Golgi compartment (1). With the creation of vesicles it is then transferred to the plasma membrane, where the vesicles fuse. Once at the cell surface, APP is rapidly internalized (2) and can follow two ways: either recycle back to the surface through the endocytic compartments or go to the lysosomes for degradation (3) (from Thinakaran and Koo, 2008).

Like APP, the Notch receptor is also cleaved from more than one enzyme during its transportation from the ER to the cell surface, and these modifications are pivotal for the signaling (Figure 7).

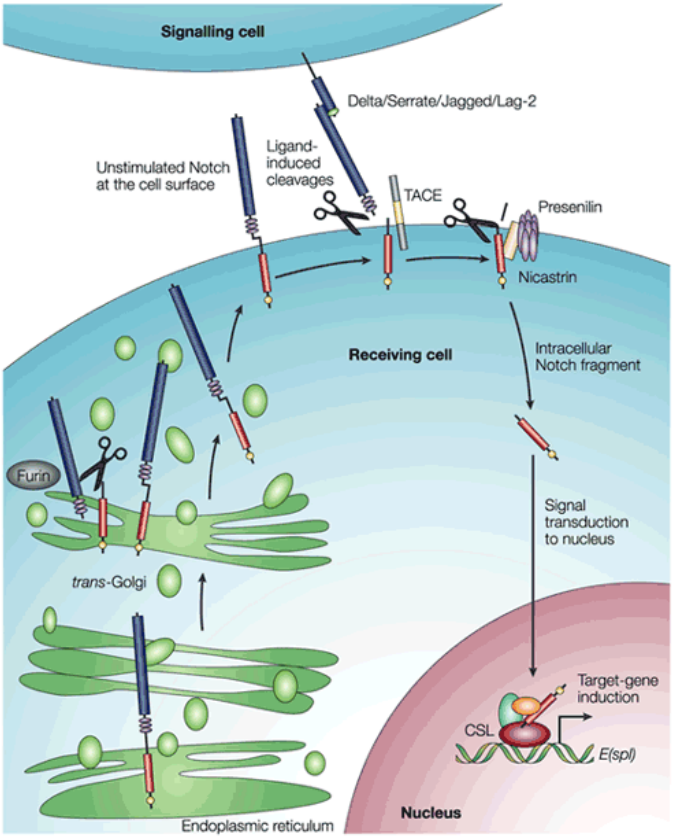


Figure 7. After being synthesized in the ER, Notch is trafficked via the secretory apparatus, where it is cleaved in the *trans*-Golgi by a furin-like convertase. The two fragments that are formed are noncovalently connected and the heterodimer moves to the plasma membrane. There, ligands of the Delta/Serrate/Jagged family from the signaling cell are stimulating the Notch receptor and its ectodomain shedding by the TACE/ADAM (a disintegrin and metalloproteinase/tumour necrosis factor-α-converting enzyme) metalloproteinases. The final step of the pathway is the proteolysis of the remaining carboxy-terminal fragment by γ-secretase and the liberation of the Notch intracellular domain (NICD), which will interact with transcription factors of the CSL family (C promoter binding factor/Suppressor of Hairless/Lag-1) and regulate the expression of Notch target genes (from Fortini, 2002)

Notch is a 300 kDa a type I transmembrane protein, which is first cleaved by furin-like protease at the S1 position in the Golgi compartment. The two fragments from two different S1 cleaved Notch molecules are reassembled as a heterodimer and this is the form present at the plasma membrane. A subsequent necessary interaction with a ligand induces S2 cleavage by a disintegrin/metalloprotease, generating the notch extracellular truncation (NEXT)/Notch C-terminal fragment. NEXT is then processed by γ-secretase complex at the S3 site that corresponds to ε-cleavage of APP (Ikeuchi and Sisodia, 2002) to liberate Notch Intracellular

Cytoplasmic Domain (NICD). NICD translocates to the nucleus where it acts as a regulator of transcription (De Strooper *et al.*, 1999, Struhl and Greenwald, 1999).

The similarities in the proteolysis of APP and Notch together with the common γ-secretase cleavage and the dependence of Notch signaling on NICD liberation make clear the necessity of careful γ-secretase activity regulation, if this strategy is to be used for therapeutic purposes.

2.6 *Drosophila melanogaster* as a model

For the beginning of this project we used *Drosophila melanogaster* as our model organism to perform genetic screens. *Drosophila melanogaster* (Figure 8) has rapidly gained a lead role as a model for human diseases, including neurodegenerative ones, such as Alzheimer's disease, tauopathies and Parkinson's disease. The reasons for this fact are varying between the short generation period and the conservation of genes from the fly to human.



Figure 8. The photo shows a female Drosophila melanogaster on the left side and a male on the right. Characteristic is the smaller size of the male fruitfly and the darker backbody (photo from Exploratorium).

Specifically, relevant to modeling neurodegenerative disease is the complexity of the *Drosophila* brain and its regional separation. Moreover, it has been stated that around 75% of human genetic loci associated with disease have a homologue in the fruitfly (Reiter *et al.*, 2001), while the functional conservation of many genes is noteworthy. Certainly, in parallel to similar cell biology, as important is the fact that *Drosophila* has a ten-day generation time, producing plenty of progeny, while the maintenance of this organism is relatively easy. All these advantages would not have been of use if genetic manipulation of *Drosophila* were unfeasible. However, the abundance of genetic tools in the fly renders it ideal for wide genetic screens for pathways and proteins that have a role in disease progression (Cauchi and van den Heuvel, 2006). In addition, the APP-like protein that is present in *Drosophila* does not contain the Aβ domain (Rosen DR *et al.*, 1989), which makes possible the expression of the human

APP without any background Aβ production. As far as the other AD-related proteins are concerned, *Drosophila* carries a conserved functional γ-secretase complex, but not β-secretase, however the chimaeras containing human C99 (β-secretase product) can circumvent this absence (Fossgreen *et al.,* 1998). Several studies have provided evidence that some of the protein functions and mechanisms associated with AD are conserved in the fruitfly. For example, mutations of the dPS (*Drosophila* presenilin) gene lead to defects in Notch signaling and consequent developmental problems (Ye *et al.,* 1999, Struhl and Greenwald, 1999). It needs to be noted that Notch was firstly identified in *Drosophila melanogaster* and the pathway is greatly conserved, controlling pattern formation and other developmental events (Morgan, 1917, Lai, 2004). Additionally, even though the exact function of human APP is still under study, one of the functions that have been suggested is the one of receptor of kinesin in vesicles. Similarly, the *Drosophila* APPL has also been shown to act as such (Gunawardena *et al.,* 2001). Another example is the fact that, parallel expression of human APP and β-secretase in the fly retina causes neurodegeneration of the tissue accompanied by amyloid plaque formation (Greeve *et al.,* 2004).

In summary, it has become clear through the years and studies that *Drosophila melanogaster* is an exemplary model organism, rich in genetic tools, which can assist towards the identification of disease-related genes or drugs.

2.7 Aims of my Ph.D. thesis

Studies on γ-secretase complex have proved that this enzyme is involved in the proteolysis of several type I transmembrane proteins. Nevertheless, because of its role in the generation of amyloid beta (Aβ) and the progression of Alzheimer's disease scientists in the AD field have put it in the centre of vast research, resulting in an improvement of current understanding about the complex's assembly and function. Inhibition or reduction of γ-secretase cleavage of APP leads to decreased Aβ generation and has the potential, therefore, to modify the course of the disease. However, it is essential to avoid interfering with the γ-secretase cleavage of Notch in order to avoid Notch deficit-mediated side effects. The vital signaling properties of Notch and their dependence on γ-secretase are a critical reason why application of γ-secretase inhibitors for therapeutically purposes has been hampered. Whether the Notch cleaving property is independent of that of APP and how it is regulated and could be manipulated still remains undetermined. To circumvent this obstacle, one could search for molecules-targets that affect APP metabolism and Aβ generation, without directly targeting γ-secretase.

In line with this, we set the following objectives:

- The identification of molecules that could affect Aβ generation without disturbing Notch signaling. For this purpose we used *Drosophila melanogaster* as a model organism rich in genetic tools.

- The validation of the detected candidate genes as γ-secretase modulators and the analysis of their effect on APP proteolysis. And for this we used a human cell line and rat primary hippocampal cultures.

Section 3

Materials and Methods

3. Materials and Methods

3.1 *Drosophila melanogaster*: Maintenance, Stocks, Genetics, Biochemistry and Molecular Biology

All *Drosophila melanogaster* stocks were maintained at 18ºC or 25°C and were raised on standard cornmeal-yeast agar medium.

3.1.1 Strains

GMR-APP-GAL4,UAS-GRIM/Cyo

These flies were a kind gift of Dr. Ming Guo. This fly carries a chimeric human APP protein comprised of the C99 transmembrane fragment of APP, just downstream of the β-secretase cleavage site, fused to the yeast transcription factor GAL4-VP16 (GV), and the cell-death reporter gene GRIM, cloned under the GAL4 responsive transcriptional cassette (Upstream Activating Sequence or UAS). The use of the GMR promoter allows for eye-specific expression of GRIM (Guo *et al.*, 2003). *Cyo* stands for the dominant marker that is present in the balancer chromosome and causes the curly wings.

GMR-GAL4,UAS-G/RPR/TM3

The chimeric protein G/REAPER (G/RPR) contains the N-terminus of GRIM and the C-terminus of the protein REAPER, which is another cell-death activator. Expression of this protein leads to caspase-dependent cell death, independently of APP cleavage. (also kindly provided by Dr. Ming Guo) (Guo *et al.*, 2003). *TM3* is on the balancer chromosome and is a marker for larger alters and more shoulder hair of the fly.

APP-GAL4,UAS-GFP/Cyo

Isogenic stock containing the C99-GV construct together with the UAS-cd8-GFP reporter gene. The cd8 permits for targeting of the protein to the membranes, while a heat shock promoter induces the expression of the C99-GV protein exclusively in adult tissues.

Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT80M/TM2 and

Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT82M/TM6b

This strain carries the C99-GV construct in combination with the UAS-cd8-GFP reporter. Due to the application of the eyFLP/FRT technique (Newsome *et al*, 2000), eye-specific expression of a recombination competent enzyme generates homozygous mutant clones only in the eyes, leaving the rest of the animal heterozygous. *TM2* is a marker for bigger alters and

more shoulder hair, while *TM6b* is a marker for short and fat body, on the balancer chromosomes.

Ne511/FM6; Df(N+)/TM2

These transgenic flies carry a null loss of function allele of Notch that produces a dominant phenotype in the morphological pattern of veins and margin of Drosophila wings. This stock was a kind gift of Dr. Marco Milan.

P(lacW) insertion lines (l(3)Sxxxxxx)

10.6kb lethal insertions in the third chromosome that cause homozygous lethal mutations in approximately 30% of the genes sited on the *Drosophila* 3rd chromosome. They contain the mini-*white* gene as a marker for detection because the eyes become red. They also include a bacterial origin of replication and the gene coding for ampicillin, thus allowing plasmid rescue experiments**.** These P-elements were previously recombined in specially engineered chromosomes that contain the yeast specific FRT sequences to allow mitotic recombination and clonal analysis. The P(lacW) insertion lines (l(3)Sxxxxxx) were purchased from the Szeged Drosophila Stock Centre:

http://expbio.bio.u-szeged.hu/fly/modules.php?name=Chromos3&op=Chromos3List

Oregon R and w^{1118}

Wild-type flies used for controls for the crosses (**w^{1118}**) and the drug treatments (**Oregon R**).

3.1.2 Crosses and scoring of phenotype

All crossings were incubated at 25°C with 75% humidity for approximately 15-20 days, in order to produce sufficient amount of progeny.

All crossings were compared to a control crossing against the wild type fly stock **w^{1118}**

The following crosses were set during the genetic screens:

P-element x GMR-APP-GAL4,UAS-GRIM/Cyo

P-element x GMR-GAL4,UAS-G/RPR/TM3

P-element x Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT80M/TM2

Or P-element x Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT82M/TM6b

P-element x Ne511/FM6; Df(N+)/TM2

For the crosses males from the P-element collection were crossed to virgins of each reporter stock. In particular, 1-2 males were placed in a tube with food with 3-6 virgins and put at

25ºC. After ten days the progeny was collected and scored for change of phenotype using a stereoscope. For the eyflp; APP-GAL4,UAS-GFP; FRT (80 or 82) reporter stock, the progeny was heat-shocked for 1h at 38°C and was left 24h in the 25°C incubator to recover. After this time the GFP expression was monitored using a stereoscope equipped with a fluorescent lamp.

3.1.3 Drug treatments

The drugs that were used are two previously characterized inhibitors of γ-secretase, DAPT and Compound 3 (Micchelli *et al*., 2003; Seiffert *et al*., 2000; Dovey *et al*., 2001). 20mM of Compound 3 and 50mM of DAPT diluted in DMSO were added in the tube with the fly food by making three holes with the pipette and pouring the drug. The tube was left overnight under the hood, in order for the drug to dry. Food containing only DMSO in the respective volumes was used for the control flies. The next day, 50 **APP-GAL4,UAS-GFP** flies were put in the tube and incubated at 25°C for 4-5 days. After these days the flies were heat-shocked for 1h at 37°C in a waterbath and were left 24h in the 25°C incubator to recover and express GFP. Relative levels of GFP expression were evaluated either under a microscope equipped with fluorescence lamp or by Western blot.

For the Western blot analysis, after the drug treatment period and 24h post heat-shock, 30 flies were homogenized in 1x PBS plus complete protease inhibitors. Upon centrifugation at 6000rpm for 10min, to pellet the non-soluble part of the fly body, the protein load was quantified with the BIO-RAD system. Equal amounts of protein for the control (only DMSO treated flies) and for the sample (drug treated flies) were mixed with 1x Laemmli sample buffer and heated in a heating block at 95ºC for 5min. Samples were loaded on a 12% SDS-acrylamide gel and after electrophoresis the proteins were transferred on a nitrocellulose membrane. For the blocking of unspecific binding sites the membrane was incubated 1h at room temperature with 5% milk blocking solution in TBS-T (25mM Tris-HCl pH 7.4, 150mM NaCl, 4mM KCl, 0.1% Tween-20) and after this overnight at 4°C with the primary anti-GFP antibody (A.v. Peptide antibody, BD Biosciences). Next day the membrane was washed 3 times with TBS-T and was incubated 1h at room temperature with the secondary rabbit antibody. The detection of GFP was accomplished with the ECL system and exposure to BioMax Light Film for Chemiluminescent Imaging (Kodak Co).

3.1.4 Inverse PCR for the identification of the P-element insertion site

The procedure for the identification of the disrupted gene included five steps:

1. Total genomic DNA isolation
2. Digestion
3. Ligation
4. PCR
5. Sequencing

Total genomic DNA isolation

30 anaesthetized flies of each P-element were placed in an eppendorf and put at -20ºC to freeze. Some minutes later, the flies were homogenized using a pestle in 100ul of solution A (Tris-HCl 0.1M, EDTA 0.1M, SDS 1% (v/v), DEPC 1%). The lysate was brought up to 200ul of solution A and was incubated at 70° for 30min. After this, 28ul of potassium acetate 8M were added and the mix was left in ice for 30min. To clear the lysate it was centrifuged for 15min at maximum speed in room temperature and the supernatant was transferred in a new eppendorf tube. Equal volume of phenol/chloroform was added, mixed and centrifuged 15min, room temperature at maximum speed. The upper phase was taken in a new eppendorf and 1/2 volume of isopropanol was added, mixed and centrifuged 5min, room temperature at maximum speed. The pelleted DNA was washed carefully with 300ul of 70% EtOH and centrifuged for 2min. After the pellet was allowed to dry for some minutes, the DNA was resuspended in 60ul of autoclaved, filtered milliQ water and stored in –20° for further use. The quality of the isolated DNA was analyzed by running 1 or 2ul of the resuspended sample in a 1% agarose gel.

Digestion

10ul of the isolated DNA were incubated at 37°C for 2 1/2 hours with the appropriate restriction enzyme (Sau3AI, HinP1I, or Msp I, see Figures 1 and 2 below for restriction enzymes and sequencing primers) and buffer in 20ul final volume. To stop the reaction the mix was heated at 80°C for 15min. The enzymes were purchased from Fermentas.

Ligation

The above restriction reaction (20ul) was incubated at 16ºC, overnight, after addition of 1ul of ligase and the appropriate ligation buffer, at a final volume of 25ul.

PCR

Reaction (40ul):

25ul DNA (whole ligation reaction)

4ul buffer 10x (without Mg)

2.4ul $MgCl_2$ (final 1.5mM)

0.02 – 0.2mM dNTP's mix

0.4uM each primer (primers are diluted 100uM in TE for the stock and 1:10 in water for use)

1ul Taq polymerase

Autoclaved, filtered milliQ water

Program:

94°C for 4min

94°C for 30sec
62°C for 30sec } *39 cycles*
72°C for 2min

72°C for 7min

4°C keep

5ul of the reaction was analyzed in 1% agarose gel and the rest was stored in -20ºC.

Sequencing

The sequencing of the PCR products was performed by the PRIMM Sequencing Facilities, using the appropriate primers from the below list:

Sp1 - CACACAACCTTTCCTCTCAACAAGCAA

Splac2 - CTTGGCACTGGCCGTCGTTTTAC

Pry2 - CTT GCC GAC GGG ACC ACC TTA TGT TAT T

Plac4 - ACT GTG CGT TAG GTC CTG TTC ATT GTT

Plac1 - CAC CCA AGG CTC TGC TCC CAC AAT

Pry4 - CAA TCA TAT CGC TGT CTC ACT CA

Plw3-1 - TGT CGG CGT CAT CAA CTC C

The sequences retrieved were blasted using the BLAST tool of FlyBase (http://flybase.bio.indiana.edu/blast/). The genes were identified according to the release 3.0 and 4.0 of the annotated *Drosophila melanogaster* genome.

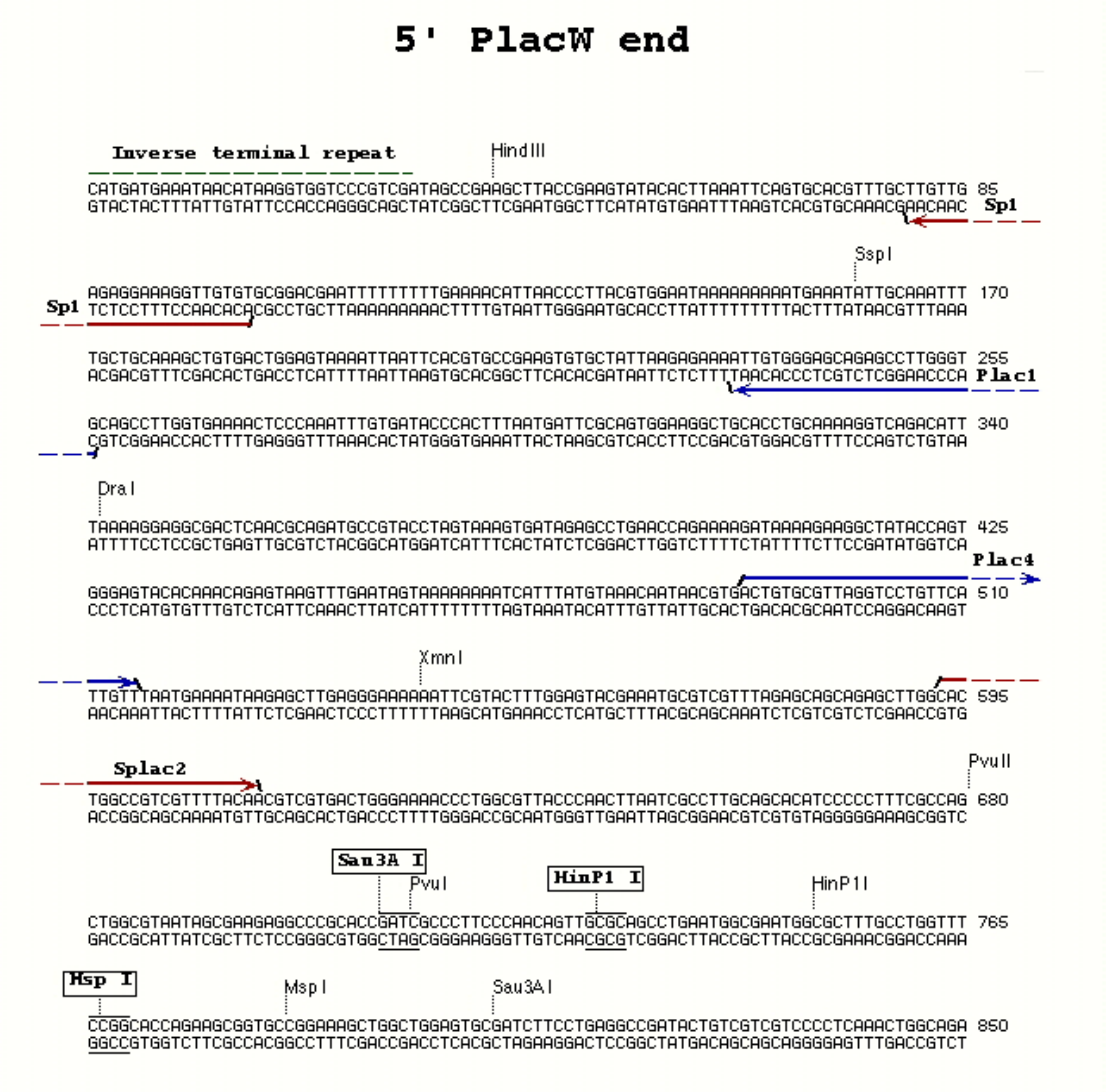


Figure 1. 5' terminal of the PlacW element. The restriction enzymes that were used are in squares and the red and blue arrows depict the primer combination used for the sequencing (taken from http://www.fruitfly.org/about/methods/inverse.pcr.html).

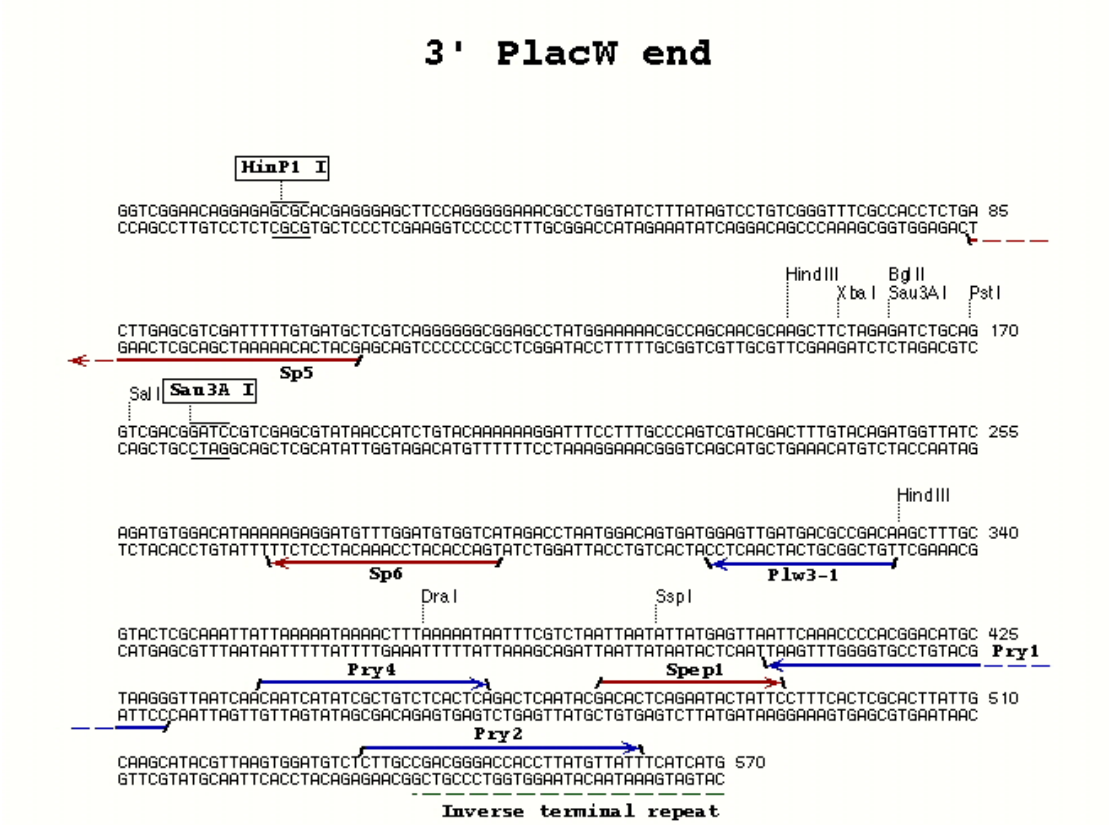


Figure 2. 3' terminal of the PlacW element. The restriction enzymes that were used are in squares and the red and blue arrows depict the primer combinations used for the sequencing (taken from http://www.fruitfly.org/about/methods/inverse.pcr.html).

3.2 Cell Biology

3.2.1 Cell lines

Human Embryonic Kidney (HEK) 293 cells and HEK293 cells stably overexpressing human APP695 were grown in Dulbecco's modified Eagle's minimum (DMEM) essential medium (GIBCO) supplemented with 10% heat inactivated fetal bovine serum (Perbio) and 100µg/ml penicillin/streptomycin (GIBCO). All cell lines were kept at 37°C with 5% CO_2.

3.2.2 Primary rat hippocampal cultures

Rat primary hippocampal cultures were prepared from embryonic day-18 brains as previously described (Kaech *et al.*, 2006). Briefly, the pregnant mother is killed by anaesthetization and cervical dislocation and the embryos are removed from the uterus under sterile conditions. The hippocampi of the embryos are dissected out and are dissociated by trypsinization and pipetting. After the cell count, $2x10^5$ cells are plated in appropriately prepared dishes. For

biochemical analysis, 12-well and 3cm plastic dishes were coated with poly-L-lysine dissolved in borate buffer at 0,1mg/ml. After an overnight incubation at 37°C the dishes are washed 2 times with sterile water and each wash is left 1h. Finally, 3ml of MEM-Horse (Minimal Essential Medium, 10% horse serum, 0.6% glucose, 1xL-glutamin) are added per 3cm dish or 1ml per well of the 12-well and they are returned to the incubator overnight. 6h after plating of the neurons the media are changed to pre-equilibrated Neurobasal medium with B27 supplement (NB-B27) (GIBCO). Neurons are kept under 5% CO2 at 37°C.

3.2.3 siRNA and plasmid transfection of cell lines

For our assays 100pmol of the siRNA oligonucletides were transfected per well in a 6-well dish using the Lipofectamine 2000 (Invitrogen) transfection reagent. One day before transfection the cells were split in the dishes in order to obtain around 80% confluency at the time of the transfection. The siRNA and 5ul Lipofectamine were each separately added to 250ul Opti-MEM (brand) and left 5min to stand in room temperature. After this time the two were carefully mixed and 20min later the complexes were added drop wise to the cells. For the plasmid transfections 4ug of DNA were transfected per well, with the difference that 10ul of Lipofectamine were used. Both, siRNA and plasmid transfections were incubated for 72h to allow for maximum efficiency of silencing or expression, respectively.

3.2.4 shRNA transfection of primary hippocampal neurons

For neuronal transfections the shRNAs of *Abca2* and *Habp4* were cloned in the pLentiLox3.7 vector (see molecular biology methods). 3DIV (days in vitro) neurons plated in a 12-well dish were transfected with 1.6ug of the plasmid and Lipofectamine 2000 for 72h.

3.2.5 Lentiviral infection of primary hippocampal neurons

For the infections primary hippocampal neurons were plated on 3cm dishes. After 3DIV the medium was removed and was replaced with 900ul of the same growth medium (NB-B27) containing the lentivirus in a dilution 1:70. The neurons were returned to the incubator at 37°C, 5% CO2 for 7h. After this period the virus was removed and the medium was replaced back to the original and the neurons were incubated for 5days more before the assays. A lentiviral vector that just expressed the *CMV-GFP-WPRE* cassette without any shRNA was used as control (Follenzi *et al*., 2000, VandenDriessche *et al*., 2002).

3.2.6 Cell viability

To measure cell viability and proliferation upon siRNA treatments of the cell lines we used the MTT kit I (Roche). This colorimetric assay is based on the capacity of metabolic active cells to produce purple formazan crystals by processing the yellow tetrazolium salt MTT. For this assay the cells were plated in 96-well microplates and 24, 48 and 72h post transfection with the siRNA the MTT reagent was added in a concentration of 0.5mg/ml. The plate was returned in the incubator for 4h and after this period 100ul of the solubilization solution was pipetted in the wells. After overnight incubation at 37ºC, 5% CO_2 the absorbance of the formazan was measured between 550-600nm on a microplate reader (Victor3 by PerkinElmer).

3.2.7 Intracranial stereotaxic injections *in vivo*

For the *in vivo* stereotaxic injections in the hippocampus we used female APP/PS1 Tg/w mice of 2 months old (Radde *et al.,* 2006). The mice were anesthetized with a mix of ketalar (50mg/ml), rompun 2%, atropine (0.5mg/ml) (1ml of each plus 1ml of saline), at a dose of 5ul/g. The cranium was revealed with a midsagittal incision and the coordinates for injection in the CA1 area of the hippocampus were found using the Mouse Brain Atlas.

2ul of the lentiviral construct expressing the shRNA of each, ABCA2 or HABP4, was injected with the rate of 0.2ul/min. The control mice were injected with the lentivirus empty vector. For analysis of Aβ secretion the mice were sacrificed 10days post-injection. The injected area of the hippocampus was dissected and homogenized in Tissue Protein Extraction Reagent (T-Per, Pierce), and after 1h centrifugation at 100000xg at 4ºC the supernatant was analyzed with the hAβ40 or hAβ42 ELISA kit (The Genetics Company).

3.3 Biochemistry

3.3.1 Enzyme-Linked ImmunoSorbent Assay (ELISA)

This assay is based on the formation of a "sandwich complex" between a monoclonal antibody already pre-coated on the microplate, the Aβ peptide in the sample and another biotinylated antibody against another epitope of Aβ. Indirectly, a Biotin-Streptavidin link couples this complex to an enzyme, which in turn converts a chromogen substrate to a colored product. The Aβ concentration is correlated to the absorbance of this product at 450nm. A

standard curve prepared using a synthetic Aβ peptide is included in the assay for the calculation of the concentration of Aβ in the sample.

For Aβ peptide detection in HEK293 cells overexpressing APP695 the cells were transfected with siRNA and the medium was replaced with fresh 48h post-transfection. Cells were grown for another 24h and 50ul of the media were assayed for Aβ load using the hAβ40 or hAβ42 ELISA kit (The Genetics Company). Rodent Aβ secretion was assayed using the Aβ40 ELISA kit (Wako) 72h post-transfection and 120h post-infection of primary hippocampal neurons, loading 10ul of the media.

3.3.2 Western blot

Lysis of cells

72h post-transfection the media were aspirated from the plates and the cells were washed once with ice cold PBS. The cells were scraped in STEN lysis buffer (1xSTEN: 50 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 0.2 % NP40; STEN-lysis buffer: 1% Triton X100, 1% NP40, complete protease inhibitors in 1xSTEN), incubated on ice for 30min and clarified by a 30min centrifugation at 13200rpm. The amount of protein in the lysate was evaluated with the BIO-RAD Protein Assay.

Electrophoresis and blotting

For the analysis of protein levels and expression, the samples were prepared with equal amounts of protein in Laemmli sample buffer (0.2M Tris pH 6.8, 0.04M DTT, 40% glycerol, 8% (w/v) SDS, 0.4% (w/v) bromophenol blue) and were boiled 5min at 95ºC to denature the proteins. The samples were loaded on SDS-acrylamide gel and were allowed to separate at 200V in a Mini-PROTEAN II tank (BIO-RAD). After electrophoresis the proteins were transferred on a 0.45um pore diameter nitrocellulose transfer membrane (Whatman, Schleicher & Schuell) at 250mA for 1h in a Mini Trans-Blot tank (BIO-RAD) and were incubated for 1h at room temperature in blocking solution (5% powder milk in 0.2 M phosphate, 1.5 M NaCl, 0.1% Tween-20 (PBS-T)). After the blocking, the membrane was placed in a bag containing a sufficient amount of the primary antibody in the appropriate dilution (see Antibodies section) and was incubated overnight at 4°C, shaking. Next day the membrane was washed 3 times with PBS-T and was incubated 1h at room temperature with the secondary antibody. For the detection of the proteins the ECL solutions were applied (GE

Healthcare), and the visualization was performed in a Fujifilm LAS3000 Image reader. Quantification of protein expression was done using the ImageJ software.

3.3.3 Immunoprecipitation from lysates and media

Immunoprecipitation of Aβ peptide and secreted APPα (APPsα)

For immunoprecipitation of intracellular Aβ the lysates were prepared as described above for the western blot analysis. Aβ secreted and APPsα were immunoprecipitated from media of the cells. 48h post-transfection the media of the cells were replaced by 1ml of fresh complete DMEM and were returned in the incubator for another 24h. At 72h lysates or conditioned media were placed in a 1.5ml eppendorf tube and the volume was filled up with STEN lysis buffer. The samples were then incubated rotating overnight with 0.5ul 6E10 and 40ul of protein G sepharose beads (Amersham). The following day the beads were washed twice with STEN-NaCl (STEN buffer with 500mM NaCl), and once with STEN buffer. Between each wash the tubes were mixed (without vortexing) and centrifuged 3min at 7000rpm at 4ºC to pellet the beads. After the last wash all the buffer was carefully removed with a 26G needle and the beads were resuspended in 10ul NuPAGE sample buffer (Invitrogen). After boiling the samples were loaded on a 4-12% gradient NuPAGE Bis-Tris Gel (Invitrogen) and mounted in an XCell SureLock Novex Mini-Cell (Invitrogen). After separation the proteins were transferred on nitrocellulose transfer membrane (Whatman, Schleicher & Schuell) using the Mini Trans-Blot chamber (BIO-RAD). In order to enhance the Aβ signal the membrane was afterwards boiled in 1xPBS for 5min and subsequently incubated for 1h at room temperature in 5% milk blocking solution and overnight with the required primary antibody. For the secreted Aβ and APPsα the detection antibody was the 6E10, while for the intracellular peptide the detection antibody was the 4G8. Next day, the mouse secondary antibody was applied for 1h at room temperature and the ECL system (GE Healthcare) was used for the detection of the immunoprecipitated proteins.

Immunoprecipitation of full length APP, carboxy-terminal fragments (CTFs) and total secreted forms of APP (APPs)

For the analysis of the APP proteolytic products we used metabolic labeling of APP (see Pulse-Chase experiments section). The membrane-tethered CTFs and the total secreted fragments of APP were immunoprecipitated from the lysates and the media of the transfected cells, respectively. Lysates were again prepared as described above. The immunoprecipitation

procedure was the same as for the Aβ peptide with the following alterations: CTFs, APPfl and APPs were precipitated with the APP-Ct antibody (Sigma) (CTFs) and the 22C11 N-terminal antibody (Chemicon) (APPfl, APPs). Radiolabeled proteins were visualized after exposure on Hyperfilm autoradiography films (Amersham).

3.3.4 Antibodies

For immunoprecipitation and detection of Aβ and secreted APPα the 6E10 antibody (Sigma) or for Aβ alone 4G8 (Sigma) was used. APP C-terminal fragments as well as AICD were precipitated with APP Ct antibody (Sigma). Total secreted APP and APP full-length were detected with the 22C11 N-terminal antibody (Chemicon). Presenilin was detected with the SB129 antibody, Nicastrin with the 9C3 antibody (Esselens *et al.*, 2004), APH1a with the B80.3 (Nyabi *et al.*, 2003) and PEN2 (Nyabi *et al.*, 2003) with the B126.2 antibody. γ-tubulin was detected using Sigma antibody. For the detection of myc-tagged proteins 9E10 antibody of Santa Cruz was used.

3.3.5 Pulse-chase experiments

Pulse-chase experiments are used in order to track a cellular procedure and it involves the sequential radioactive labeling (pulse) of the cells, when a radioactive molecule is used by the cell in the procedure of interest, and the return to the supply of non-radioactive molecule (chase). In our case 72h post siRNA transfection HEK293 stably overexpressing APP695 were starved with 1ml of starvation medium lacking methionine in order to stop translation for 45min and labeled with radioactive methionine/cysteine for 2h. For the proteolytic products of APP the cells were chased for 4h and lysates and media were analyzed, by immunoprecipitation, for CTF generation and APPs secretion, respectively. To monitor APP maturation through the early secretory pathway cells were lysed at 0, 15, 30, 60 and 120min and APP was precipitated with the APP-Ct antibody. Radiolabeled proteins were visualized with phosphoimaging.

3.3.6 Luciferase assays

This method is based on the principle that once gal4-VP16 is liberated from a reporter construct it will translocate to the nucleus, bind to the UAS (Upstream Activating Sequence) and activate luciferase expression. Firefly luciferase catalyzes luciferin oxidation to oxyluciferin. Converting the chemical energy of luciferin oxidation through an electron

transition, forming the product molecule oxyluciferin, produces light. For our assays the reporter constructs that were transfected in combination with the siRNA oligonucletides targeting our candidate genes were the following:

- **C99-Gal4-VP16** and **UAS-luciferase** for the AICD generation evaluation (Serneels *et al.*, 2005)
- **NotchΔE-Gal4-VP16** and **UAS-luciferase** for NICD production evaluation (Serneels *et al.*, 2005)

After 48h the transfected HEK293 cells were lysed and processed according to the manufacturer's instructions (Promega). Briefly, after a PBS rinsing the cells were scraped and lysed in 1x Lysis buffer provided in the assay kit. The lysates were cleared by centrifugation and part of the supernatant was mixed with the Luciferase Assay Reagent. Emitted light was measured with the microplate reader (Victor3 by PerkinElmer). γ-secretase cleavage of APP or Notch was determined as the luciferase activity of the γ-secretase-dependent variant (C99-Gal4-VP16, NotchΔE-Gal4-VP16) minus the activity of the luciferase alone.

3.3.7 Secretase assays

For the quantification of the enzymatic activity of α-, b- and γ-secretase, the respective Activity Kits (R&D Systems) were applied. In these assays the secretase activity is determined by adding a secretase-specific peptide conjugated to the reporter molecules EDANS and DABCYL. Before cleavage, the physical proximity and the identical absorption wavelength of the two molecules (495nm) does not allow detection of the fluorescent emissions of EDANS. Secretase cleavage causes separation of EDANS and DABCYL and release of a fluorescent signal. This fluorometric reaction is directly proportional to the secretase activity and can be measured with a microplate reader. HEK293 cells overexpressing APP695 were transfected with the siRNA oligonucleotides and 72h later the cells were washed once and scraped gently in 1x PBS, and collected by brief centrifugation at 300g. Pelleted cells were lysed by adding 1x Cell Extraction Buffer, incubation on ice for 30min and centrifugation at 10,000g for 1min. 50ug of protein, 50ul of 2x Reaction Buffer and 5ul of the secretase-specific Substrate were loaded on a black microplate in duplicates. The plate was incubated for 2h in the dark at 37ºC and emitted light was measured at 495nm after excitation at 355nm(Victor3 by PerkinElmer). No cell lysate and no substrate wells were used as negative controls.

3.4 Molecular Biology

3.4.1 RNA isolation

The isolation of RNA from cells, human brain and mouse brain was achieved with the RNeasy Plus Kit (Qiagen). The cells were lysed using the RTL lysis buffer provided in the kit. For the isolation of RNA from mice, C57BL/6 animals of 1, 3, and 20months old were sacrificed by cervical dislocation and brains were isolated. Cerebellum, cortex and hippocampus were separated and homogenized in the RNeasy Plus lysis buffer. Human brain samples were also homogenized in the same buffer. The quantity of the RNA was determined using the NanoDrop spectrophotometer (Isogen Lifescience). The quality of the RNA was analyzed on a 1% agarose gel.

3.4.2 Reverse transcription for cDNA preparation

The cDNA was prepared from the RNA with the RevertAid First Strand cDNA Synthesis kit from Fermentas. The reaction mixture was the following:

500ng RNA

1ul random hexamer primer (0.2µg/ul)

DEPC-treated water to 12µl

The reaction was incubated at 70°C for 5min and after chilling on ice the below components were added:

4ul 5x reaction buffer

1ul RiboLockTM Ribonuclease Inhibitor (20u/ul)

2ul 10mM dNTP mix

After 5min at 25ºC 1ul of the RevertAidTM M-MuLV Reverse Transcriptase (200u/ul) was added and the mixture stayed another 10min at 25°C. The reaction was finally incubated at 42°C for 60min and stopped by heating at 70°C for 10min.

3.4.3 Real-Time PCR

The Real Time Polymerase Chain Reaction allows the simultaneous detection and quantification of the target DNA. This is achieved by utilizing fluorescent dyes that intercalate with the double-stranded DNA and this DNA is quantified in each cycle. In our case we used the SYBR green as the intercalation dye.

The reaction was set as follows:

6ng RNA (calculated from the amount used for the reverse transcription)

500nM each of the primer

1x reaction buffer (SYBR Green PCR MasterMix, RT-SN2X-005+WOUN, Eurogentec)

Autoclaved and filtered milliQ water to 25ul final reaction volume

The plates were the MicroAmp Optical 96-Well Reaction Plates with Barcode and the respective Optical Adhesive Films (Applied Biosystems). The PCR machine was an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The quantification is performed against a control housekeeping gene, namely b-actin. The primers were designed with the online tool of Invitrogen, OligoPerfect Designer, and are the following:

Primers for the mouse genes

APP770 F	AAG AAC TTG CCC AAA GCT GA
APP770 R	GTC TCT CAT TGG CTG CTT CC
Ddx17 F	AGG AGT ATG CTG CTG GCA CT
Ddx17 R	CAG ATC GGC CTA TCC CAC TA
Ddx5 F	CAG CAA GTG GCT GCT GAA TA
Ddx5 R	ACG AAT CTG TGG TCC TTT GG
PS1 F	GGT ACC CAA GAA CCC CAA GT
PS1 R	CTC CCA CTC CTC ACT GAA GC
Habp4 F	TGA TAC AGA GCC ACC TGC AC
Habp4 R	GAA CTT TGG CTG CAG ACT CC
HNRPR F	CCA GAA GCA CTG CGT ATG AA
HNRPR R	CTC TCA TTG GAG GTG GCA TT
E2f3 F	ATG ATA CGT CCC TCG GTC TG
E2f3 R	GGC TCA GGA GCT GAA TGA AC
IDB1 F	TCC TGC AGC ATG TAA TCG AC
IDB1 R	GTG GTC CCG ACT TCA GAC TC
TIF1a F	CTC TGG CTC TTC CAG CAA AC
TIF1a R	TGG CTC CAG CAT GAC TAC TG
Tmcc2 F	CAG CTG AAC GAC CTG ACT GA

Tmcc2 R	CAG CTC CTG CTT TAG GTT GG
Rbpms F	ACA CGA AGA TGG CCA AGA AC
Rbpms R	ACA GTG TTG GGC AGA GGA GT
Armet F	CAT TGG AGC CAC AGA TGA TG
Armet R	GGC TTC GAC ACC TCA TTG AT
Prim2a F	CCA AGC CTA AGT GGG ATC AA
Prim2a R	CAT CAG CAA AGG GGA CCT TA
Elovl1 F	TTG TCC TGG TCT CAC TGC AC
Elovl1 R	GGA TGA TGA TGG GGT ACT GG
Ttk F	ACC AAG CAC ATG TTC ATC CA
Tttk R	GTA ATC GCG TAG CGA GAA GG
Ccna2 F	GTC TCT TTA CCC GCA GCA AG
Ccna2 R	TGA TTC AAA ACT GCC ATC CA
Abca2 F	CCT GCT CTT CAC CTC TGA CC
Abca2 R	CCA AAG GAT GCT GGG ATA GA
Atp5g2 F	TGG GAC TGT TTT TGG GAG TC
Atp5g2 R	CCA GAA TCG CGT AGG AGA AG
Pairbp1 F	CCC CGT GAA AGA AGA TTT G
Pairbp1 R	GCC GTT CGA TAA TCG GTC TA
b-actin F	GAC GGC CAG GTC ATC ACT AT
b-actin R	AAG GAA GGC TGG AAA AGA GC

Primers for the rat genes

Abca2 F	GCA CCG AGC TCT CCT ACA TC
Abca2 R	TGT GCT CCA ATT GCT GAA AG
b-actin F	GTC GTA CCA CTG GCA TTG TG
b-actin R	ACC CTC ATA GAT GGG CAC AG
Habp4 F	ATG ACA ACA GGG GGA CAG AC
Habp4 R	CCT GTA GGA CCT TCG CTC TG

Primers for the human genes

hActin-b F	GGC ATC CTC ACC CTG AAG TA
hActin-b R	AGG TGT GGT GCC AGA TTT TC
hHabp4 F	ACG CTT TTG ACC AGA GAG GA
hHabp4 R	ACT CCA CAT CCA CCC ATG TT
hDdx5 F	TAT GGT TGG AGT GGC ACA GA
hDdx5 R	GAC AAT GGC AGG AAG CAA AT
hPairbp1 F	CTA TTC GAG GTC GTG GTG GT
hPairbp1 R	GCC ACG AGA ATC AAA TCC AT
hABCA2 F	GTG TTC AGC AAG ATG GAG CA
hABCA2 R	CAT TGT CCA GTG TGG TCT GG
hATP5G2 F	GGC AGT CTC ATG TCC CCT TA
hATP5G2 R	TGT GTC GAT GTC CCT TGA AA

3.4.4 siRNA and shRNA design

The siRNA sequences were chosen from the predesigned siGenome siRNA collection available at www.dharmacon.com. The sequences of the duplexes were the following (5' to 3'):

hAbca2

Sense: ACAAGAAGCUGGCCCUGAAUU

Antisense: P-UUCAGGGCCAGCUUCUUGUUU

hAtp5g2

Sense: CAACAGCUCUUCUCCUACGUU

Antisense: P-CGUAGGAGAAGAGCUGUUGUU

hHabp4

Sense: GACCAAAGCCUGAGUUUAAUU

Antisense: P-UUAAACUCAGGCUUUGGUCUU

hDdx5

Sense: GCAAGUAGCUGAAUAUUU

Antisense: P-AUAUUCAGCAGCUACUUGCUU

hPairbp1

Sense: CAACCGAUUCGACCAGUUAUU

Antisense: P-UAACUGGUCGAAUCGGUUGUU

The shRNA oligonucleotides for Abca2 and Habp4 were designed to match the mouse and rat sequences and the loop sequence (underlined) was chosen according to Brummelkamp et al., 2002. *NotI-XhoI* restriction sites and *HpaI-XhoI* restriction sites were flanking the Abca2 and Habp4 shRNA sequences, respectively, for the insertion in the pLentiLox 3.7 vector (see Cloning section).

Abca2 (5' to 3')

Sense: CCC CCT CGA GGC TCT AGA GCC ATT ATC TTA GTT CAA GAG ACT AAG ATA ATG GCT CTA GAG CGC GGC CGC CCC C

Antisense: GGG GGC GGC CGC GCT CTA GAG CCA TTA TCT TAG TCT CTT GAA CTA AGA TAA TGG CTC TAG AGC CTC GAG GGG G

Habp4 (5' to 3')

Sense: TGA CCA AAG CCT GAG TTC AAT ATT CAA GAG ATA TTG AAC TCA GGC TTT GGT CTT TTT TC

Antisense: TCG AGA AAA AAG ACC AAA GCC TGA GTT CAA TAT CTC TTG AAT ATT GAA CTC AGG CTT TGG TCA

3.4.5 Cloning

The oligonucleotides encoding shRNA specific for *Abca2* (flanked with *NotI-XhoI* restriction sites) and for *Habp4* (flanked *HpaI-XhoI* restriction sites) were annealed and cloned into the corresponding restriction sites of the pLentiLox3.7 lentiviral vector backbone (Dharmacon, PerBio Science). The pLentiLox 3.7 lentiviral vector expressed the shRNAs from a U6 promoter and also contained an expression cassette composed of the cytomegalovirus promotor (*CMV*), green fluorescent protein (*GFP*) and Woodchuck hepatitis virus post-transcriptional regulatory element (*WPRE*) to independently monitor transduction efficiency *(CMV-GFP-WPRE)*.

For the annealing the following reaction was set:
10ul sense oligo (60pmol/ul)
10ul antisense
5ul NEB2 buffer (New England Biolabs)
25ul water

The annealing was performed in a PCR machine using the following program:
95ºC for 4min
65ºC for 10min
65ºC for 1min (x40, decreasing 1ºC after each cycle)
25ºC for 10min
4ºC keep
In order to create the insertion site for the shRNAs 1ug of the vector was digested with a combination of NotI-XhoI (for the Abca2) or HpaI-XhoI (for the Habp4) restriction enzymes (Fermentas) for 1h at 37ºC per enzyme, and was subsequently dephosphorylated by treating with CIAP (Fermentas) for 1h at 37ºC. The linear vector was ligated with the shRNA oligos at equimolar concentration at 16ºC overnight. The ligation was evaluated by transformation of competent E. coli cells, isolation of the plasmid (see Transformation and Plasmid amplification section) and digestion with the same restriction enzymes used for the shRNA insertion. The insert was analyzed on a 1% agarose gel.

3.4.6 Transformation and plasmid amplification

The competent cells for the transformations were the DH5a strain of E.coli from Invitrogen. 1ul of plasmid was added in the bacteria and they were incubated 30min on ice. After 45sec of heat-shock at 42ºC and 2min chilling on ice, 1ml of pre-warmed LB medium (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, pH 7.5) was added and the bacteria were left for 1h at 37ºC shaking. 50ul and 200ul were afterwards plated on agar plates containing ampicillin for drug-resistance selectivity and the plates were incubated overnight at 37ºC. The next day a single colony was picked and grown in LB medium, in a volume according to the kind of prep performed. Plasmid isolation from the culture was accomplished with the Qiagen mini, midi or maxi prep kits.

3.4.7 Lentiviral vector generation

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine (Gln), 100IU/ml penicillin, 100ug/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS, Invitrogen). Cells were grown in cell factories and/or single tray units (Nalge Nunc International) during viral vector production in medium with 10% Nu-Serum IV instead of FBS ("serum-free" medium; Becton Dickinson). To produce lentiviral vectors pseudotyped with the vesicular stomatis virus glycoprotein (VSV-G), 293T cells were transfected when 90-95% confluent using a modified calcium phosphate transfection protocol (Life Technologies) (VandenDriessche *et al*., 2002, 2007, Thorrez *et al*., 2006). Plasmid DNA was extracted using Qiagen Maxi or Mega kits. The following amount of DNA was used per cell tray: 120ug of lentiviral vector DNA, 60ug of *pMDL gag/pol RRE* helper plasmid, 60ug of Rev expressing plasmid, 60ug pCI-VSV-G envelope-encoding plasmid, which were previously described (Follenzi *et al*., 2000).

24h post-transfection, medium was removed, cells were washed with PBS and 100 ml "serum-free" medium containing 1.1mg/ml sodium butyrate (Sigma) was added per cell tray. Conditioned medium was collected every 24 hr-interval during the subsequent 2 days. After pre-filtration using a 0.45µm filter (Corning), the conditioned medium was applied on a Centricon Plus-80 centrifugal filter device (Millipore, 100 kDa nominal MW cut-off) and centrifuged for 100min at 1500rpm (10°C). The retentate could be retrieved by centrifuging for 2min at 2000rpm (10°C). A 200-fold concentration could be achieved using this method. Functional titers of the lentiviral vectors were determined by transducing 293T cells fibroblasts seeded in a 6-well plate ($3x10^5$ cells per well) with serial dilutions of the concentrated vector-containing supernatant supplemented with 8ug/ml polybrene. Since GFP was used as a marker, the number of fluorescent cells per microscopic field (5 fields) were counted 48 hours post-transfection, on which basis the total number of fluorescent cells per ml viral vector-containing supernatant could be calculated (= titer in TU/ml, transducing units per ml). Lentiviral vector titers typically fell in the range of $1\text{-}2x10^7$ TU/ml.

Section 4

Results

4. Results

4.1 *In vivo* identification of APP processing modulators

4.1.1 Genetic screens for the detection of molecules that affect APP metabolism

a. Crosses: P-element x GMR-APP-GAL4,UAS-GRIM/Cyo

Progeny scored: GMR-APP-GAL4,UAS-GRIM/P-element

To identify genes with a role in the regulation of Aβ production *in vivo* we utilized a *Drosophila melanogaster*-based screen (Guo *et al*., 2003). This consists of *Drosophila melanogaster* expressing a chimeric human APP protein comprised of the C99 transmembrane protein fragment of APP fused to the yeast transcription factor GAL4-VP16 (GV) and the cell-death reporter gene GRIM, cloned under the GAL4 responsive transcriptional cassette (UAS). Upon C99 cleavage the GV is released and translocates to the nucleus where it binds to the UAS and activates expression of the reporter gene. Expression of GRIM causes what is known as the "rough-eye" phenotype, because of the death of the ommatidia (**Fig. 1**). The presence of the GMR promoter in front of the C99 leads to the targeting of the GRIM effect only in the eyes of the animals, allowing, thus, fly survival.

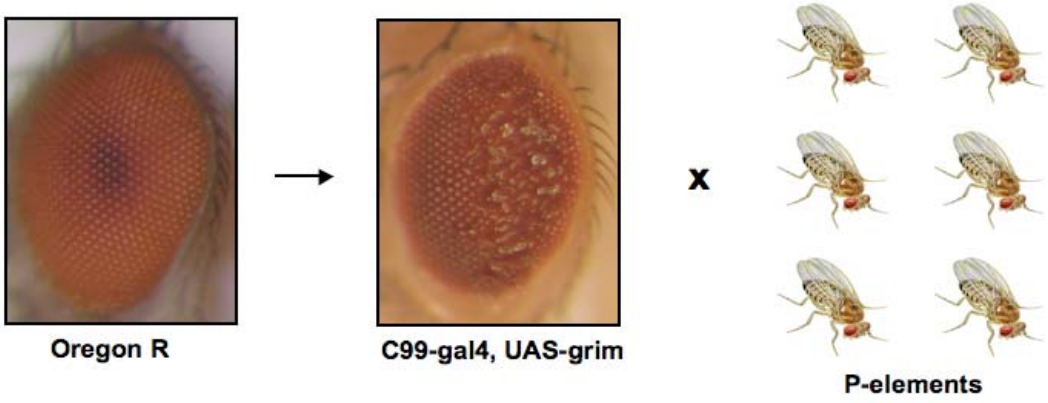


Figure 1. 776 mutants were crossed to the reporter flies that express a cell lethal gene (GRIM), specifically in the eyes. The roughness, compared to the eye texture of the wild-type Oregon R flies, is C99-cleavage-dependent.

Cyo is the dominant marker present in the balancer chromosome and makes the wings of the fly curly. The balancer chromosomes are used in order to preclude meiotic recombination of

homologous chromosomes, permitting the maintenance of mutations in heterozygosis (Fly Pushing: The Theory and Practice of Drosophila Genetics, Ralph J. Greenspan).

The C99-GAL4,UAS-GRIM flies were crossed with each of the mutants from a collection of 776 P-elements known to induce lethality in homozygosis in approximately 30% of the genes sited on the *Drosophila* third chromosome (Deak *et al*., 1997). When crossed with the reporter line, 194 mutants could produce a change in the eye cell-death phenotype. Changes were seen in eye surface texture and varied in severity. **Figure 2** illustrates the principle of the screen, and **Figure 3** provides two examples of the extreme phenotypic changes, amelioration and worsening.

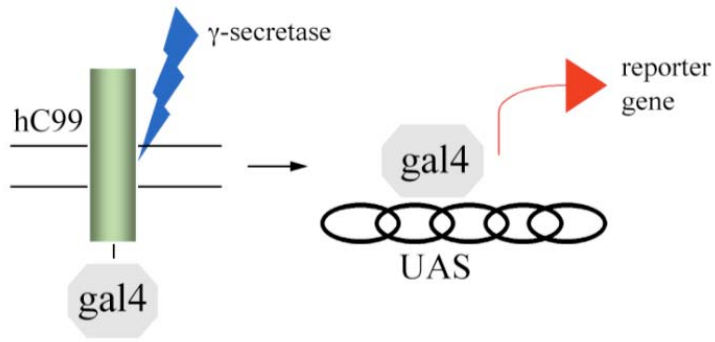


Figure 2. Schematic representation of the *Drosophila* read-out system. γ-secretase cleavage of C99-Gal4-VP16 releases the C-terminal part of C99 that is fused to the Gal4-VP16 sequence, which in turn translocates to the nucleus, binds UAS and activates reporter gene expression.

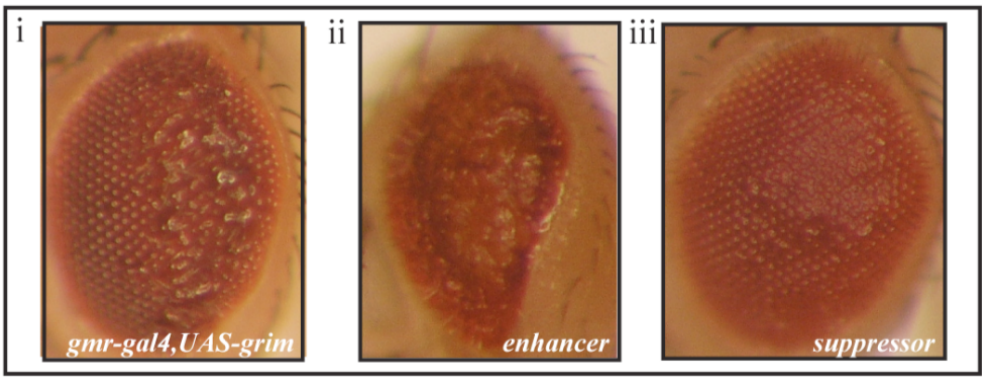


Figure 3. Analysis of the effect of mutations on a GRIM-induced rough-eye phenotype. Expression of the cell death activator reporter GRIM produces a rough-eye phenotype. P-element induced gene mutations were crossed to the reporter stock **(i)** and progeny was analyzed for enhancement **(ii)** or suppression **(iii)** of the reporter phenotype.

b. Crosses for the generation of homozygous mutant clones:

P-element x Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT80M/TM2

Or P-element x Eyflp; APP-GAL4,UAS-GFP/Cyo; FRT82M/TM6b

Progeny scored: APP-GAL4,UAS-GFP/ P-element

To further characterise the above mutants, the clonal analysis strategy was combined with an inducible readout system (**Fig. 4**). The generation of homozygous clones is a technique widely used in *Drosophila melanogaster* because it allows the study of mutations that otherwise would be lethal in homozygosis (for example mutations of essential genes). In combination with the tissue-specific targeting of the clones, in our case in the eyes, the lethality is prevented and both recessive and dominant mutations can be screened in the first generation of progeny. Also, the GFP reporter assay was chosen for the study of the mutations in homozygosis in order to avoid lethal interactions between homozygous P-elements and the cell death activator GRIM.

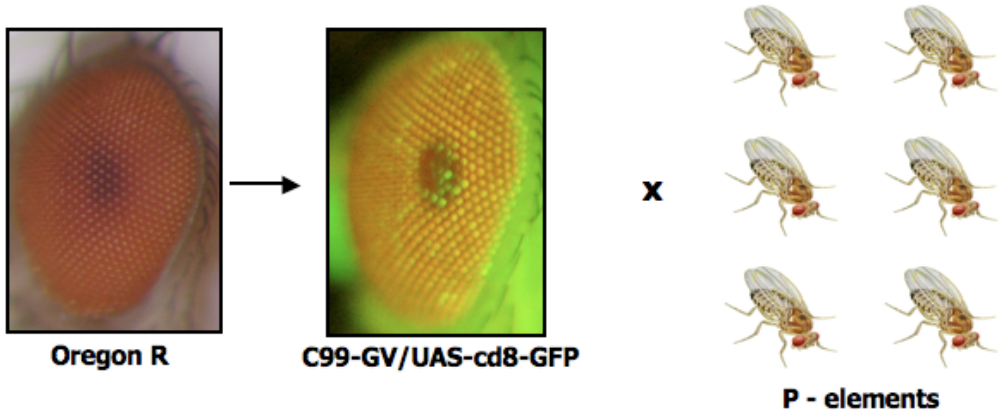


Figure 4. The positive mutants that were retrieved at the first screen were then crossed with a reporter fly that allows for the generation of mutant clones only in the eyes. The expression of GFP is again C99-cleavage-dependent and inducible by heat-shock.

We generated isogenic stocks of recombinant flies containing the C99-GAL4 construct together with the UAS-cd8-GFP reporter gene and a heat shock promoter to induce the expression of the C99-GV protein exclusively in adult tissues. For this series of experiments we also took advantage of the eyFLP/FRT technique for targeting mitotic clones to the eye, which allowed us to generate homozygous mutant clones occupying the eye in flies that also carried the C99-GV,UAS-cd8-GFP reporter system in the genetic background (**Fig. 5**). This

approach enabled us to test the effect of homozygous mutations exclusively in adult tissue, without compromising fly survival. The markers on the balancer chromosomes were either the *TM2* or *TM6b*, for larger alters and more hair shoulder or short and fat body, respectively.

These flies were then crossed with the positive clones from the previous, C99-GAL4,UAS-GRIM screening, and progeny bearing homozygous mutant clones in their eyes were collected during five days, heat shocked for 1h at 38ºC and scored 24h later for analysis of suppression or enhancement of GFP expression (**Fig. 6)**. This screen reduced the total number of positives, both enhancers and suppressors to 127 mutant lines.

It should be noted that even though we did not test whether the mutations affect GFP expression alone, intuitively we can hypothesize that it does not, considering also that GFP is quite a neutral protein in the cell and rarely affects or is affected by other proteins.

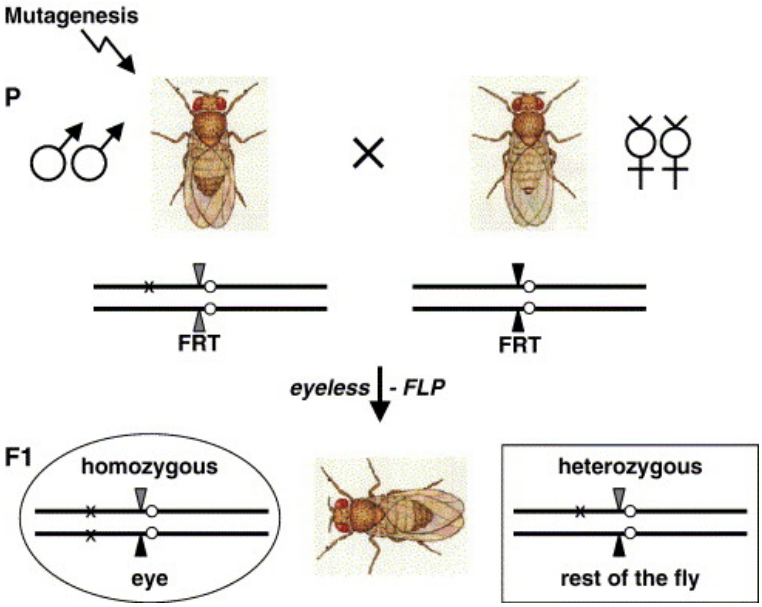


Figure 5. Male flies carrying the mutation, in our case the P-element, are crossed to females bearing the eyFLP. Both parents have the FRT. Mitotic recombination at the FRT site, and exclusively in the eyes because of the eyeless promoter (see also materials and methods), generates progeny with the homozygous mutation in the eyes and heterozygous the rest of the body. The mutation is depicted with the "x" (from Chen and Mardon, 2005).

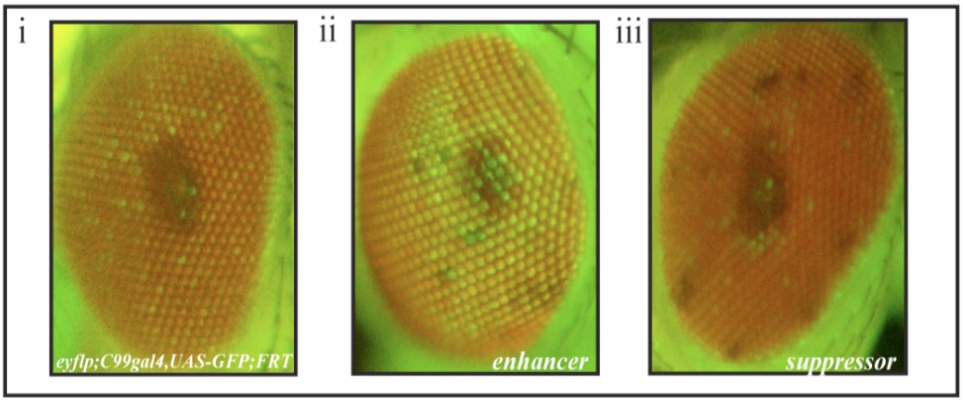
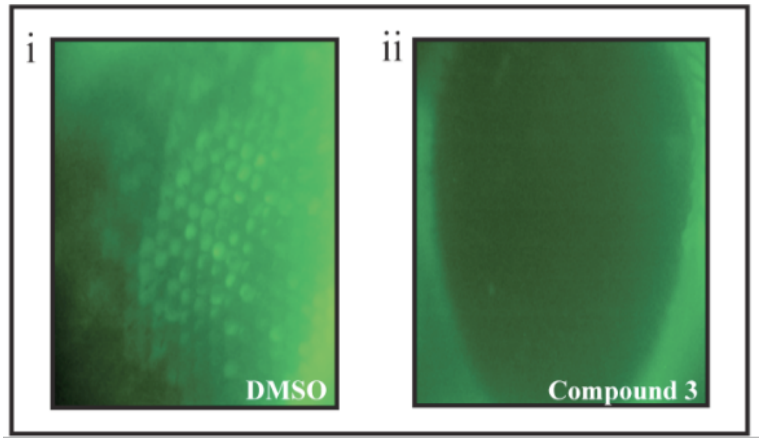


Figure 6. Clonal analysis of the mutations' effect on an inducible read-out system. For the generation of homozygous mutant clones in the eyes, flies that have the heat-shocked inducible C99-GV/UAS-cd8-GFP reporter system were crossed to mutant flies. The progeny was collected during five days, heat shocked for 1h at 38ºC and scored 24h later for suppression or enhancement of GFP expression. Panels **(ii)** and **(iii)** depict examples of enhanced and decreased GFP expression, respectively, compared to flies that do not carry any mutation **(i)**.

To demonstrate that GFP levels represent a valid indicator of γ-secretase activity, C99-GAL4,UAS-cd8-GFP adult flies were fed with two well characterized, potent and specific γ-secretase inhibitors, Compound 3 and DAPT, during five days at 20mM and 50mM, respectively (Micchelli *et al*., 2003, Seiffert *et al*., 2000, Dovey *et al*., 2001). The drugs were added in the fly food and the flies were left at 25ºC for 5 days. Then, C99-GAL4 expression was induced by heat shocking at 37ºC for 1h, and the levels of GFP measured 24h later. In this assay clear suppression of GFP levels was obtained (**Fig. 7**) compared to flies fed only with DMSO. Thus, mutants that gave altered GFP levels represent good candidates of true modulators of γ-secretase in photoreceptor neurons.

A



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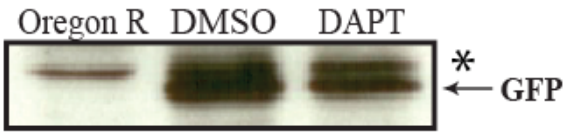


Figure 7. Analyses of correlation of GFP expression with γ-secretase activity. (A) Flies were fed with 20mM Compound 3 and DMSO as control for 5 days. 24h after heat shock at 38ºC for 1h the GFP expression was evaluated under a microscope with a fluorescent lamp and was found reduced in the Compound 3 treated flies. **(B)** Flies were fed with 50mM DAPT and DMSO as control for 5 days. After this time the flies were heat shocked at 38ºC for 1h and lysates from whole flies were prepared 24h later. DAPT treatment could decrease GFP expression compared to DMSO fed flies. Oregon R flies were used as negative control of GFP expression. The band marked with the asterisk is a non-specific band, therefore also appears in lysate from wild-type, non-GFP carrying flies.

c. Control crosses with a constitutive rough-eye phenotype:

P-element x GMR-GAL4,UAS-G/RPR / TM3

Progeny scored: GMR-GAL4,UAS-G/RPR/P-element

In order to exclude the possibility that the rough-eye phenotype we used as a read-out in the first genetic screen is due to genetic interaction of GRIM with the P-element or activation of any apoptotic pathway, we performed a screen testing the mutations against a reporter fly that presents the rough-eye phenotype independent of C99 cleavage. This fly strain carries the chimeric protein G/REAPER (G/RPR), which contains the N-terminus of GRIM and the C-

terminus of the protein REAPER, another cell-death activator. The GMR–GAL4,UAS–GRIM flies, initially intended to be used as false positive controls, are reported to be lethal (Guo *et al.,* 2003). For that the chimeric protein G/RPR was chosen. Expression of this protein leads to caspase-dependent cell death, independently of APP cleavage (**Fig. 8**).

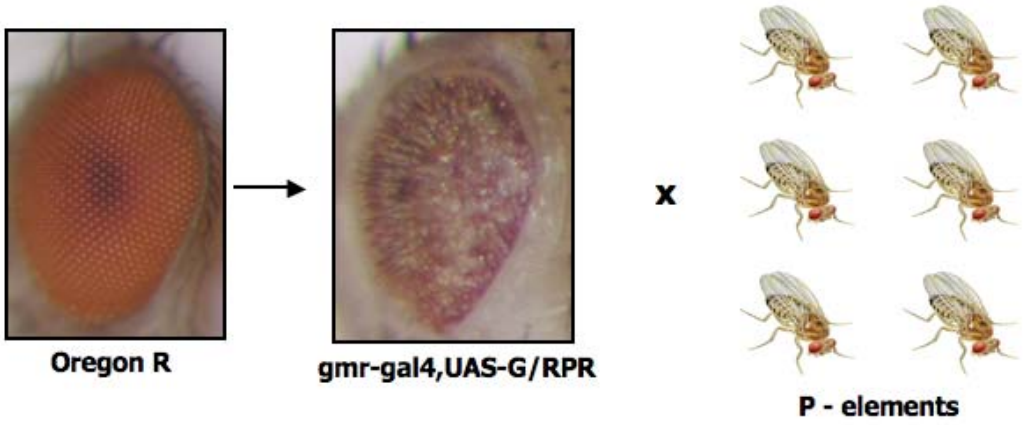


Figure 8. As a control screen to determine if the mutations indeed affect the cleavage of C99 or merely activate other apoptotic pathways, we crossed the P-element mutants to a reporter fly that expressed constitutively in the eyes a chimeric protein with the N-terminus of GRIM and the C-terminus of REAPER. REAPER is also a cell-death activator. The expression of this chimeric protein is independent of C99 cleavage and, therefore, should not be affected by mutations.

TM3 in the genotype shows the marker on the balancer chromosome, which stands for larger alters and more shoulder hair. For the screen the positive mutants of the C99-GAL4,UAS-GRIM screen were crossed to the GMR-GAL4,UAS-G/RPR stock and the ones able to alter the rough-eye phenotype (74 mutants) were excluded from further studying **(Fig. 9).**

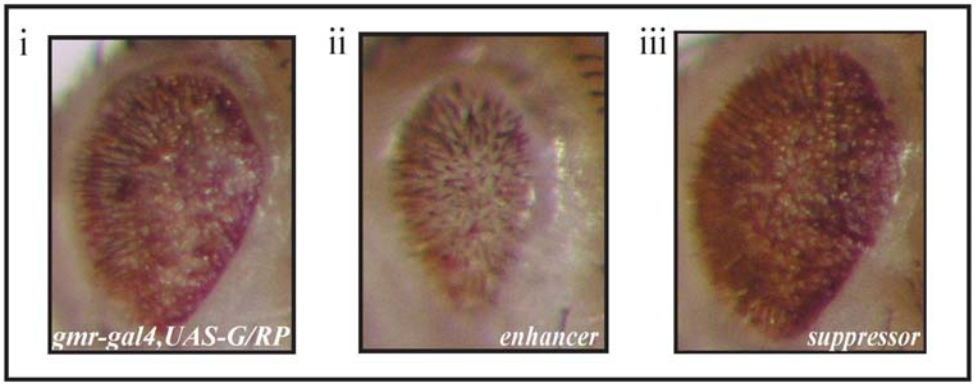


Figure 9. Analysis of the effect of mutations on a C99 cleavage-independent rough-eye phenotype. Constitutively expressed G/RPR leads to caspase-dependent cell death **(i)**. Mutants that altered this phenotype as depicted in panel **(ii)** and **(iii)** were considered as false-positive modulators of C99 cleavage and were omitted from further studying.

4.1.2 Analysis of the effect of putative modulator P-elements on Notch signaling

Crosses: P-element x Ne511/FM6; Df(N+)/TM2

Progeny scored : Ne511/FM6; P-element /TM2

In *Drosophila*, the formation of the wing is a complicated process that requires Notch signaling. Specifically, Serrate and Delta that are the Notch ligands in the fly activate Notch, which in turn triggers the transcription (through the Suppressor of Hairless) of the *vestigial*, *wingless* and *cut* genes (Sotillos *et al.,* 1997, Glise *et al.,* 2002, Kankel *et al.,* 2007). Therefore, it becomes obvious that affecting the Notch pathway would result to a phenotype at the wing. Additionally, *Notch* is a haplo-insufficient gene and loss of one copy produces a dominant mutant phenotype (Celis and Bray, 2000). Ne511 is a null, loss-of-function allele of *Notch* that produces, in heterozygosity, a dominant phenotype in the morphological pattern of veins and margin of *Drosophila* wings (Murata *et al.,* 1996, Glise *et al.,* 2002, Kankel *et al.,* 2007) (**Fig.10**).

After the completion of the three aforementioned screens we were having 74 mutant lines, out of which 55 were acting as enhancers and 19 as suppressors. To identify which of these mutants have substrate specificity, in terms of not perturbing the Notch pathway, all positive clones from the previous screen were crossed with the flies carrying this null allele of *Notch* (**Fig. 10**). *TM2* is a marker for larger alters and more shoulder hair present in the balancer chromosome.

Although we recognise that this screening was not the most stringent for Notch signaling analysis, it was sufficiently good to make an initial narrowing down of the number of genes to later test in mammalian cells as possible modulators with substrate specificity. In fact, this screening reduced to 16 the number of mutants that did not affect Notch signaling.

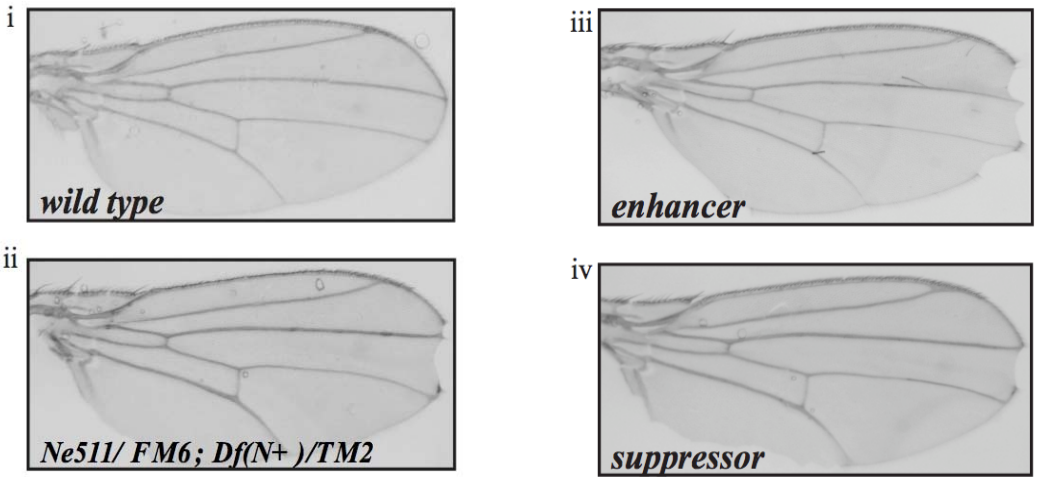


Figure 10. Evaluation of the effect of the positive candidates on Notch signaling pathway. Ne511/FM6;Df(N+)/TM2 flies contain a null allele of Notch, which in heterozygous state produces a dominant phenotype in the wings characterized by nicking of the wing margin **(ii)** compared to wild type w1118 flies **(i)**. Panel **(iii)** shows an example of a modulator that worsened the phenotype, while panel **(iv)** a modulator that did not.

4.1.3 Identification of the P-element insertion site and affected gene in the candidate lines

The P-element is inserted in a random position in the 3rd chromosome. So, in order to identify which gene is interrupted by this insertion we isolated the genomic DNA and digested it with restriction enzymes specific for cutting once in the insert and expected to cut at least once in the genome. The linear fragment, containing part of the 3' or 5' end of the insert and part of the interrupted gene, was then ligated and amplified by PCR. The PCR product was sequenced using primers either for the 3' end or the 5' end, and the Drosophila gene was determined with the BLAST tool of FlyBase. We could identify 16 genes, 8 enhancers and 8 suppressors of APP cleavage. **Table 1** provides the names and accession numbers of the 16 genes isolated from the screens.

Table 1. Summary of the identified genes

Enhancers of read-out phenotype in Drosophila				Suppressors of read-out phenotype in Drosophila			
Drosophila gene	Human homolog	Chromosomal location (human)	Accession Number (RefSeq) (human)	Drosophila gene	Human homolog	Chromosomal location (human)	Accession Number (RefSeq) (human)
Emc	*Id1*	20q11	NM_002165	*CG11844*	*Pairbp1*	1p31	NM_001018067.1
Bonus	*Trim24*	7q32-q34	NM_003852.3	*CG11844*	*Habp4*	9q22.3-q31	NM_014282.2
CG1021	*Tmcc2*	1q32.1	NM_014858.2	*Rm62*	*Ddx5, Ddx17*	17q21 22q13.1	NM_004386.2 NM_001098505.1
Couch Potato	*Rbpms*	8p12-p11	NM_001008710.1	*CG17838*	*Hnrnpr*	1p36.12	NM_001102397.1
Arp-like	*Armet*	3p21.1	NM_006010.2	*E2f*	*E2f3*	6p22	NM_001949.2
DNAPrim	*Prim2a*	6p12-p11.1	NM_000947.2	*CG33970*	*Abca2*	9q34	NM_001606
CG31522	*Elovl1*	1p34.2	NM_022821.2	*CG1746*	*Atp5g2*	12q13.13	NM_001002031
Ttk	*Zbtb45*	19q13.43	NM_032792.2	*cycA*	*Ccna2*	4q25-q31	NM_001237.2

4.2 Spatial and temporal expression in the mouse brain

Because of the possible therapeutical perspective, only the suppressors of read-out phenotypes in Drosophila (therefore putative enhancers of γ-cleavage) were utilized for further characterization. Since our candidate genes were identified in Drosophila, before we proceed to the mammalian validation we analyzed the expression levels in the mouse brain. For that we used real-time PCR to quantify and compare the levels of expression separately in the cortex, the cerebellum and the hippocampus at 1, 3 and 20 months of age. We found that for some genes the expression was relatively low, while another observation was that there was not any significant variation in the expression of the genes in the different brain regions, as well as no distinction between the 1 and the 3 months old mice (data not shown). Nevertheless, since aging is a major risk factor for the development of AD we also compared the expression of these genes between young (1m) and old (20m) mice. Interestingly, most of the enhancers of APP cleavage were found to increase in the aged brain **(Fig. 11)**.

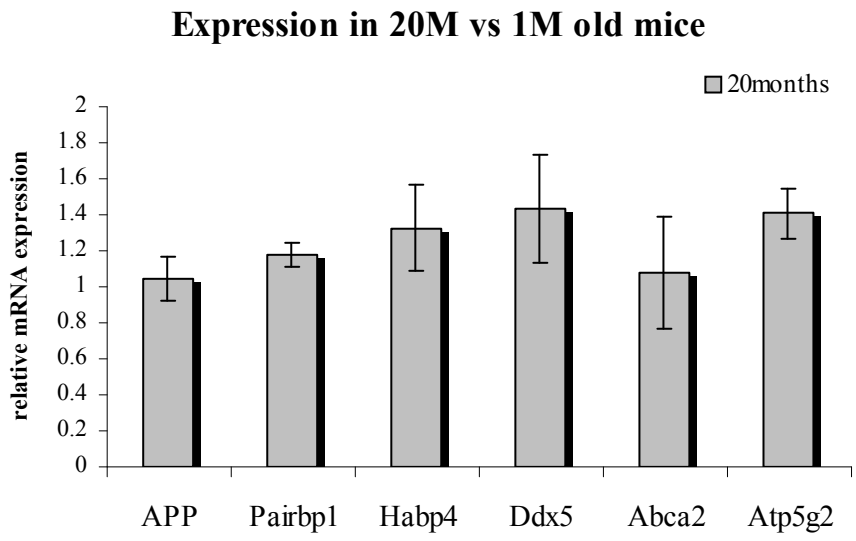


Figure 11. Temporal expression of the candidate genes in the mouse brain. The graph displays the relative amount of mRNA of *APP*, *Abca2*, *Ddx5*, *Atp5g2*, *Habp4*, and *Pairbp1* in brains of 20 months old mice as quantified with real-time PCR. The graph depicts the relative amount of mRNA of the genes in 20months (bars) old mouse compared to 1month (set as 1) old mouse. The bars represent the average expression in the three areas tested, namely the cortex, the cerebellum and the hippocampus.

In summary, the proteins we found significantly expressed in the mammalian brain and, hence, we focused on were the following:

_ ***Pairbp1*** (Plasminogen Activator Inhibitor RNA-Binding Protein 1) has been proposed to have a role in the regulation of mRNA stability, since it binds the 3'-end of the SERPINE1/PAI1 mRNA at a region that is involved in message decay (Heaton *et al.,* 2001). The PAI1 protein interacts with tissue plasminogen activator (TPA) and is part of the fibrinolysis system. Pairbp1 has also been shown to interact with the human chromatin-remodeling factor CHD-3 (chromo-helicase-DNA-binding domain protein-3) and has therefore implicated in nuclear chromatin remodeling (Lemos *et al.,* 2003). Recent studies show that Pairbp1 binds Progesterone (P4) receptor membrane component-1 (PGRMC1) and together function as a membrane receptor for P4, mediating it s antiapoptotic action (Peluso *et al.,* 2005 and 2008). Moreover, knockdown of Pairbp1 in PC12 cells can block neurite formation and lead to cell death (Kobayashi et al., 2009).

_***Habp4*** (Intracellular hyaluronan-binding protein 4): component of the brain extracellular matrix (Huang *et al.,* 2000).

_***Ddx5*** (or p68) is an RNA helicase and an RNA-dependent ATPase that belongs to the DEAD box family of proteins (Hloch *et al.,* 1990). Members of this family share regions of great homology and are implicated in RNA metabolism, such as RNA translation, ribosomal assembly and splicing (Iggo *et al.,* 1991). They are ubiquitously expressed. Ddx5 has been shown to interact with the androgen receptor and be overexpressed in prostate cancer (Clark *et al.,* 2008), while its interaction and crosstalk with Runx2 have also implicated it in osteoblast specification and maturation (Jensen *et al.,* 2008).

_***Abca2*** (ATP-binding cassette sub-family A member 2): member of the ABC family of transporters, suggested role in lipid transport in the brain (Davis *et al.,* 2004).

_***Atp5g2*** (ATP synthase lipid-binding protein) is a mitochondrial multipass membrane protein, component of the mitochondrial respiratory chain that produces ATP from ADP (Dyer *et al.,* 1993).

In order to evaluate whether the mRNA for these genes is present particularly in the hippocampus, which is one of the brain areas affected by Alzheimer's disease, and because it was also in our later goals to use hippocampal cultures to study the effect of the genes, we also performed a real-time PCR with RNA isolated from these primary cells. **Figure 12** shows that the mRNA for the candidate genes was present in the hippocampal cells.

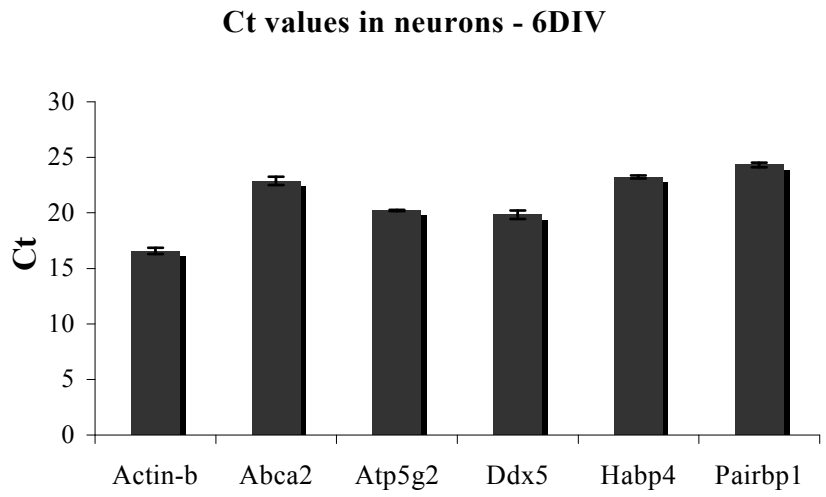


Figure 12. Expression of the candidate genes in primary hippocampal cells. 6DIV neurons were lysed and RNA was isolated. The graph shows the cycle at which the fluorescence crosses the threshold (cycle threshold or Ct) for each gene, in comparison to the housekeeping gene actin-b (one of the most abundant proteins in the cells). Actin-b has a Ct of around 18 and in general, cycle thresholds below 30 are considered a significant indication for the presence of an mRNA.

Furthermore, in order for this study to be of any potential therapeutic relevance, the expression of the genes needed to be also determined in human brain samples, as shown in **Figure 13**.

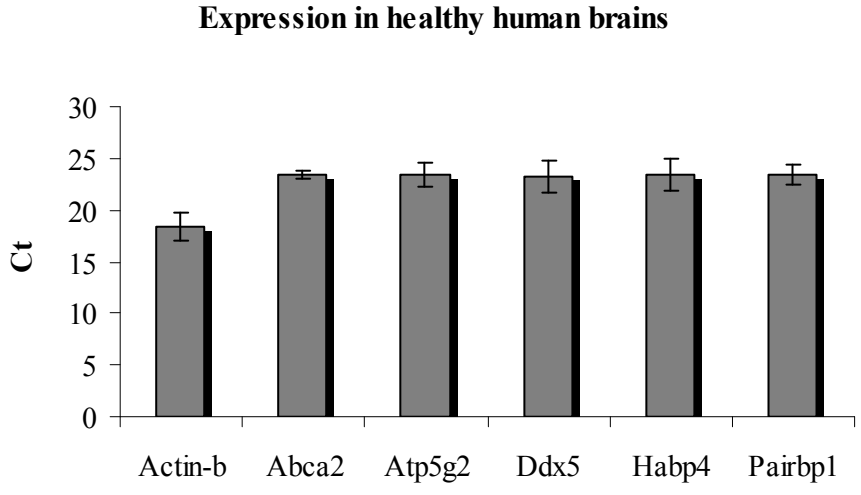
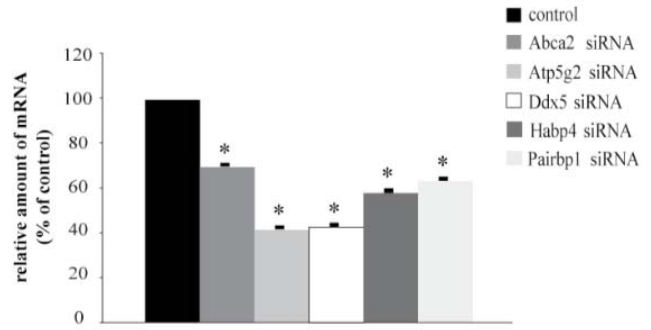


Figure 13. Expression of the candidate genes in human brain. Healthy human brain samples were analysed by real-time PCR for the expression of the genes of interest. The Ct displayed (<30) is indicating that *Abca2*, *Ddx5*, *Atp5g2*, *Habp4*, and *Pairbp1* are indeed expressed. The actin-b is used as a control housekeeping gene and because of its abundance in the cells it has a low cycle threshold (Ct).

4.3 Decreased Aβ secretion in mammalian cells with reduced levels of *Pairbp1, Habp4, Ddx5, Atp5g2* and *Abca2*

Since it is widely accepted that disease progression in AD could be delayed or even prevented by reducing Aβ production (Cummings *et al.*, 2007), the *Drosophila* genes that reduced γ-secretase cleavage of APP were taken forward to mammalian validation studies. To determine the extent to which wild type *Atp5g2, Ddx5, Pairbp1, Habp4* and *Abca2* act as positive regulators of Aβ production in mammalian cells, each was knocked down by sequence specific siRNAs in HEK293 cells stably expressing human APP (APP695). The efficiency of the siRNA knockdown was evaluated by Real Time PCR, which revealed a reduction at the mRNA level ranging between 40 to 60% (**Fig. 14A**). In such cells, ELISA-based analysis of Aβ levels in the medium, at 72h post-transfection of the siRNA, showed a significant decrease in Aβ40 for all targeted genes, varying from 30 to 70% ($(p < 0.05)$ **Fig. 14B**). Likewise the secretion of Aβ42 was significantly decreased compared to controls (**Fig. 14C).**

A



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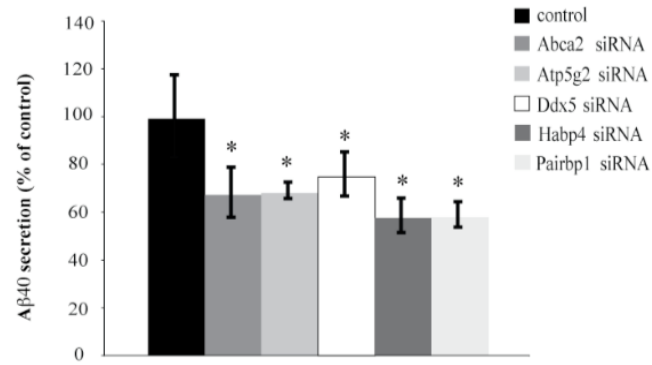

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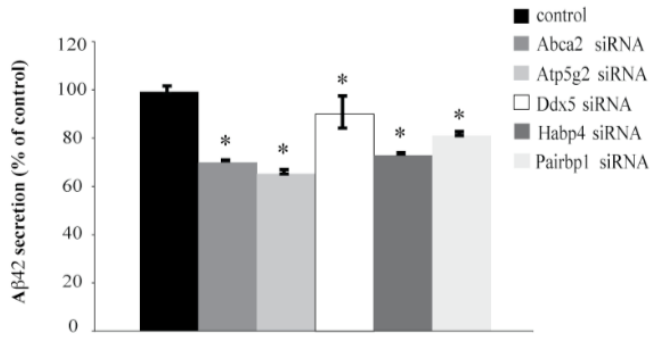


Figure 14. Effect of the depletion of the candidate genes on Aβ secretion in mammalian cells. (A) HEK293 cells overexpressing APP695 were transfected with siRNA specifically directed against the different candidate genes. The efficiency of the siRNA reduction was analyzed by quantitative PCR 72h after siRNA transfection. The graph depicts the relative amount of mRNA of the genes as percentage compared to the control that is set to 100%. **(B-C)** Media of HEK293 cells overexpressing APP695 transfected with the siRNAs against the enhancers were collected 72h post-transfection and secreted Aβ40 and Aβ42 load was evaluated by ELISA and found significantly decreased in treated cells. Values represent means of three independent experiments ±SD. Significance was calculated with the Student's t-test. *p values are: *Abca2*: 0.026, *Atp5g2*: 0.019, *Ddx5*: 0.049, *Habp4*: 0.009 and *Pairbp1*: 0.008 for Aβ40; and *Abca2*: 0.00007, *Atp5g2*: 0.00006, *Ddx5*: 0.04, *Habp4*: 0.0001 and *Pairbp1*: 0.0004 for Aβ42.

Irrespective of the different intensities of blockade, which could be attributed to the relative levels of siRNA knockdown for each gene, or their site of action in the different cell compartments where APP resides, the data are consistent with the genetic screen results suggesting that each of these genes could regulate Aβ generation/release. To strengthen this conclusion, we next analyzed Aβ generation by peptide immunoprecipitation/western blot. **Figure 15** confirms that siRNA knockdown of any of these five genes reduced the amount of secreted Aβ. We consider it unlikely that Aβ reduction was due to off-target action of the siRNA because, first, it occurred with all 5 genes and, second, because the effects on Aβ exactly matched the effect produced by the true loss of these genes, *in vivo* (reduced γ-cleavage).

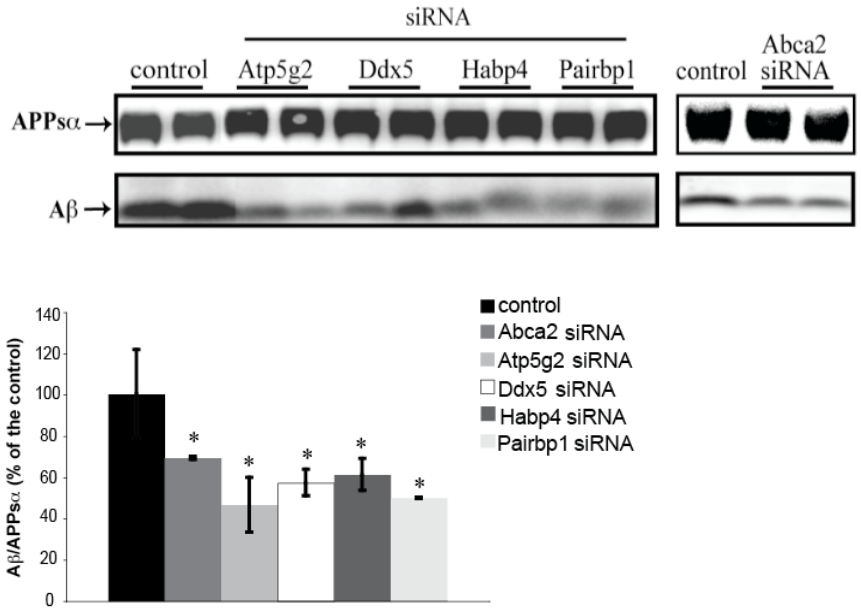


Figure 15. Analysis of Aβ secretion by immunoprecipitation. Media of HEK293 cells overexpressing APP695 treated with the specific siRNAs against the enhancers were collected 72h post-transfection and secreted Aβ was determined by immunoprecipitation with the 6E10 antibody. Detection of Aβ was achieved again with the 6E10 antibody. APPsα can be detected at the same time by 6E10 and, thus, can be used as a normalization control. As depicted by the quantification of the ratio Aβ/APPsα, knockdown of the enhancers could reduce Aβ secretion significantly. Values represent means of three independent experiments ±SD. *p values were calculated with the Student's t-test and are: *Abca2*: 0.017, *Atp5g2*: 0.047, *Ddx5*: 0.05, *Habp4*: 0.007 and *Pairbp1*: 0.04.

To rule out the possibility that the effect we observe on Aβ generation is a result of increased cell lethality because of the siRNA treatment we performed an MTT assay, which determines the viability and proliferation capacity of the cells. We found no adverse effect of the siRNA treatments upon cell viability or proliferation, at 24, 48 or 72h post-transfection **(Fig. 16).**

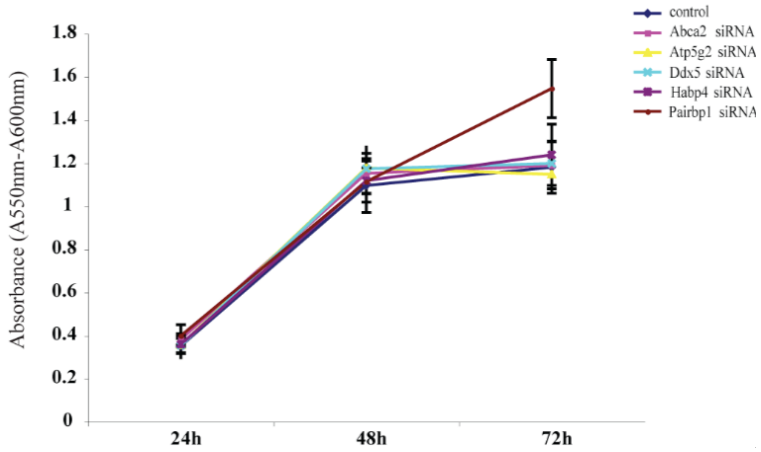


Figure 16. Evaluation of cell viability upon siRNA treatment. Cell viability was determined by the MTT assay, at 24, 48 and 72h post-transfection with the collection of siRNAs. As is depicted in the graph, none of the oligos was able to promote cell death. Values represent means of three independent experiments ±SD. *(p values <0,05).

4.4 Evaluation of γ-secretase activity

Our next step was to evaluate whether reduced production of Aβ is a result of a direct γ-secretase defect. To test this possibility we measured total γ-secretase activity (see Material and Methods) in cells treated with the siRNA for each of the five genes. Briefly, this method is quantifying enzymatic activity after lysis of the cells by adding a synthetic peptide, perfect substrate for the enzyme. Hence, any intracellular effectors or transport events that might influence the activity are not monitored. This study revealed a lack of major inhibitory effect on general γ-secretase activity (**Fig. 17**), suggesting that these genes are not just general regulators of γ-activity, and most probably affect Aβ production through an indirect mechanism.

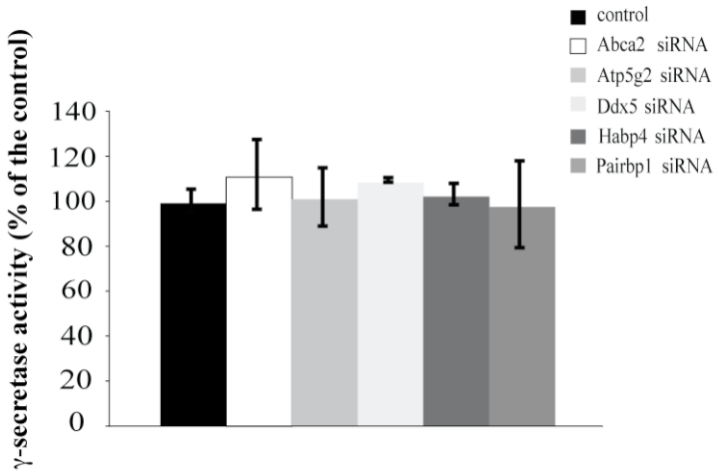
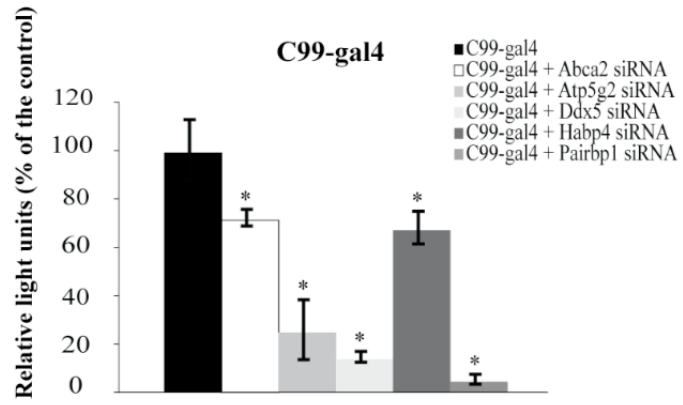


Figure 17. Analysis of general γ-secretase activity. HEK293 cells overexpressing APP695 were transfected with the siRNA against the different candidate genes and were lysed 72h using the lysis buffer included in the kit (R&D Systems, see materials and methods). The efficiency of γ-secretase activity that was present in these lysates was evaluated by the addition in the lysate of a synthetic peptide that is perfect substrate for γ-secretase. With this method, which is independent of the cell environment, the general activity of the enzyme was found unaffected.

4.5 Knockdown of *Abca2* and *Habp4* differentially affect APP and Notch processing

Before Aβ is produced, a small intracellular fragment, AICD (APP intracellular domain) is generated by a γ-secretase-like intramembranous ε-cleavage. The function of this domain has been a controversial issue with some studies appointing to AICD a signaling role and some arguing against it (Cao and Sudhof, 2001, Nakaya and Suzuki, 2006, Waldron *et al.,* 2008). In order to simulate better the read-out system in Drosophila and take also into consideration other intracellular factors that might be involved in the regulation of APP metabolism we analyzed the γ-secretase activity by evaluating the levels of AICD production in HEK cells using a luciferase assay. The cells were co-transfected with two different constructs, APPC99-GAL4 and UAS-luciferase, in combination with each of the specific siRNA oligonucleotides for our candidate genes. AICD generation was monitored 72h later and revealed that knockdown of each of the five genes resulted in a reduction of γ-cleavage of APP. In fact, reduction of the cellular levels of each of the 5 candidates decreased AICD generation by between 30 and 90% **(Fig. 18A**).

A



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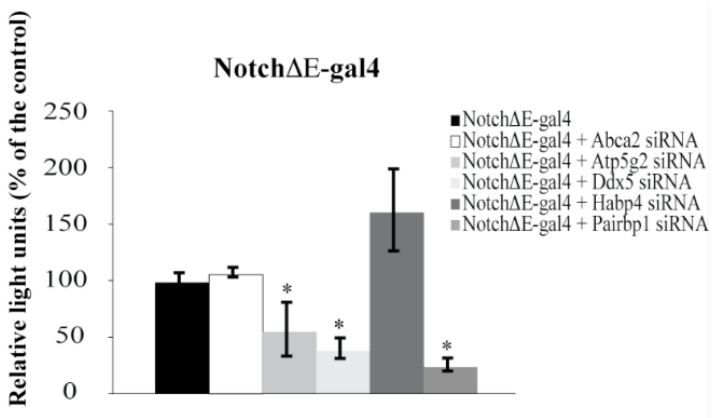

Figure 18. Assessment of γ-secretase activity on AICD and NICD using a luciferase assay. HEK293 cells were co-transfected either with C99-Gal4-VP16, UAS-luciferase and the siRNAs, or with NotchΔE-Gal4-VP16, UAS-luciferase and the siRNAs or Gal4-VP16 and UAS-luciferase as control. The luciferase activities of the γ-secretase-dependent variants (C99-Gal4-VP16, NotchE-Gal4-VP16) were determined. All five genes reduced AICD generation, while only *Atp5g2, Ddx5* and *Pairbp1* were able to decrease NICD release significantly. For C99-Gal4-VP16 (n= 9; *p values all <0,01) and for NotchE-Gal4-VP16 (n=6; *p values all <0,05). Values are presented as means ± SD and significance was assessed using Student's *t* test.

Since the main goal of the project was the identification of modulators that would not interfere with Notch signaling, we used the same methodology to analyze NICD (Notch intracellular domain) production **(Fig. 18B).** HEK cells were in this case co-transfected with the NotchΔE-GAL4 and UAS-luciferase constructs together with the siRNAs and NICD generation was determined 72h later. Importantly, this assay revealed differences in the contribution of these genes to the processing of APP and Notch**.** In contrast to AICD, NICD generation was differently affected. Knockdown of *Atp5g2*, *Ddx5* and *Pairbp1* showed a

significant reduction of NICD generation; siRNA to *Abca2* showed no inhibition of NICD production and knockdown of *Habp4* resulted in an increase in NICD production (**Fig. 18B**). The differences found with Notch and APP cleavage further prove that at least two of the five genes are not just general regulators of γ-activity. Furthermore, in line with recent studies (Tolia *et al.,* 2008, Thathiah *et al.,* 2009) we also observe that the proteolysis of these two proteins can be differentially modulated.

4.6 β-secretase and α-secretase activities

Apart from γ-secretase there are two other enzymes participating in the proteolysis of APP, α-secretase and β-secretase. To investigate whether our candidate genes have an additional effect on these two enzymes we first assayed the effect of their knockdown on α- and β-secretase cleavage of APP. HEK293 cells stably expressing APP695 were treated for 72h with the siRNA for each of the genes under study, radiolabeled with [^{35}S] methionine, and APP and its proteolytic processing derivatives were immunoprecipitated from cell lysates and conditioned media, utilizing antibodies directed against APP intracellular and extracellular domains, respectively. Compared with cells overexpressing APP alone, cells co-expressing APP and any of the different siRNAs presented no significant alteration in CTFβ/α ratio (**Fig. 19**).

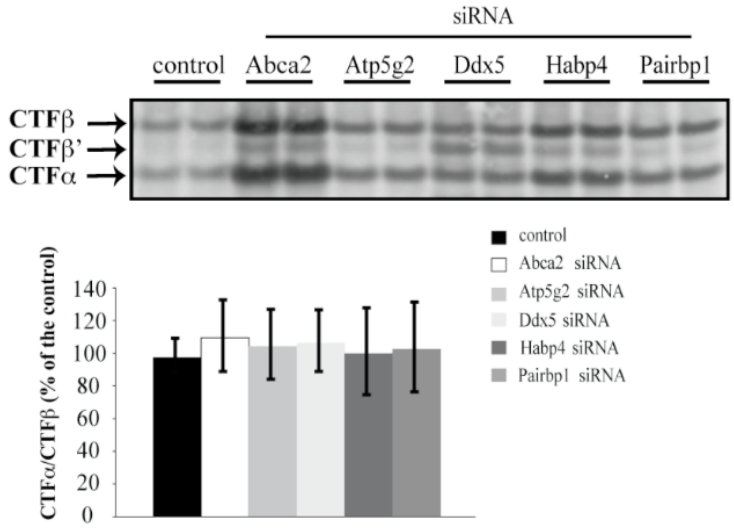

Figure 19. APP CTF ratio is not affected by knockdown of the candidate genes. HEK293 cells expressing APP695 were transfected with the specific siRNAs. 72h post-transfection the cells were pulse labeled with [^{35}S]methionine for 2h. Cells were chased for 4h, and CTFs were immunoprecipitated with antibody APP-Ct from cell lysates**.** Following SDS-PAGE, radiolabeled proteins were detected by phosphoimaging. As shown by the quantification the ratio CTFα/CTFβ was not altered upon gene knockdown.

Furthermore, knockdown did not significantly alter the levels of total secreted APP (APPs) (**Fig. 20**). To specifically detect APPsα, media were also immunoprecipitated with an antibody against the Aβ domain. Cells transfected with the siRNAs for the different genes did not produce a significant change on this fragment either further suggesting that α-secretase is not influenced, while the Aβ was again decreased (**Fig. 20**).

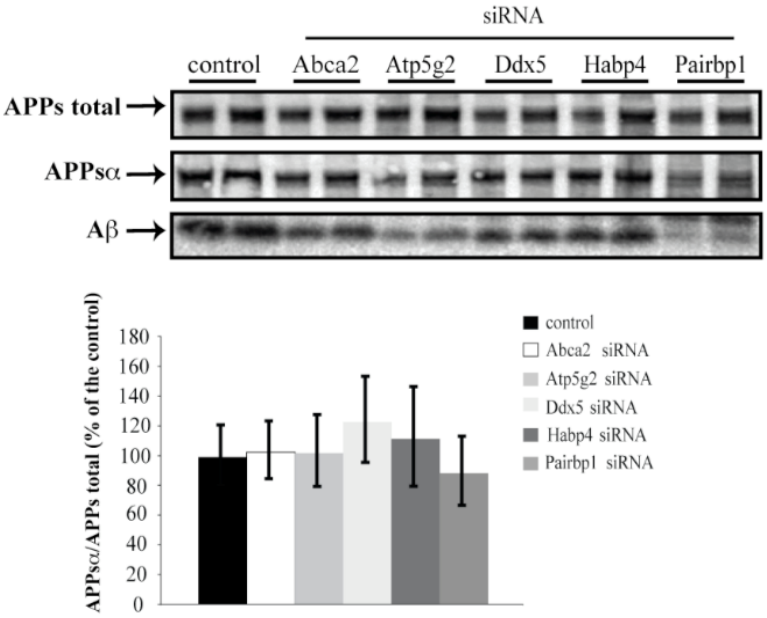

Figure 20. Secreted forms of APP are not affected by knockdown of the candidate genes. 72h post-transfection with the different siRNAs HEK293 expressing APP695 were pulse labeled with [^{35}S]methionine for 2h and chased for 4h. Secreted APP was immunoprecipitated from the media of the cells with the antibody 22c11 that recognizes both APPsα and APPsβ (termed APPs total, first panel). An immunoprecipitation with antibody 6E10 that selectively detects APPsα and Aβ (second and third panel, respectively) was also performed. Precipitates were separated by SDS-PAGE, and radiolabeled proteins were detected by phosphoimaging. The quantification of the ratio APPsα/APPs total showed that the secretion of the various APPs forms was not affected.

Additionally, we investigated the effect of gene knockdown on β-cleavage enzymatic activity, by a β-secretase specific assay: no major changes in this enzymatic activity were observed for any of the different siRNA treatments (**Fig. 21**). Altogether these results suggest that the enzymatic activities of α- and β-secretase are not affected by knockdown of our candidate genes and that this is not the reason of Aβ secretion reduction.

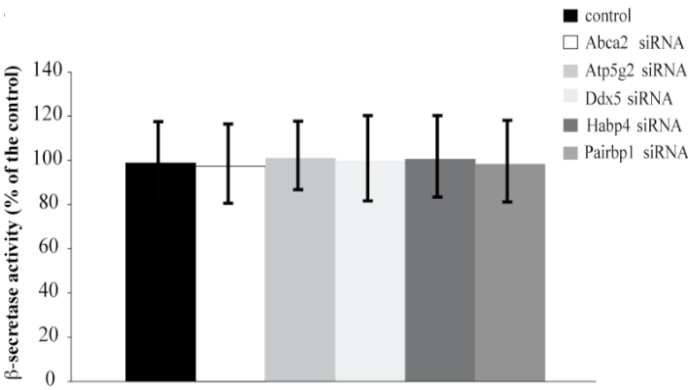

Figure 21. Analysis of β-secretase activity. Lysates of HEK293 cells expressing APP695 and transfected with the collection of siRNAs were analyzed 72h post transfection for β-secretase activity on a synthetic substrate using a commercial kit (R&D Systems, see materials and methods). This assay further indicated that β-secretase activity remains normal when the candidate genes are knocked down.

4.7 Intracellular Aβ

It is well established that the majority of the Aβ peptide is produced inside the cell and is secreted afterwards (Ida *et al.*, 1996, Thinakaran and Koo, 2008). Therefore, to evaluate whether the reduced extracellular peptide corresponds to an increased retention in intracellular pools we measured generation and retention of Aβ inside the cells. HEK293 cells were co-transfected with C99 and the siRNAs against the target genes. 72h later the peptide was immunoprecipitated and intracellular Aβ was found significantly decreased in cells fed with the siRNAs (**Fig. 22**), indicating that the lower levels of Aβ upon knockdown reflect a true effect in peptide generation and are probably not due to increased extracellular degradation.

It has to be noted here that for this experiment the C99 fragment of APP (product of β-secretase and substrate for γ-secretase) needed to be transfected in HEK293 cells, in order to boost up the intracellular Aβ levels, since the endogenous intracellular Aβ was almost impossible to detect. However, because the transfection was transient, not all the cells in all the wells were expressing the same amount of C99, which is visible also in the blot. So, the levels of intracellular Aβ were normalized to the total amount of C-terminal stubs, and this is the reason why in the graph the difference is more obvious than in the blot.

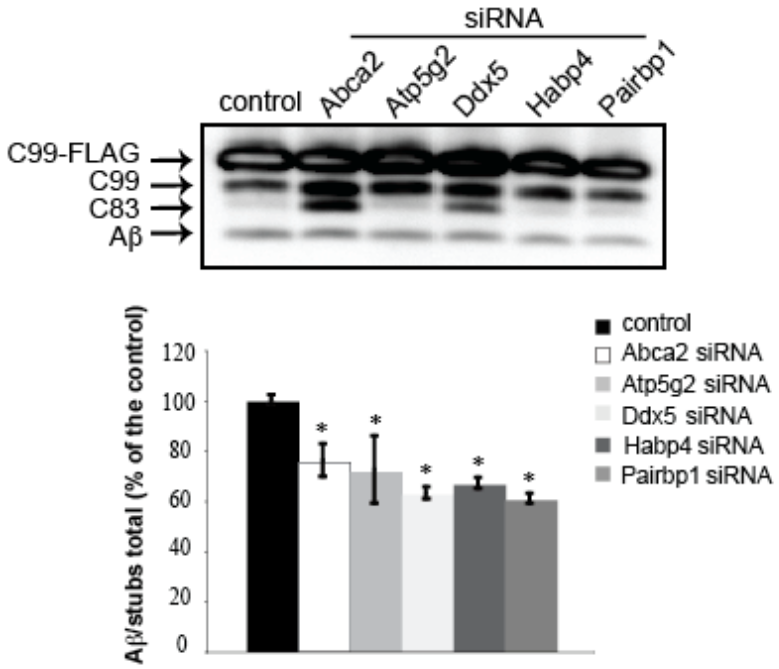

Figure 22. Evaluation of intracellular levels of Aβ peptide. HEK293 cells were co-transfected with Flag-CTFβ (Flag-C99) and the different siRNAs targeting the candidate genes. We used CTFβ and not APP overexpression to increase the intracellular Aβ levels. 72h post-transfection cells were lysed and levels of intracellular Aβ were detected by Western blot using the antibody 4G8. Aβ levels were normalized against all CTFs (Flag-C99, endogenous C99 and C83) and were found reduced in all treated cells. Values represent means of three independent experiments ± SD, *(p values all <0,01; Student's *t* test).

4.8 Expression levels of γ-secretase complex components

As described in the introduction, γ-secretase is a multiprotein enzyme comprising of four necessary constituents, namely presenilin (PS), nicastrin (Nct), Aph1 and Pen2. The presence of all four proteins is essential for the assembly of an active enzymatic activity, while the expression and stability of the components is tightly regulated by each other. Thus, a change in protein levels could reflect an altered enzymatic activity. Therefore, and in line with the previous result about the non-affected general γ-secretase activity, we went further to evaluate whether the expression levels of the four components of γ-secretase is changed. Western blot analysis revealed that siRNA reduction of the candidate genes in HEK cells overexpressing APP695 did not change dramatically the expression of Presenilin, Aph1, Nicastrin and Pen2 **(Fig. 23)**. A small tendency to increase Nct and Aph1 in the *Ddx5* and *Pairbp1* siRNA treatments could justify the affected gamma-cleavage also of Notch, however we were not observing this increase consistently in our experiments.

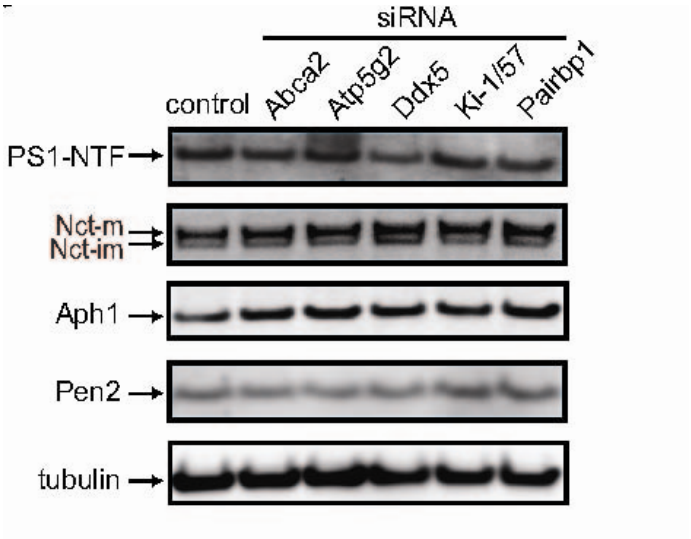

Figure 23. Lysates from HEK293 cells overexpressing APP695 and transfected with the candidate siRNAs were analysed 72h later for the expression levels of the components of γ-secretase, namely Presenilin N-terminal fragment (PS1-NTF), Nicastrin (Nct), Aph1 and Pen2. Note that NCT appears as double band corresponding to the mature (m) and immature (im) protein. Tubulin was used as loading control.

4.9 Intracranial stereotaxic injections of *Abca2* and *Habp4* shRNA

To test how valuable the predictions from the *Drosophila* and HEK293 assays might be for the brain scenario, the suppression of the identified genes on Aβ secretion was investigated in human APP expressing transgenic mice (Radde *et al.,* 2006). To obtain inhibition we engineered lentivirus expressing the shRNAs for *Abca2* or *Habp4* (see Materials and Methods). These two genes were chosen because of their lesser effects upon Notch cleavage. Stereotaxic injection of these viruses in the hippocampus led to the infection of a considerable number of neurons yet the virus did not spread away from the injected area. In fact, ELISA analysis of injected hippocampi revealed a 10% reduction of Aβ secretion in one experiment, in comparison to empty virus injected brains, and no differences with controls in two other experiments. One possibility for the lack of a clear and consistent reduction would be failure of our virus to spread beyond the injected area, with the subsequent contamination of our sample with non-infected, highly expressing neurons. Alternatively, the presence of the mutation in APP responsible for its high Aβ generation might have affected the subcellular distribution of the protein, or its capacity to interact with its physiological modulators.

4.10 Reduced levels of *Abca2* and *Habp4* in primary rat hippocampal neurons decrease Aβ secretion

Because of the ambiguous result *in vivo* we used the same lentivirus to infect rat hippocampal neurons *in vitro*. The advantage of using this system is twofold: i) APP synthesis is at basal physiological levels (De Strooper *et al.,* 1995) and ii) 50% of the neurons can be infected with moderate amounts of virus (**Fig. 24A**). Hippocampal neurons maintained *in vitro* for 3 days were infected with lentivirus coding for shRNA to *Abca2*, to *Habp4* or simply expressing GFP, and Aβ levels were then measured in the medium 5 days later, at a time when neurons reach maturity *in vitro* (Craig and Banker, 1994). ELISA analysis revealed a decrease in Aβ production of 20% or 40% after the suppression of *Abca2* or *Habp4*, respectively, compared to control neurons infected with the same vector expressing only GFP (**Fig. 24B**). Although to a lower level, Aβ production was also reduced when neurons were transfected with these genes' shRNAs by a lipofection method (**Fig. 24C**). The differences in Aβ reduction upon *Abca2* or *Habp4* knockdown may reveal simple differences in protein levels after knockdown. Alternatively, they indicate that *Habp4* plays a more fundamental role in the control of Aβ production.

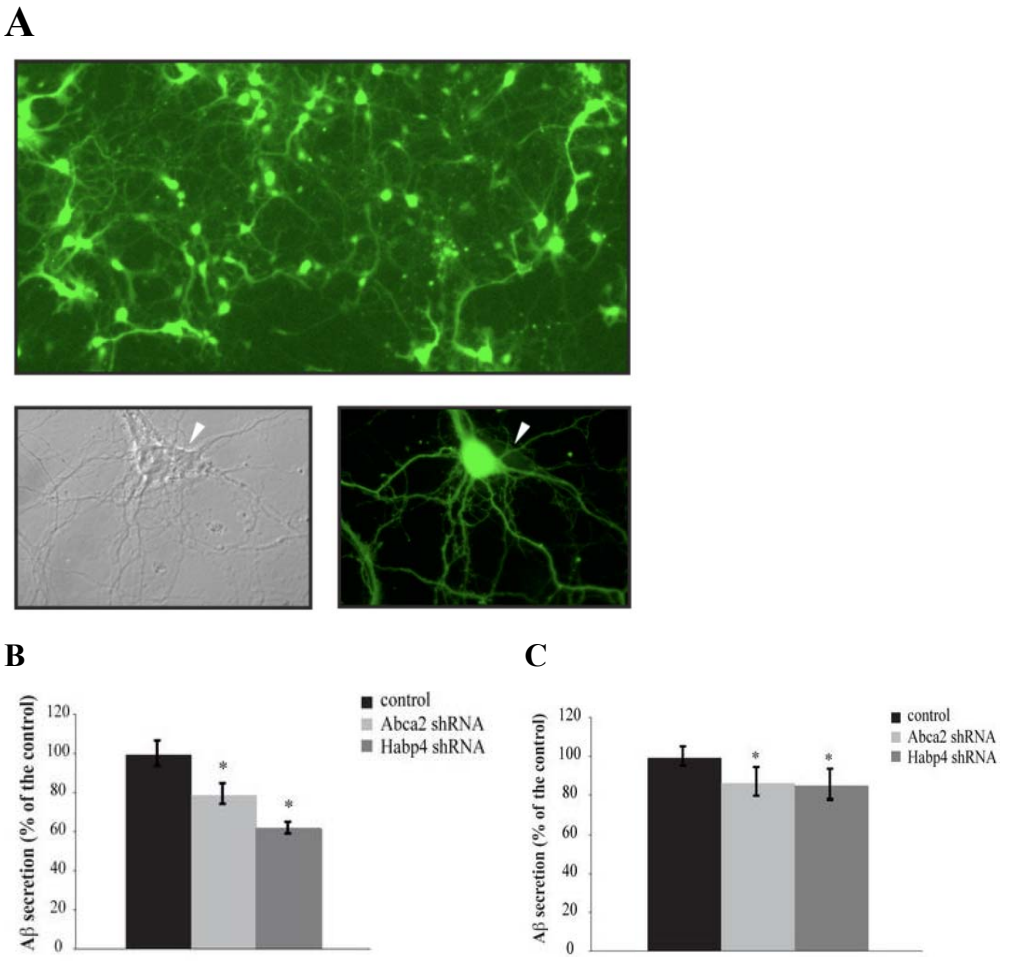

Figure 24. Knockdown of *Abca2* and *Habp4* in primary rat hippocampal neurons decreases Aβ secretion. 3DIV primary rat hippocampal neurons were incubated for 7h with the virus 1:70 diluted in growth medium. After this time the medium was changed and the cells were returned in the incubator for 120h. **(A)** The lentiviral infection efficiency of the infection with the *Abca2*, *Habp4* and empty vector of the hippocampal neurons was evaluated by microscopy. The percentage of infected neurons for all conditions was approximately 50%. The upper panel shows a representative field of the infected neurons, while the two lower inserts depict in higher magnification two neurons, one infected and one not (GFP and phase contrast, arrowhead points the non-infected cell). **(B)** Aβ secretion in the media was analysed by ELISA and was found significantly decreased in the *Abca2* and *Habp4* infected cells. Values are presented as means of three assays ± SD * (p values <0,05). **(C)** Rat hippocampal neurons were transfected after 3DIV with the shRNAs against *Abca2* and *Habp4*, cloned in the pLentiLox3.7 vector, using the Lipofectamine 2000. 72h later the media were collected and the secreted Aβ was measured by ELISA (see Material and Methods). Although the shRNA transfection was able to reduce significantly the Ab secretion, the percentage of reduction was not as pronounced as the one obtained using the lentiviral approach. Values are presented as means of three assays ± SD. * (p values <0,05).

4.11 APP maturation is not affected by siRNA reduction of the target genes

Source of the Aβ peptide is APP, a much larger glycoprotein initially synthesized as an immature type I protein that later undergoes a post-translational maturation process, which involves various modifications, like glycosylation, in the early secretory pathway (Tomita *et al.,* 1998, Takahashi *et al.,* 2008). Since we found Aβ secretion reduced after siRNA knockdown we went on to study if this is a result of an improper early secretory trafficking of APP, which would lead to a defect in its maturation. For that we used pulse-chase experiments and determined how APP is maturing through time, between 0min and 120min. HEK293 overexpressing APP were treated for 72h with the siRNAs against our candidate genes. After this time cells were radiolabeled for 2h and lysed at 0, 15, 30, 60 and 120min and APP was precipitated with the APP-Ct antibody. As **Figure 25** depicts we observed that APP is maturing in a manner comparable (progressive decrease of the immature form and respective increase of the mature) to the non-treated cells, suggesting that impaired APP maturation and early trafficking though the ER and the Golgi are not the reason for lower Aβ generation.

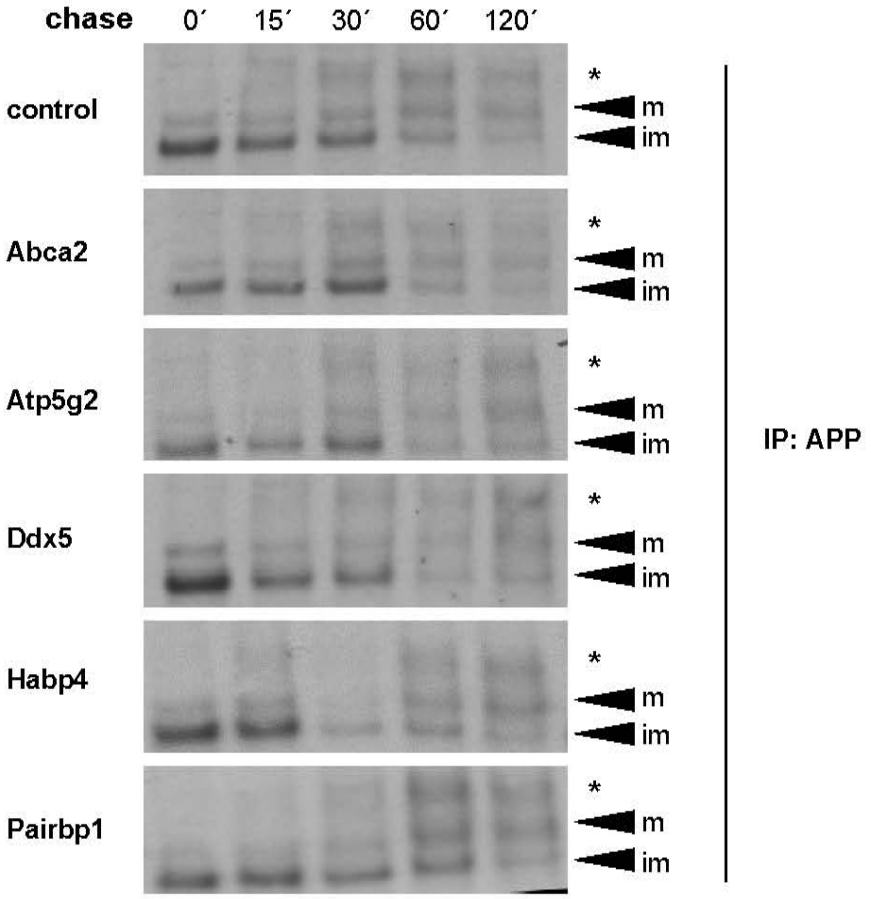

Figure 25. APP maturation pattern is not affected in siRNA treated cells. HEK293 cells expressing APP695 were transfected with siRNA against the enhancers. 72h post-transfection cells were pulsed for 2h with [^{35}S]methionine and chased for the different time points between 0min and 120min. As shown in the image, after immunoprecipitation of APP full length from the lysates using the APP C-t antibody, the pattern of maturation (progressive decrease of the immature form and respective increase of the mature APP) was similar for all treatments and did not differ compared to the control. The asterisk denotes a non-specific band.

Section 5

Discussion

5. Discussion

5.1 Identification of APP metabolism modulators

The modulation of the APP-cleaving enzymes (α-, β- and γ-secretase) or APP metabolism itself in order to decrease Aβ production and prevent its neurotoxic effects is a goal of many scientists in the Alzheimer disease field and one of the basic therapeutic approaches the pharmaceutical companies seek (Cummings *et al.,* 2007, Tang *et al.,* 2007). Several genetic screens of various kinds have been performed using either Drosophila, human cDNA libraries, fusion proteins, RNAi libraries and more that are progressively increasing the knowledge surrounding the regulatory mechanisms of APP proteolysis (van de Hoef *et al.,* 2009, Thathiah *et al.,* 2009, Yang *et al.,* 2009, Schobel *et al.,* 2008, Zhang *et al.,* 2007, Yang *et al.,* 2006). In a recent high-throughput functional genomics screening, using adenoviruses encoding potential drug targets, Thathiah and colleagues discovered that GPR3, an orphan G protein–coupled receptor that constitutively elevates cyclic adenosine monophosphate levels through adenylate cyclase activation, could affect Aβ generation and γ-secretase complex formation without any apparent effect on Notch processing, indicating that these two biological events can be separated and differentially regulated (Thathiah *et al.,* 2009). This fact also strengthens our objective to identify molecules that can modulate APP metabolism without influencing Notch signaling.

5.2 Genetic screens using *Drosophila melanogaster* as a model

The use of *Drosophila melanogaster* is a tactic that has been widely adopted to model human neurodegenerative diseases (Mugit and Feany, 2002, Cauchi and van den Heuvel, 2006, Bilen and Bonini, 2005). Not only the genetic tools for in vivo experiments are numerous and the generation time short, but *Drosophila* presents a variety of external features that can be altered and rather easily analyzed. Concerning the field of Alzheimer's disease particularly, the γ-secretase complex and activity are highly conserved between vertebrates and the fruitfly. The eyes of *Drosophila*, which was our main system, are extensively used in genetic screens because they are not necessary for the viability and fertility of the fly. Importantly, it is known that γ-secretase is present, conserved and functional in the fly eyes, and proof that it is essential there is the γ-secretase – dependent cleavage and subsequent signaling of Notch receptor.

In this study we used *Drosophila melanogaster* as our starting point towards the identification of APP γ-cleavage modifiers that would ideally not affect Notch signaling. Although

Drosophila has γ-secretase activity it contains no APP, making it, thus, a suitable model to investigate γ-cleavage of transgenically expressed human APP. We used a commercial collection of 776 P-elements and performed three separate screens that led us to the detection of genes affecting C99 metabolism. For one screen we utilized the read-out model prepared by Guo and colleagues that combines the human C99 fragment with the GAL4-UAS system for reporter gene expression. The rough-eye phenotype caused by the expression of the reporter gene GRIM reflects C99 cleavage and can be used as a read-out of alterations of this process. In parallel to this screen we tested these mutants against a *Drosophila* model expressing constitutively GRIM, independently of C99 cleavage, avoiding, hence, the false positive results (mutants). In another approach the mutants were tested in eye-specific homozygosis using GFP as the output. With the combination of these strategies we could identify a considerable number of enhancers and suppressors of γ-secretase activity.

Nevertheless, the importance of γ-secretase-dependent Notch signaling in synaptic function and other biological events is one of the key reasons why therapeutical inhibition of the enzyme has been hindered. Taking this into consideration we assessed whether our positive candidates would also interfere with Notch signaling. Eventually, mutations that had an effect on Notch pathway were excluded from further characterization.

We isolated 16 genes affecting the γ-mediated APP cleavage, with human orthologues, some with well described and some with nearly unknown biological functions, and we could subsequently determine that only 5 of them where present in the mammalian brain in significant levels: *Abca2, Atp5g2, Ddx5, Habp4,* and *Pairbp1*. Encouragingly, one of the identified genes, *Abca2*, has been previously implicated in AD (Chen *et al*., 2004), and variations in the *Abca2* gene haven been described as risk factors for AD (Mace *et al.*, 2005) strengthening the validity of the approach taken and positively influencing the possible relevance of the other genes identified. On the side, one could note that the genes that were not further studied were not completely absent from the brain, just in relatively low levels, which might have to do with their function and cell-cycle relation. This could hint that APP metabolism is affected by cell cycle. In fact, in a very recent study (Myre *et al.,* 2009) the authors use the Differentiation-Inducing Factor-1 (DIF-1) from Dictyostelium, which slows cell cycle progression through G0/G1, and analyze its effect on APP proteolysis. They find decreased levels of secreted APP, mature APP, C-terminal fragments and Aβ40 and 42.

It is important to mention that, as Guo and colleagues also comment, the APPC99 reporter flies were designed to identify factors that regulate γ-secretase cleavage. One, however, has to point out that a screening with this type of read-out model cannot discriminate between direct

γ-secretase activity modulators (i.e. genes that affect the levels, stability, composition, activity and/or subcellular localization of the different components of the γ-secretase complex) and indirect modulators (i.e. genes that affect the expression levels, maturation, trafficking and subcellular localization of the C99–GAL4 reporter). Moreover, molecules that regulate proteolysis of the reporter outside the γ-secretase target site (i.e. ε-cleavage) could be also identified by this approach. In any event, a gene whose suppression reduces the generation of Aβ, by direct or indirect participation, without altering to a great extent the cleavage of Notch, will be of relevance to a better understanding of Alzheimer's disease mechanisms and of potential therapeutic use.

5.3 Analyses of the effect of the candidate genes in mammalian systems

The first step of the validation of these candidate genes in a mammalian model was to analyze the effect of their knockdown in a human cell line. Interestingly, all five genes were shown to reduce Aβ secretion, a result that was in agreement with the effect we scored in the *Drosophila* screen. Even though further work is certainly required in order to dissect the exact mechanistic details of how these genes function as positive regulators of Aβ production, our work does provide with a number of mechanistic insights. In particular, we could not detect any major change in the levels of APP, indicating that the action of these genes is not at the level of transcription of the substrate. Additionally, the maturation of APP was not disturbed, suggesting that the early secretory pathway is not the place where these genes exert their regulatory role. Moreover, these genes' knockdown did not affect the levels of expression of each of the individual components of the γ-secretase complex, hence excluding a role at the transcriptional level of these molecules either. These two are an important piece of information, especially for the future analysis of the role of *Habp4*, which has been reported to play a role in transcriptional activation (Nery *et al.*, 2006).

Apart from γ-secretase, α- and β-secretase have also been considered as therapeutic targets against AD: α-secretase, because of its participation in the non-amyloidogenic pathway and β-secretase, because of its involvement in Aβ production. Even though the *Drosophila* screens were not allowing for detection of α- or β-secretase activity alterations, it was still important to us to analyze whether these two enzymes function normally in the mammalian system. However, knockdown of the candidate genes did not affect APP proteolysis by these enzymes, and this was mainly indicated by the unaffected CTF and APPs ratios.

5.4 γ-secretase assays and selection of *Abca2* and *Habp4*

The reduction in Aβ secretion led us initially to hypothesize that the genes where affecting γ-secretase activity, either by altering directly the enzymatic activity, (like for example the nonsteroidal anti-inflammatory drugs, or NSAIDs) or by interfering with the complex's formation or localization, (like it has been suggested for Rer1p and GPR3) (Thathiah *et al.,* 2009, Spasic *et al.,* 2007, Chen *et al.,* 2006, Weggen *et al.,* 2003). A cell-free γ-secretase assay did not reveal any changes in activity. Further indication that the genes do not act as general modulators of γ-secretase activity was that the levels of the components and the formation of the mature enzyme were not altered. We therefore went on to test the same parameter with a cell-based approach in a system that was recapitulating the *Drosophila* readout (Serneels *et al.,* 2005). In this case the cellular environment and other third factors that might assist the effect of the genes are present. In fact, knockdown of all five genes resulted in a decrease of Aβ as well as AICD generation, arguing also against the possibility that they play a role in conferring specificity towards γ- or ε-cleavage differently (Chen *et al.,* 2006). On the other hand, only *Habp4* and *Abca2* did not affect either S3-cleavage or NICD generation.

5.5 *Abca2* and *Habp4* in the literature

5.5.1 Abca2

Abca2 belongs to the family of ATP-binding cassette (ABC) transporters that are responsible for the transport of molecules across the biological membranes. These proteins are highly conserved and 48 identified transporters of the family exist in human (Mack *et al.,* 2008). In particular, Abca2 has a molecular weight of approximately 270kDa and is mainly found in the brain. Several studies have shown that it is located mostly in oligodendrocytes, with an endosomal/lysosomal pattern, but a subset of neurons containing Abca2 has also been reported (Zhao *et al.,* 2000, Kaminski *et al.,* 2001, Vulevic *et al.,* 2001, Saito et al., 2007). Abca1, which is a member of the same family, has been extensively studied and a role in cholesterol efflux and Tangier disease is well established (Rust *et al.,* 1999, Orso *et al.,* 2000). Due to structural similarities of the two proteins, the connection between Abca2 and lipid transport was also analyzed (Mack *et al.,* 2006, Kim *et al.,* 2008). In fact, it was shown that Abca2 is sterol-responsive and when Abca2 was overexpressed unesterified cholesterol was detected in lysosomes and the expression of cholesterol-responsive genes was increased (Kaminski *et al.,* 2001, Davis *et al.,* 2004). Nevertheless, studies using knockout mice for

Abca2 have reported that the cholesterol levels were not altered implying an intracellular rather than a general role. On the other hand, the same knockout animals presented features indicative of an implication of Abca2 in the myelination process, like neurological shaking and increase of the myelin thickness in the spinal cord. Other defects included increased GM1 ganglioside and decreased sphingomyelin during aging supporting further the involvement of Abca2 in lipid transport (Mack *et al.,* 2007, Sakai *et al.,* 2007).

Apart from its role in lipid homeostasis, Abca2 has also been found to offer resistance to several anti-cancer molecules, like estramustine and be present in various tumor cell lines (Laing *et al,* 1998, Vulevic *et al,* 2001. In addition, HEK293 cells overexpressing Abca2 displayed higher resistance to treatment with AAPH (2,2′- azobis - (2-amidinopropane)) that causes oxidative stress by generating free radicals (Chen *et al.,* 2004).

What is interesting for our purposes is the fact that Abca2 has already been connected to Alzheimer's disease related proteins, co-localizing with APP and Aβ in neuroblastoma cells. Furthermore, overexpression of Abca2 in the same study was shown to increase Aβ production, strengthening like this our results (Chen *et al.,* 2004). In the same line, a single nucleotide polymorphism (SNP) has been reported as a genetic risk factor for early-onset AD, with the effect being ethnicity-dependent, affecting Caucasians and Western Europeans (Mace *et al.,* 2005, Wollmer *et al.,* 2006).

5.5.2 Habp4/Ki-1/57

Habp4, also known as Ki-1/57, was initially identified as a 57kDa antigen that cross-reacted with an antibody against CD30, and that is associated with kinase activity (Kobarg *et al.,* 1997). In 2000 it was shown that this protein could also bind hyaluronan and other negatively charged glycosaminoglycans and being the fourth member of iHABPs to be identified, after CDC37, RHAMM/IHABP and P32, it was named Habp4 (hyaluronan-binding protein 4) (Huang *et al.,* 2000). It contains numerous hyaluronan binding motifs that are conserved between human, mouse and chick, however the biological significance of this binding and the general role of this protein is not determined yet (Huang *et al.,* 2000). Some steps have been made towards this by studies that show (a) Habp4/Ki-1/57 directly interacting with the adaptor protein RACK (Nery *et al.,* 2004), (b) being phosphorylated by PKC in activated tumor cells (Nery *et al.,* 2004), (c) an interaction with chromo-helicase DNA-binding domain protein 3 (CHD3) that is involved in transcription regulation (Lemos *et al.,* 2003), (d) interaction with the protein arginine-methyltransferase-1 (PRMT1), making it a potential substrate for arginine-methylation that is suggested to be involved in nuclear export/import and interaction with nucleic acids or proteins (Gary and Clark, 1998, Xu *et al.,* 2001, Passos

et al., 2006), and (e) an interaction with MEF2C (myocyte enhancer factor 2C) in the rat myocardium (Kobarg *et al.,* 2005). Nevertheless, the importance of these findings and potential additional roles of Habp4/Ki-1/57 remain to be established.

5.6 The effect of *Abca2* and *Habp4* knockdown in primary hippocampal neurons

Naturally, since Alzheimer's disease is a human disorder and one of the major brain regions affected is the hippocampus, we proceeded to validate the effect of *Habp4* and *Abca2* knockdown (because of their lesser effect on NICD production) in a more relevant mammalian system, the rat primary hippocampal neurons. For the knockdown we applied lentiviral infections of particles expressing the shRNAs against these genes, a tactic that led to a significant percentage of infected neurons and a more pronounced decrease of rat Aβ secretion, compared to the standard lipofection-based shRNA transfection. This result further suggested that *Abca2* and *Habp4* present a regulatory effect towards APP metabolism, not only in a cell line but also on the endogenous APP of a neuronal system.

5.7 Conclusions

In summary, our work combined the strengths of *Drosophila* genetics with conventional biochemical and cell biology studies in mammalian cells, allowing us to gain information for the insect identified genes, namely *Pairbp1*, *Habp4, Ddx5, Atp5g2* and *Abca2,* in a context more similar to the human disease: mammalian cells and hippocampal neurons. We could demonstrate that γ-cleavage of APP was reduced by the knockdown of each of these genes. Interestingly, we detected clear selectivity in terms of the effects on the production of Notch intracellular cytoplasmic domain (NICD). Only the knockdown of *Ddx5*, *Atp5g2* and *Pairbp1* reduced NICD levels but knockdown of *Habp4* and *Abca2* did not. The fact that we could retrieve genes, which preferentially influence APP C99 γ-cleavage, suggests that there could be putative mechanisms that γ-secretase uses in order to discriminate between its substrates. Certainly, γ-secretase has approximately 55 known substrates to date, therefore the specificity of *Abca2* and *Habp4* needs to be further addressed. Initial experiments performed in our laboratory suggest that another two proteins-substrates for γ-secretase, namely N-cadherin and Nrg, are most probably not affected. Nevertheless, eventually, elaborate *in vivo* studies will be necessary in order to see if manipulation of these genes is having phenotypic consequences (i.e. vitality, development) that would maybe suggest a defect on another substrate.

Further work is also needed to prove that the reduction of *Abca2* or *Habp4* produces, *in vivo*, the same effect on Aβ production and lack of an effect on Notch cleavage as shown *in vitro*. Confidence that this could be the case comes, on the other hand, from the observations that i) null mutants of *Abca2* and *Habp4 in vivo* (*Drosophila*) did not dramatically change Notch phenotype and ii) that the anti-Aβ effect upon knockdown was also observed in hippocampal neurons *in vitro* that closely resemble in numerous aspects the same neurons *in vivo*. Another important task for the future is the analysis of the fine mechanisms by which *Abca2* and *Habp4* regulate γ-cleavage, specifically in regard to the specificity towards APP. In this regard however, ongoing work in the laboratory demonstrated that *Habp4* might participate in Aβ production through the control of APP recycling/residence at the plasma membrane and early endosomes. In fact, *Habp4* knockdown resulted in reduction of APP at the plasma membrane together with increased rate of internalization and concentration in juxta-nuclear structures. It has previously been shown that *Habp4* interacts with PKC, which is known to be involved in the trafficking of many membrane proteins, including γ-secretase substrates like E-cadherin (Le *et al.,* 2002, Nery *et al.,* 2006). Moreover, it was shown that stimulation of PKC with phorbol esters increased the endocytosis of E-cadherin (Le *et al.,* 2002). Taken together, these data give hints for how *Habp4* might affect APP metabolism, but further work is needed in order to define the mechanism by which *Habp4* controls APP surface and endosomal dynamics.

The situation with *Abca2* appears less clear, though initial results suggest that it may modulate Aβ production by controlling the levels of membrane lipids, essentially sphingomyelin and cholesterol via its lipid transport properties. Recent data obtained in our lab are showing that *Abca2* knockdown resulted in a decrease in membrane cholesterol, while also sphingomyelin was reduced. However, this lipid change did not affect the levels of APP or PS1 at the surface or intracellular compartments, suggesting that decreased Aβ production upon *Abca2* knockdown could be due to retained APP in membrane microdomains not accessible to the action of the γ-complex. In fact, *Abca2* has been suggested to contribute to AD by regulating the cholesterol homeostasis and its suppression led to a reduction of sphingomyelin in knock out mice, indicating that it may control APP cleavage via assuring the appropriate composition of the microdomains where γ-secretase components and APP need to interact (Bartzokis, 2004, Sakai *et al.,* 2007). In line with that, previous work from my laboratory as well as others has demonstrated that membrane lipids play a key role in APP cleavage (Ledesma *et al.,* 2003, Abad-Rodriguez *et al.,* 2004, Crestini *et al.,* 2006,

Osenkowski *et al.,* 2008). An interesting and informative approach would be the analysis of Aβ production in the *Abca2* knockout background in mice. This would not only strengthen the relevance of our results *in vivo*, but would also offer additional evidence for the putative implication of lipid changes on APP metabolism. Furthermore, since there is now an available commercial collection of siRNA-carrying *Drosophila melanogaster* one could cross these flies with humanAPP-expressing flies (or C99, since *Drosophila* does not have a beta-secretase to process full length APP) and gather more information about the effect of the fly homologs on Aβ secretion. These experiments would "backwards" prove our concept and the value of *Drosophila* as a model.

However, until the above indications are strongly validated I find that at this point the fairest conclusion would be that *Abca2* and *Habp4* participate in Aβ generation by acting as cellular regulators of either secretase-substrate intracellular localization-recognition, or of intracellular trafficking efficacy of APP or the γ-secretase components, or involved in sorting and recycling in different membrane compartments. These different possibilities are schematized in Figure 1. On the other hand, we think that the identification of these two genes is of biological and possible social relevance *per se*, assisting towards the definition of the role of APP and how these genes may participate in APP processing. Certainly, only future work will establish how these genes work precisely and whether or not their suppression can be of any use to prevent or treat this disease.

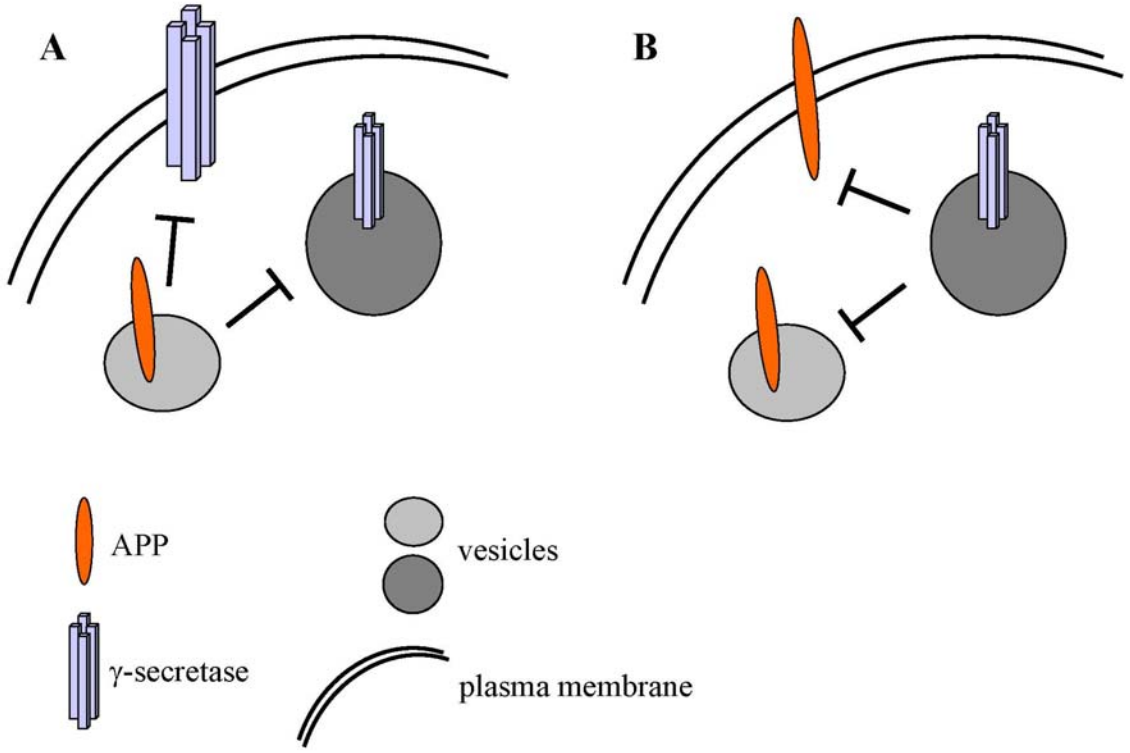

Figure 1. Working model for *Abca2* and *Habp4* mechanism of action. There are areas in the plasma membrane and intracellular vesicles where the γ-secretase complex and APP reside. We hypothesize that *Abca2* and *Habp4* act either indirectly, by affecting trafficking of APP or the γ-secretase components, or the sorting and recycling in different compartments, or by interfering with a third factor that might be involved in the aforementioned procedures. This leads to an impaired interaction of the substrate with the enzyme and consequently to the decreased Aβ and AICD production that we observe with our results.

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