# Physiological role of amyloid precursor protein during neural development

Rakesh Kumar Banote

Department of Psychiatry and Neurochemistry
Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg


UNIVERSITY OF GOTHENBURG

Gothenburg 2017

Physiological role of amyloid precursor protein during neural development

rakesh.k.banote@neuro.gu.se

ISBN 978-91-629-0212-4

Printed in Gothenburg, Sweden 2017
Ineko, Gothenburg

To Tulasi and my dear parents

# Physiological role of amyloid precursor protein during neural development

Rakesh Kumar Banote

Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology
Sahlgrenska Academy at University of Gothenburg, Sweden

## ABSTRACT

Amyloid precursor protein (APP) is a type-one membrane-spanning protein with a large extracellular N-terminal domain and a small intracellular C-terminal domain. APP first gained interest due to its involvement in the pathogenesis of Alzheimer's disease (AD). Its proteolytic processing liberates the neurotoxic amyloid-beta (Aβ) peptide that accumulates in the amyloid plaques, characteristic of AD. Thus, APP has been intensively studied for its amyloidogenic properties with less focus on its normal cell biological roles. APP is an evolutionarily conserved protein involved in biological processes including neuronal migration, synaptogenesis, synaptic function and plasticity. Still, it is unclear what role APP plays in the development of specific neuronal cell types in the central nervous system. The aim of this thesis was to examine the physiological functions of the zebrafish Appb, a highly conserved homologue of human APP, during neural development. Through a knockdown approach, we found that Appb is required for the patterning and outgrowth of motor neurons in the spinal cord as well as for the synapse formation at the neuromuscular junctions (NMJs), thus essential for the formation of normal locomotor behavior. We also show the cell-specific utility of Appb in the hindbrain-specific Mauthner cell (M-cell) development that our data indicate is mediated through a Notch1a-dependent mechanism. To confirm the function of Appb we generated an *appb* mutant carrying a homozygous non-sense mutation in exon 2. Although the smaller size of mutants was similar to morphants, mutants appeared morphologically normal after 48 hrs post-fertilization (hpf), suggesting that the genetic deficit

is compensated for, potentially by other App family members or by modifications of other genes, such as Notch. Lastly, to get a deeper insight into molecular pathways affected by Appb, we determined the proteomic consequence of Appb down-regulation and provided crucial information on proteins and pathways that are differently expressed when the expression of Appb is modulated.

In summary, we report on an essential role of Appb during neural development in the spinal cord and hindbrain and provide a link between Appb and other proteins and pathways. We believe that the zebrafish model used here provided appreciable knowledge in gaining insights into APP function and the described studies above will significantly contribute to our understanding of this complex protein during neural development.

**Keywords:** Amyloid precursor protein-b function, zebrafish, spinal cord, motor neurons, hindbrain, Mauthner cell, development, mass spectrometry, proteomics

**ISBN:** 978-91-629-0212-4

# SAMMANFATTNING PÅ SVENSKA

Amyloid precursor protein (APP) är ett membranspännande protein med stor extracellulär N-terminal domän och en liten intracellulär C-terminal domän. Proteolytisk klyvning av APP frigör den nervcells-toxiska Aβ peptiden vars ackumulation leder till amyloida plack karaktäristiska för Alzheimer's sjukdom. Denna amyloidogena egenskap hos APP har varit mål för intensiv forskning under många år medan dess normala cellbiologiska funktion rönt mindre fokus. APP är ett evolutionärt konserverat protein involverat i biologiska processer så som nervcells-migration, synapsbildning, synapsfunktion och plasticitet men dess roll vid specificering av olika nervcells-typer i centrala nervsystemet är mindre väldefinierad. Målet med följande avhandling var att studera den fysiologiska funktionen hos Appb, en välkonserverad homolog i zebrafisk, under nervcellsbilning. Nedreglering av Appb resulterade i hämmad utväxt och mönsterbildning av motornervceller i ryggraden samt felaktig synapsformation mellan nervcell och skelettmuskel. Vi kunde även visa att Appb är krävs för utveckling av Mauthner cellen (M-cell) via en Notch beroende mekanism. För att ytterligare undersöka funktionen av Appb, skapade vi zebrafiskar med en homozygot noll-mutation i *appb* genen. Dessa mutanter saknar Appb proteinet och påvisar tidiga defekter under utvecklingen. Trots vissa likheter utvecklades mutanterna, till skillnad från fiskar med nedreglerat Appb, normalt efter 48 timmar. Våra resultat stödjer att avsaknaden av Appb till viss del kan kompenseras av andra proteiner i APP-familjen. Slutligen kvantifierade vi skillnaden i proteiner hos kontroll fiskar och fiskar med nedreglerat Appb för att skapa bättre förståelse för de bakomliggande molekylära mekanismer vilka påverkas av Appb. Vi kunde bekräfta en rad redan etablerade signaleringsvägar samt identifiera förändringar i många nya proteiner. Dessa signalvägar och proteiner kräver dock ytterligare verifiering innan en direkt koppling kan fastställas. Sammantaget visar vi på en essentiell roll för Appb under centrala nervsystemets utveckling samt visar preliminära resultat på underliggande molekylära mekanismer. Vi tror att zebrafisken som modellsystem har gjort det möjligt att studera mekanismer hos APP vilka tidigare ej varit genomförbara och att de resultat vi beskriver ovan nämnvärt bidrar till vår förståelse av den komplexa samverkan som krävs för bildandet av ett normalt nervsystem.

# LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Abramsson A, Kettunen P, **Banote RK**, Lott E, Li M, Arner A, Zetterberg H. The zebrafish amyloid precursor protein-b is required for motor neuron guidance and synapse formation. Dev Biol. 2013; 15;381(2):377-88.

II. **Banote RK**, Edling M, Eliassen F, Kettunen P, Zetterberg H, Abramsson A. β-Amyloid precursor protein-b is essential for Mauthner cell development in the zebrafish in a Notch-dependent manner. Dev Biol. 2016; 1;413(1):26-38.

III. **Banote RK**, Edling M, Şatır TM, Burgess SM, Chebli J, Abramsson A, Zetterberg H. Characterization of β-amyloid precursor protein-b zebrafish mutants during early development. Manuscript, 2017.

IV. Abramsson A, **Banote RK**, Gobom J, Hansson KT, Blennow K, Zetterberg H. Quantitative proteomics analysis of amyloid precursor protein hypomorphic zebrafish (*Danio rerio*) embryos using TMT 10-plex isobaric labeling. Manuscript, 2017.

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| ACN | Acetonitrile |
| AD | Alzheimer's disease |
| ADAM | A disintegrin and metalloproteinase |
| AICD | Amyloid precursor protein intracellular domain |
| APLP | Amyloid precursor protein-like proteins |
| APOER2 | Apolipoprotein E receptor 2 |
| APP | Amyloid precursor protein |
| Atp2a1 | ATPase sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ transporting 1 |
| Aβ | Amyloid beta |
| BACE1 | Beta-site APP-cleaving enzyme |
| BCA | Bicinchoninic acid |
| BCIP | 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt |
| BSA | Bovine serum albumin |
| CamKIIα | Calmodulin-dependent protein kinase II |
| CNS | Central nervous system |
| Crebbpb | CREB-binding protein-b |

| | |
|---|---|
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| CSF | Cerebrospinal fluid |
| CTF | Cytoplasmic tail fragment |
| CuBD | Copper-binding domain |
| DAPT | (N-[N-(3,5-difluorophenacetyl-l-alanyl]-S-phenylglycine-t-butyl ester) |
| DCC | Deleted in colorectal carcinoma |
| DMSO | Dimethyl sulfoxide |
| DR6 | Death receptor 6 |
| E1/2 | Ectodomain1/2 |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| Epha2 | Eph receptor A2 |
| ErbB4 | Erb-b2 receptor tyrosine kinase 4 |
| FAD | Familial Alzheimer's disease |
| FDR | False discovery rate |
| fgf3 | Fibroblast growth factor 3 |
| Fn1b | Fibronectin 1b |

| | |
|---|---|
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GluR | Glutamate receptors |
| GSK3β | Glycogen synthase kinase 3 beta |
| GSMs | γ-secretase modulators |
| HBD | Heparin-binding domain |
| her6 | Hairy-related 6 |
| hoxb1a | Homeobox B1a |
| HRP | Horseradish peroxidase |
| IAA | Iodoacetamide |
| IL6 | Interleukin 6 |
| JNK | C-Jun N-terminal kinase |
| Kif4 | Kinesin family member 4 |
| KO | Knockout |
| KPI | Kunitz-type protease inhibitor |
| LC-MS | Liquid chromatography–mass spectrometry |
| Lnfg | Lunatic fringe |
| LRP1 | Lipoprotein receptor related protein 1 |
| LTP | Long-term potentiation |

| | |
|---|---|
| MO | Morpholino oligonucleotides |
| MS-222 | Tricaine methanesulfonate |
| NaCl | Sodium chloride |
| NBT | Nitro-blue tetrazolium chloride |
| NEP | Neprilysin |
| neurog1 | Neurogenin 1 |
| NFT | Neurofibrillary tangles |
| NICD | Notch intracellular domain |
| NPCs | Neural progenitor cells |
| PBS | Phosphate-buffered saline |
| PET | Positron emission tomography |
| PFA | Paraformaldehyde |
| PSEN | Presenilin |
| PTCH1 | Patched1 |
| PTU | 1- phenyl-2-thiourea |
| SAD | Sporadic Alzheimer's disease |
| sAPPα | Soluble amyloid precursor protein-α |
| sAPPβ | Soluble amyloid precursor protein-β |

| | |
|---|---|
| Sdc2 | Syndecan 2 |
| SDS | Sodium dodecyl sulfate |
| TALEN | Transcription activator-like effector nucleases |
| TEAB | Triethylammonium bicarbonate |
| TFA | Trifluoracetic acid |
| TMT | Tandem mass tag |
| TNF | Tumor necrosis factor |
| TP53 | Tumor protein p53 |
| VLDL | Very-low-density lipoprotein |
| WISH | Whole-mount *in situ* hybridization |

# 1 INTRODUCTION

## 1.1 Alzheimer's disease

### 1.1.1 History

Age-related mental illness in elderly has been described throughout history. However, in 1901, a 50-year-old woman, Auguste Deter, had been noticed by her husband with disturbances of memory, progressive confusion, sleep disorders and aggressiveness. She was admitted to Frankfurt Psychiatric Hospital in November 1901, where she was diagnosed by a German physician, Dr. Alois Alzheimer. With an interest in the symptomatology and progression of the illness of Auguste Deter, Dr. Alzheimer followed and documented the development of her disease. The disease gradually worsened and the Auguste died in April 1906. Following this, Dr. Alzheimer continued his investigation on her brain both morphologically and histologically after the autopsy and reported results on symptoms and histopathological findings at a meeting held in Tübingen on November 3, 1906. After a year, he published his research as a case report in 1907 (Alzheimer, 1907).

The *post mortem* examination of the diseased brain displayed atrophy, including neurofibrillary tangles and deposition of a special substance in the cortex called "military bodies", currently known as amyloid plaques (Alzheimer et al., 1995). Later in 1910, Dr. Emil Kraepelin for the first time used the term "Alzheimer's disease" for this condition in the 8$^{th}$ edition of his book *Psychiatrie*.

### 1.1.2 Epidemiology

Today Alzheimer's disease (AD) is the most common form of irreversible dementia, accounting for up to 75% of all cases. The disease is most common in the elderly. According to the Center for Disease Control, the number of people over the age of 65 will increase from 7% to 12% by 2030 worldwide (CDC, 2003; Qiu et al., 2009) with peaks in developed countries. As of 2015, about 47 million people worldwide are living with dementia of which most are suffering from AD (Baumgart et al., 2015; Prince et al., 2015), a number that is expected to triple by 2050 (Prince et al., 2013). Likewise, about 5-7 million new cases of AD are reported each year (Alzheimer'sAssociation, 2016; Qiu et

al., 2009; Reitz and Mayeux, 2014). Thus, there is a growing geriatric population of patients with AD; enormous resources are needed for the appropriate care of these patients. Considering that the societal cost of dementia is more than $215 billion every year in the US (Hurd et al., 2013; Hurd et al., 2015) and over $600 billion worldwide (Wimo et al., 2013). Therefore, dementia represents an emerging health care priority for the global society. Therefore, dementia represents an emerging health care priority for the global society.

### 1.1.3 Neuropathology

A gross visual examination of AD brains shows cortical atrophy along with the enlargement of sulci and ventricles (Selkoe and Podlisny, 2002). The medial temporal and occipital lobes along with the primary motor, sensory and visual cortex are particularly vulnerable and show increased neurodegeneration in AD patients. The atrophy which is usually first observed in the hippocampus and entorhinal cortex, is mainly due to degenerating neurites (Terry et al., 1991) and the symmetrical dilation of the lateral ventricles a result of the loss of brain tissue (Perl, 2010). Pathologically, senile plaques and neurofibrillary tangles are the main lesions of AD.

The senile or amyloid plaques are extracellular deposits of amyloid beta (Aβ) peptides (mainly peptides ending at amino acid 42 of the Aβ sequence, $A\beta_{42}$), produced after the sequential cleavage of amyloid precursor protein (APP) by β- and γ-secretases (discussed in section 1.2.4) (Serrano-Pozo et al., 2011b). Amyloid plaques are formed and accumulate preferably in the cerebral cortex (Braak and Braak, 1991). Two different types of plaques, dense and diffuse, are commonly present. A staining dye specific for misfolded, β-pleated rich Aβ is often used to visualize the former of these plaque categories. The dense plaques are often found in AD patients, contain activated astrocytes and microglial cells, and are associated with synaptic and neuronal loss (Urbanc et al., 2002; Vehmas et al., 2003). However, diffuse plaques are commonly present in non-demented cognitively normal elderly (Dickson and Vickers, 2001). Thal et al. described 5 stages of amyloid deposition, that starts from the frontal and temporal lobes (stage 1); proceeding to cover allocortical brain regions (entorhinal cortex, hippocampus and amygdala) (stage 2); subsequently the subcortical nuclei including striatum and hypothalamus (stage 3); brainstem structures including reticular formation, substantial nigra,

superior and inferior colliculi (stage 4) and finally shields cerebellar Aβ deposits (Thal et al., 2002). Although amyloid deposition does progress in a staged manner throughout particular regions of the brain, it is however important to note that this neither correlates with the severity of AD-related symptoms nor the progression of the disease (Nelson et al., 2009; Serrano-Pozo et al., 2011a).

In addition to senile plaques, neurofibrillary tangles (NFTs) are another histopathological hallmark of AD. The major component of NFTs is the microtubule-associated protein tau. Tau is a 68kD protein that is associated with microtubules, where it provides stabilization of microtubules (Dehmelt and Halpain, 2005). In disease conditions, tau become hyperphosphorylated, severely misfolded and aggregate into tangles (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b). Silver staining (also known as the Gallyas technique), or phospho-tau-specific AT8 and PHF1 antibodies are used to detect NFTs (Braak et al., 2006; Braak and Braak, 1991). NFTs are also found in other diseases such as Parkinson's and other tauopathies (Rajput et al., 1989; Wisniewski et al., 1979). The intraneuronal aggregation of NFTs is suggested to be the main cause of axonal and dendritic breakdown (Serrano-Pozo et al., 2011b). The stereotypical spatiotemporal progression of NFTs correlates well with the severity of the cognitive decline (Braak and Braak, 1991), however, whether NFT is a required precursor of the neuronal loss in AD is still controversial (Selkoe and Hardy, 2016; Serrano-Pozo et al., 2011b).

### 1.1.4 The amyloid cascade hypothesis

The amyloid cascade hypothesis was first proposed in 1991 (Hardy and Allsop, 1991; Selkoe, 1991). This hypothesis postulates that Aβ accumulation in the brain is the primary event driving AD pathogenesis (Hardy and Selkoe, 2002; Selkoe and Hardy, 2016). The aggregation of Aβ is induced by missense mutations in APP or the γ-secretase components, presenilin 1 or 2 genes that accelerate the APP processing to form $A\beta_{42}$ and cause aggressive forms of familial AD (FAD) (Citron et al., 1992; Goate et al., 1991; Hardy, 1997; Scheuner et al., 1996). A variety of factors influence the development and occurrence of sporadic Alzheimer's disease (SAD); it is primarily caused by environmental factors (Henderson et al., 2009), but may involve genetic risk factor such as the ε4 variant of the apolipoprotein E (*APOE*) gene (Polvikoski et al., 1995). The amyloid cascade hypothesis explains that the imbalance

between Aβ production and clearance is the prime cause of the disease. This imbalance gradually increases the $A\beta_{42}$ levels in the brain, resulting in oligomerization that finally initiates synaptic dysfunction and neuronal loss (Figure 1). Moreover, increased deposition of Aβ may trigger alterations and formation of neurofibrillary tangles (Hardy and Higgins, 1992).

Although the relevance of the amyloid cascade hypothesis has been a target for concern (reviewed in (Selkoe, 2011), it has had great influence on the understanding of the onset of AD and the main model guiding the development of new therapies for AD. However the lack of efficient treatment indicates that there may be missing links between Aβ build-up and clinical expression of the disease that still need to be uncovered. The central role of APP dysregulation in AD pathogenesis enforced the direction towards understanding its physiological functions (van der Kant and Goldstein, 2015). Though the role for most of the metabolized fragments have been suggested, we are far from understanding their biological significance. The way by which APP co-ordinates functions between the full length and the metabolized fragments is not well characterized. Furthermore, while Aβ induced synaptic dysfunction and neuronal loss in AD is established, the effect of interacting proteins on APP processing is still not defined (Nicolas and Hassan, 2014).

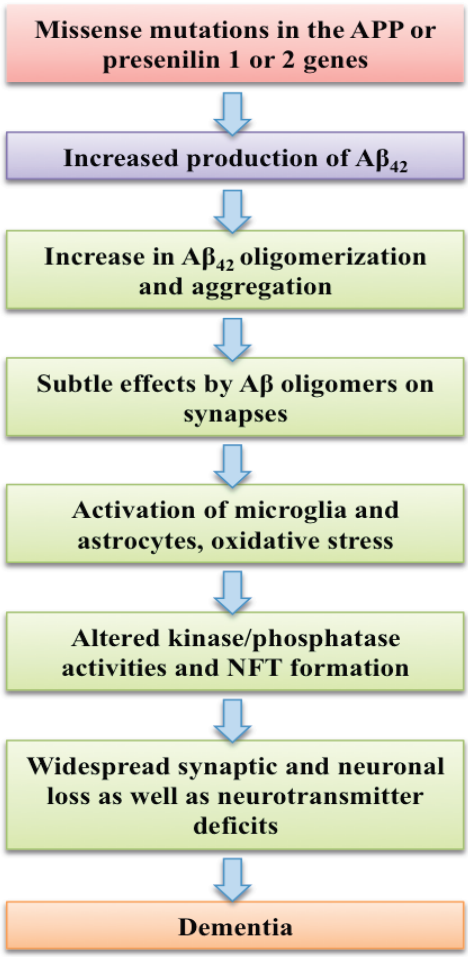


*Figure 1: The schematic describing the biological steps of the amyloid cascade hypothesis. The sequence of pathogenic events is caused by the toxicity of Aβ oligomers. Oligomeric toxicity results in inflammatory responses, synaptic disruption and subsequent hyperphosphorylation of tau. This causes progressive synaptic and neuritic injury and eventual neuronal cell death.*

## 1.2 The amyloid precursor protein

APP was discovered 30 years ago as the origin of the toxic Aβ peptide fragments, produced upon proteolytic processing (Goldgaber et al., 1987; Kang et al., 1987; Tanzi et al., 1987). However, before its discovery APP was already known as a coagulation factor (nexin-II) but it turned out later that the nexin-II and APP were the same protein (Van Nostrand et al., 1989). APP is encoded by a single gene on the distal arm of chromosome 21q21. An enormous number of genetics, biochemical and animal studies provide strong

evidence that APP-derived Aβ peptides initiate a cascade triggering synaptic dysfunction, neurodegeneration and ultimately memory impairment (Selkoe and Hardy, 2016). Genetic studies on autosomal dominant AD provide the strongest evidence implicating APP in AD. More than 200 different mutations have been identified in either the *APP* or presenilin-1 or -2 genes, which enhance APP cleavage and increase the amount of aggregation longer forms of Aβ (a relative increase in $A\beta_{42}$) (reviewed in (Tanzi, 2012)). For example, the 'Swedish' mutation near the β-secretase cleavage site leads to increased production of all Aβ forms, whilst the 'London' mutation, near the γ-secretase site, results in a relative increase in $A\beta_{42}$ in relation to shorter, C-terminally truncated and more hydrophilic Aβ forms (*e.g.*, $A\beta_{38}$ and $A\beta_{40}$) (Citron et al., 1992; Goate et al., 1991; Mullan et al., 1992; Tamaoka et al., 1994). Regarding presenilin mutations, a recent study showed that these may result in a partial loss of function of γ-secretase; this multiprotein complex degrades type I membrane proteins by sequential cleavages and γ-secretase with mutated presenilins can cleave at the 42$^{nd}$ amino acid but reaches amino acids 40, 39, 38 and 37 less effectively (Xia et al., 2015). The end result is a relative increase in aggregation-prone $A\beta_{42}$ in relation to, *e.g.*, $A\beta_{38}$. Moreover, Aβ-independent contribution of APP in AD-related neurodegeneration has been shown (Cheng et al., 2016; Pimplikar et al., 2010; Robakis, 2011).

Whilst the roles of APP (and its breakdown products) in AD pathogenesis have been intensely studied, its physiological function(s) remain poorly understood. In the path to cure AD, intensive efforts have been made to target APP cleavage to prevent Aβ generation or to enhance Aβ clearance but no clinically significant benefit of these approaches has come up yet (Abbott and Dolgin, 2016; Selkoe, 2011). Also, the basic biological function of APP has not yet been defined completely. Hence, elucidating the physiological function of APP would help in better understanding of the potential side effects of reducing APP and its processing to deliver successful therapies.

### 1.2.1 The APP family

APP is a member of an evolutionarily conserved family of type I transmembrane proteins (Figure 2). In mammals, two paralogues of APP, the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2) have been identified (Goldgaber et al., 1987; Wasco et al., 1993). APLP1 and APLP2 share high degree of sequence homology to APP and have a similar

organization of their protein domains (Slunt et al., 1994), however, unlike APP, these proteins do not contain Aβ domain (Bayer et al., 1999). Zebrafish have two homologues for the human APP *i.e.* Appa and Appb but only one orthologue to the APLPs *i.e.* Aplp1 and Aplp2 (Liao et al., 2012a; Musa et al., 2001). Details on zebrafish App are described below. APP-like proteins have been identified in invertebrates such as APPL in fruit fly (*Drosophila melanogaster*) (Luo et al., 1992; Rosen et al., 1989) and APL-1 in roundworms (*Caenorhabditis elegans*) (Daigle and Li, 1993), each carrying one gene encoding for an APP-like protein. While, the fly APPL and worms APL-1 are similar to the mammalian counterpart with a large extracellular and a small intracellular domain, the Aβ domain seems not comparable (Nicolas and Hassan, 2014; Prüßing et al., 2013). These observations indicate that only the conserved motifs rather than the non-conserved Aβ sequence, likely determine the physiological functions among the species and Aβ might have appeared during evolution to fulfill a role in complex neuronal networks.

### 1.2.2 Structure

APP consists of a large extracellular N-terminal domain and a small intracellular C-terminal domain. Structurally, APP was predicted to act as a cell-surface receptor (Kang et al., 1987) or as a growth factor (Rossjohn et al., 1999), after its discovery. The large ectodomain contains an E1 (cysteine-rich globular) domain, an acidic domain, an E2 (a helix-rich) domain and the N-terminal part Aβ sequence, extending into the transmembrane domain (Figure 2) (Reinhard et al., 2005). The small intracellular C-terminal, also known as APP intracellular domain (AICD), consists of phosphorylated sites and a YENPTY interaction motif.

The E1 domain consists of two distinct regions, the heparin-binding domain (HBD) and the copper-binding domain (CuBD). The HBD domain contains disulfide bonds, hydrophobic surface patch (hydrophobic pocket) that dimerizes in the presence of heparin (Gralle et al., 2006; Hoefgen et al., 2014). These hydrophobic regions are important sites of protein-protein interactions (Rossjohn et al., 1999). C-terminal to the HBD is the copper/metal binding domain, which can bind to several metal ions (Bush et al., 1993). Copper (II) binding and reduction is suggested to be a main function of this domain (Multhaup et al., 1996). Linked to E1 is an acidic region, rich in glutamic acid and aspartic acid residues, that is not connected with any secondary structure

formation or functional significance (Kang et al., 1987). Following the acidic region is the Kunitz-type protease inhibitor (KPI) and the OX-2 domain that are removed through alternative splicing and present only in larger isoforms (Figure 2). The shorter APP695 isoform is predominantly expressed in neurons accounting for the primary source of APP in brain (Sisodia et al., 1993). KPI containing APP is ubiquitously expressed, including non-neuronal cells (microglia and astrocytes) (Rohan de Silva et al., 1997) and in platelets to influence blood coagulation by the regulation of serine protease (Van Nostrand et al., 1990). The E2 domain contains a random coil region (RC), a second heparin site including number of putative metal-binding sites that maintains the rigid conformation of E2 (Dahms et al., 2012). The metal-binding site of E2 domain interacts with ferroportin that plays a role in cellular iron export (Duce et al., 2010). The Aβ domain resides at the end of the E2 domain and is situated partly within the ectodomain and partly within the transmembrane domain (Figure 2). The last small cytoplasmic C-terminal is a highly conserved domain among APP family members that is involved in intracellular interactions (Kerr and Small, 2005; Schettini et al., 2010). Together, this structure of APP provides insights into the similarities and dissimilarities between APP family members. Including intracellular domain, APP ectodomain (with E1 and E2 regions) is conserved, but Aβ domain is highly divergent (Walsh et al., 2007). These conserved domains between families enable them to form dimers to promote intracellular adhesion (Kaden et al., 2009; Soba et al., 2005).

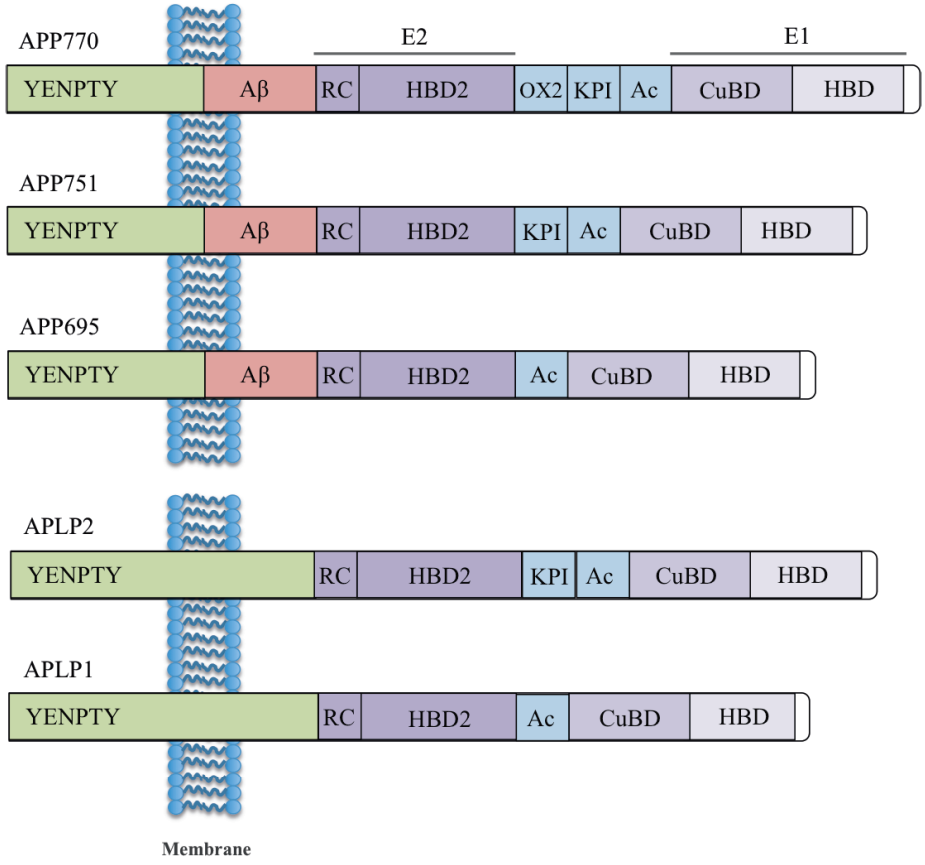


*Figure 2: Overview of the domain structure and isoforms of APP. All APP family members contain a multi-domain structure. They share conserved extracellular domains and the YENPTY motif in the carboxyl terminus but Aβ is unique for APP. Alternative splicing of APP leads to multiple isoforms, including APP770, APP751 and APP695. HBD: Heparin binding domain. CuBD: Copper binding domain. Ac: Acidic region, OX2: OX2 antigen domain. KPI: Kunitz-type protease inhibitor domain. HBD2: Heparin binding domain 2. RC: Random coil. YENPTY: Protein interaction motif.*

### 1.2.3 Expression

The mammalian *APP* gene contains 18 exons that give rise to many isoforms thorough alternative splicing (ranging from 365 to 770 amino acids) (Yoshikai et al., 1990). The most commonly expressed variants consist of 695, 751, and 770 amino acid residues, referred to as APP695, APP751 and APP770,

respectively. While all exons are included in the APP770 isoform, exon 8 encoding the OX2 domain is missing in APP751 and both exons 8 and 7 (encoding the KPI domain) are spliced out in the APP695 isoform (Tanzi et al., 1988; Weidemann et al., 1989). APP is ubiquitously expressed in the neural tissues (brain and spinal cord), as well as in a variety of non-neural tissues such as muscle (smooth, cardiac and skeletal), kidney, lung, pancreas, immune system (thymus and spleen), skin, intestine thyroid and prostate gland (Puig and Combs, 2013). APP695 is the major neuronal isoform (Kang et al., 1987) while APP770 and APP751, although present in neuronal cells, are mostly expressed in non-neural cells (as above) (Ohyagi et al., 1990; Rohan de Silva et al., 1997; Sisodia et al., 1993). More uncommon splice variants, such as the L-APP (leukocyte-derived APP) and APP639 lack exon 15 and exons 2, 7 and 8 (Beyreuther et al., 1993; Tang et al., 2003). APP693 is predominantly expressed in fetal tissues and adult liver (Tang et al., 2003), whereas L-APP splice variants (APP752, APP733, APP696, APP677) are expressed in astrocytes, microglia and in leukocytes (Beyreuther et al., 1993; Konig et al., 1992). Similar to APP, APLP2 is enriched in neuronal tissues including multiple tissues and organs, whereas APLP1 is expressed predominantly in the brain (Cappai et al., 2007; Shariati and De Strooper, 2013). During embryonic development, expression levels of each *APP* family member increase progressively (Huang and Jiang, 2011; Lorent et al., 1995). The widespread distribution and sequence homology of the *APP* gene family members suggest an important role of APP that may be reiterated in the development and homeostasis of many different tissues and organisms.

### 1.2.4 Proteolytic processing

APP is sequentially processed by secretases whereby extracellular and intracellular fragments are formed. This posttranslational processing can either be amyloidogenic to release an Aβ peptide or non-amyloidogenic if the cleavage precludes Aβ formation (shown in Figure 3) (Thinakaran and Koo, 2008). In the non-amyloidogenic pathway, the cleavage of APP is initiated within the Aβ region by α-secretase, thereby preventing Aβ production. This cleavage releases an extracellular APPα fragment (sAPPα) and a C-terminal fragment (CTFα) of 83 amino acids also known as C83 that is retained in the plasma membrane. The CTFα is subsequently cleaved by γ-secretase to generate a p3 peptide ($A\beta_{17-42}$) and the cytoplasmic APP intracellular domain (AICD) (Blennow et al., 2006; O'Brien and Wong, 2011). In contrast, the

amyloidogenic pathway is initiated by β-secretase (BACE1) cleavage of APP at the N-terminal of the Aβ domain. This cleavage produces a large extracellular sAPPβ fragment and a membrane integral C-terminal fragment (CTFβ) of 99 amino acids residues, C99. Similar to CTFα, CTFβ is processed by γ-secretase to release Aβ peptides into the extracellular space and AICD into the cytosol (Gu et al., 2001). In addition to the established amyloidogenic pathway, recent work by Andrew *et al*. report on additional cleavage of APP by δ-secretase, η-secretase and meprin β to liberate Aβ peptides (reviewed in (Andrew et al., 2016). These results indicate a growing complexity of APP processing that challenges our understanding of the role of APP in AD pathology further. Similar to APP, APLP1 and APLP2 undergo sequential cleavage by β- and γ-secretase but do not generate Aβ peptides due to sequence differences (Eggert et al., 2004; Walsh et al., 2007).

The α-secretases are members of the ADAM (a disintegrin and metalloproteinase) family of enzymes, including ADAM9, ADAM10, ADAM17 and ADAM19, which are expressed and linked to cell membrane (Allinson et al., 2003; Koike et al., 1999; Lammich et al., 1999). The α-secretases process APP in addition to many other substrates such as epidermal growth factor (EGF), interleukin 6 (IL6), cadherins, tumor necrosis factor (TNF), and Notch (Haass et al., 2012). Its activity can be regulated by the activation of protein kinase C (PKC). Activation of PKC by stimulation of muscarinic acetylcholine receptors or treatment with phorbol esters increases the production of sAPPα and decreases the Aβ generation in cells (Caporaso et al., 1992; Nitsch et al., 1992; Zhu et al., 2001). The activity of α-secretases depend on the cellular location; being constitutive activity at the cell surface (Parvathy et al., 1999), and with a regulated activity in the Golgi compartments (Skovronsky et al., 2000).

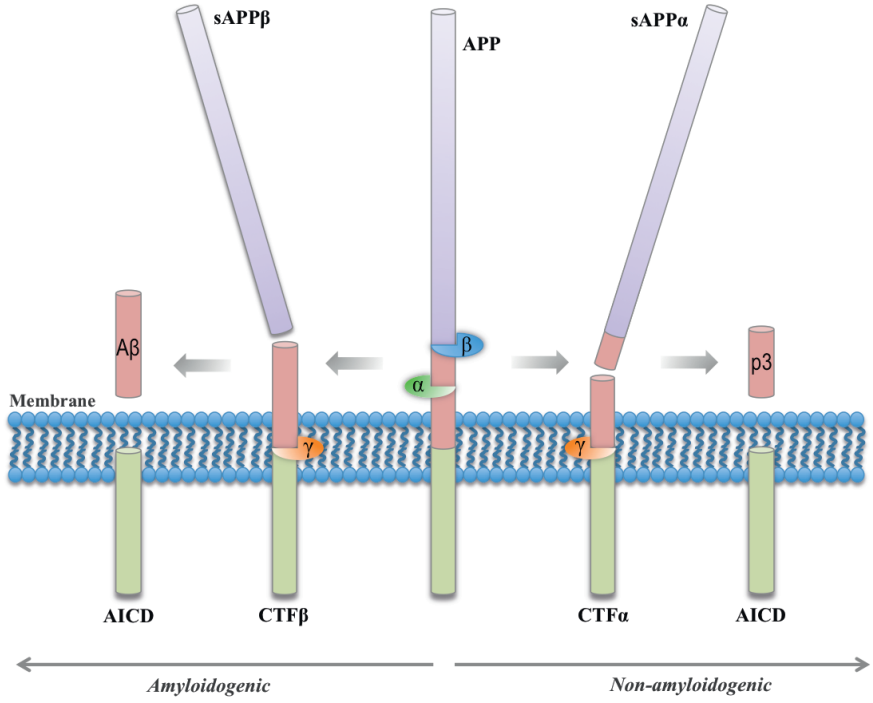


*Figure 3: Schematic illustration of the proteolytic processing of APP. Sequential proteolytic processing of APP occurs either in an amyloidogenic pathway, that produces Aβ (left) or in a non-amyloidogenic pathway (right), which generates p3 peptides and thereby precludes Aβ production.*

Beta-site amyloid precursor protein cleaving enzyme-1 (BACE1) is a transmembrane aspartyl protease with active sites in the extracellular space, also called Asp-2 and memapsin-2 with ubiquitous expression in the brain, especially in the neurons. β-secretase cleavage of APP occurs within the Golgi apparatus and endosomes due to its higher activity in acidic environments (Vassar, 2005). BACE1 initiates Aβ generation, therefore its inhibition has been proposed as good target candidate to prevent Aβ formation (Cole and Vassar, 2007; Vassar and Kandalepas, 2011). However, the identification of a broad range of additional BACE1 substrates (Hemming et al, 2009) and the role of BACE1 in important biological pathways has highlighted the risk of potential side effects (Barao et al., 2016; Mullard, 2017). The β-secretase family consists of an additional member, BACE2, expressed mainly in non-

neural cells. This secretase cleaves within the Aβ domain and is thereby not the part of the amyloidogenic pathway (Solans et al., 2000; Yan et al., 2001).

The third class of secretases, the γ-secretase is also an aspartyl protease, consisting of four subunits: PSEN (presenilin 1 or presenilin 2), NCSTN (nicastrin), APH1 (anterior pharynx defective 1) and PEN2 (presenilin enhancer 2) (Bai et al., 2015; Lu et al., 2014). PSEN1 and PSEN2 are the catalytic subunits, nicastrin works as a substrate receptor, APH1 serves as a scaffold and PEN2 acts as an enhancer of catalytic activity (De Strooper, 2003; Dries and Yu, 2008; Shah et al., 2005; Zhang et al., 2014). γ-Secretase cleaves APP at multiple sites within the transmembrane domain, generating Aβ peptides of different lengths (De Strooper et al., 2012; Sanders, 2016; Zhao et al., 2005; Zhao et al., 2004). However in familial AD, FAD-linked *APP* or *PSEN1* or *PSEN2* mutations predominantly increase the ratio between $A\beta_{42}$ and shorter Aβ peptides, such as $A\beta_{40}$ and $A\beta_{38}$ (Selkoe and Wolfe, 2007).

In addition to APP, an extensively studied substrate of γ-secretase is the Notch receptor (De Strooper et al., 2012; Hartmann et al., 2001). Cleavage of Notch by γ-secretase releases Notch intracellular domain (NICD) that translocates to the nucleus of the cell and regulates gene activity. Also, many other substrates of γ-secretase have been reported, such as ErbB4 (erb-b2 receptor tyrosine kinase 4), E-cadherin, N-cadherin, ephrin-B2 and CD44 (Haapasalo and Kovacs, 2011; Hemming et al., 2008). Inhibition of γ-secretase activity was a major target of AD therapeutics but has been hampered by severe side effects. Recently, the focus has instead been on identifying APP-selective γ-secretase modulators (GSMs), that maintain the Notch-cleaving activity (Golde et al., 2013). However, this remains challenging and yet to be completed.

### 1.2.5 Functions

APP is a complex multifunctional protein and beyond its contribution to AD pathology, it has been attributed many putative biological functions (van der Kant and Goldstein, 2015). Moreover, its multiple cleavage sites, numerous cleavage products and several roles in the central nervous system (CNS) make it challenging to understand its complete set of functions (Guo et al., 2012; Nhan et al., 2015; Wolfe and Guenette, 2007). In this section we summarize the studies related to the functions of APP and its cleavage products in the nervous system.

*Neurogenesis and neural development:* During early development, the expression pattern of APP in neuroblasts and neurons in the neural tube suggests a role in neurogenesis, including neural proliferation, differentiation and axonal outgrowth (Sarasa et al., 2000; Trapp and Hauer, 1994; Yasuoka et al., 2004). APP transgenic mice have revealed the possible contribution of APP in neural progenitor cells (NPCs) proliferation. J20 mice overexpressing human *APP* with two mutations linked to familial AD (the 'Swedish' and 'Indiana' mutations) showed a 2-fold increase in the number of proliferating stem cells in the dentate gyrus and subventricular zone (SVZ) at an age of 3 months (Jin et al., 2004; Lopez-Toledano and Shelanski, 2007). However, decreased proliferation was observed in the APP overexpressing mice at the later ages (Dong et al., 2004; Donovan et al., 2006; Naumann et al., 2010), suggested as a result of Aβ accumulation (Lopez-Toledano and Shelanski, 2007; Shu et al., 2015). Moreover, a study by Hu et al., showed that while cells derived from *App* KO mice exhibited decreased neuronal differentiation, NPCs overexpressing APP derived from Tg2576 showed greater potential to differentiate into neurons (Hu et al., 2013).

Processing of APP generates fragments that have been associated with distinct and different functions. The secreted sAPPα fragment robustly stimulates proliferation of NPCs when added to cultured cells (Baratchi et al., 2012; Hayashi et al., 1994; Ohsawa et al., 1999b). This observation was supported by a study of Demars et al., showing that the reduced NPCs proliferation following α-secretase inhibition was recovered by the addition of sAPPα (Demars et al., 2011b). Similarly, the ectodomain of APLP2 can also stimulate NPCs proliferation, but not APLP1 (Caille et al., 2004), suggesting redundancy between secreted sAPPα and sAPLP2. While sAPPα has a primary function in proliferation, sAPPβ seems mostly involved in differentiation and neural development (Chasseigneaux et al., 2011; Freude et al., 2011). In human embryonic stem cells, sAPPβ induced a rapid neural differentiation compared to sAPPα (Freude et al., 2011).

*Neurite outgrowth and guidance:* APP has been shown to play a role in neurite outgrowth and guidance by increasing neurite length and branching, either independently or via interactions with other proteins such as disabled-1 (Hoareau et al., 2008; Milward et al., 1992). Reports from *in vitro* studies showed that APP products such as sAPPα/β and AICD are involved in promoting neurite outgrowth (Chasseigneaux et al., 2011; Zhou et al., 2012).

Interestingly, cell surface interaction between reelin and APP through the E1 domain of APP has been shown to have important effects on neurite development in cell culture (Hoe et al., 2009). In support of APP playing a role in neurite outgrowth and guidance, are studies suggesting that APP interact with axon guidance cues such as semaphoring (Magdesian et al., 2011), netrin (Lourenco et al., 2009) for path finding during development and the DR6 (death receptor 6) for axon pruning (Nikolaev et al., 2009). Studies in different organisms collectively support a role of APP in neurite outgrowth and guidance. Transgenic mice lacking APP display commissural axon guidance defects characterized by thickened axon bundles and subtle defects in axon extension (Magara et al., 1999; Rama et al., 2012). Moreover, the authors provide evidence that APP is expressed in the growth cone during commissural axon navigation and coordinate with the deleted in colorectal cancer (DCC) complex to mediate netrin 1-dependent axon guidance (Rama et al., 2012). Likewise, *Appl* null flies display developmental defects in axonal growth and guidance in mushroom bodies (a cell population involved in learning and memory), by modulating Wnt-planar cell polarity (PCP) pathway (Soldano et al., 2013). *apl-1* knockout *C. elegans* show disrupted molting and morphogenesis, resulting in larval lethality; a phenotype that could be rescued by the extracellular domain of APL-1 (Hornsten et al., 2007). Additionally, several studies have proposed that APP can modulate synaptic function and neurite outgrowth via cell adhesion properties (Ando et al., 1999; Baumkotter et al., 2012; Coburger et al., 2014; Muller and Zheng, 2012; Qiu et al., 1995; Soba et al., 2005; Thinakaran and Koo, 2008; Turner et al., 2003b). For example, APP can form trans-dimers in the presence of heparin for cell-cell contacts (Dahms et al., 2010; Gralle et al., 2006), a mechanism that was proposed for the stabilization of synapses by APP (Wang et al., 2009).

*Synaptogenesis:* APP is expressed in both pre- and post-synaptic terminals during development where it is needed for proper formation and maintenance of neuronal synapses. During mammalian development, the APP level persists after birth and reaches peak levels by the second postnatal week, overlapping with the time of completion of synaptic connections and brain maturation (Loffler and Huber, 1992). Noticeably, increased expression of APP was found in mitral cells of the olfactory bulbs and also in post-synaptic boutons of the neurons coming in contact with mitral cells dendrites (Clarris et al., 1995), suggesting a role for APP in synapse formation or maintenance. Not only is

the location of APP expression important but also the adequate level. Overexpression of APP leads to increased synaptic density (Lee et al., 2010), whereas lack of APP such as in *App* knockout mice results in a lower number of spines (Dawson et al., 1999) and synapses (Tyan et al., 2012). Additionally, APP is involved in the regulation of NMJs. In a study by Wang et al., *App/Aplp2* double KO mice exhibited poorly formed NMJs (Wang et al., 2009), which was confirmed by reduced number of synaptic vesicles and impaired synaptic transmission. However, the *App/Aplp2* double KO is embryonic lethal, which may be explained by the severities in synaptic deficits (Wang et al., 2005b). The behavioral deficits in *App* knockout mice, such as reduced grip strength and locomotor activities, have been described as defects in synaptogenesis and neuromuscular junctions (Ring et al., 2007; Zheng et al., 1995). *Drosophila* with *Appl* knockout show only subtle behavioral changes but have clear defects in the maintenance of synaptic boutons at NMJs (Luo et al., 1992).

### 1.2.6 Protein interactions

APP has been shown to interact with several proteins and receptors to perform its functions. One of those is the Notch receptor.

Notch is a large transmembrane type 1 receptor that plays an essential role in the maintenance of neural stem/progenitor cell pools by enhancing symmetric division during development (Alexson et al., 2006). Notch controls neuronal differentiation and cell fate through lateral inhibition (Geling et al., 2004; Schweisguth, 2004; Shimojo et al., 2011), whereas APP increases the cell differentiation of neural progenitors (Kwak et al., 2011). While a crosstalk between these two proteins has been suggested (Fischer et al., 2005; Merdes et al., 2004a), a couple of studies have even provided evidence of a physical interaction of APP and Notch *in vitro* (Chen et al., 2006; Oh et al., 2005). When APP was first cloned, it was proposed to function as a cell surface receptor, mainly due to its high analogy with the Notch receptors (Kang et al., 1987). Processing of APP and Notch receptor is similar (Hartmann et al., 2001) in that they both are cleaved by α and γ-secretases (De Strooper et al., 1999; LaVoie and Selkoe, 2003; Selkoe and Kopan, 2003; van Tetering et al., 2009) to release AICD and the Notch intracellular domain (NICD), respectively. While the NICD enters the nucleus and regulates gene transcription, a similar function of AICD has only been suggested (Ebinu and

Yankner, 2002). Interestingly, Notch and APP can influence cleavage of the other by competing for γ-secretase cleavage (Berezovska et al., 2001), where absence of one may increase the signaling of the other protein. Moreover, because of the different spatiotemporal requirement of the Notch-APP interactions, the mechanism behind these interactions is not clear and needs further investigation (Chen et al., 2006; Fassa et al., 2005; Kim et al., 2007).

Reelin is a large secreted glycoprotein that controls neuronal migration and positioning during CNS development (Honda et al., 2011). Signaling by Reelin is mediated through the activation of APOER2 (apolipoprotein E receptor 2) and VLDLR (very-low-density lipoprotein) receptor, which subsequently leads to disabled-1 (Dab1) phosphorylation (D'Arcangelo et al., 1999; Howell et al., 1997; Trommsdorff et al., 1999). Reelin directly interacts with APP, where the N-terminal domain of Reelin binds to the E1 domain of APP (Hoe et al., 2009). This interaction has been shown to be critical for neurite outgrowth via both *in vivo* and *in vitro* studies (Hoe et al., 2009). The involvement of Reelin in AD has been suggested in multiple studies. Transgenic APP mice and AD patients have increased Reelin expression in the brain (Botella-López et al., 2010) that occasionally co-localize with Aβ oligomers (Doehner and Knuesel, 2010). Reelin-deficient transgenic AD mice accelerate the amyloidogenic APP processing and amyloid plaques deposition (Kocherhans et al., 2010). Treatment with Reelin-containing media for 24hrs increased sAPPα and CTFs in cells overexpressing APP and decreased $A\beta_{40/42,}$ suggesting that Reelin can increase α-cleavage of APP (Hoe et al., 2009; Hoe et al., 2006).

Interaction studies of the intracellular domain of APP have identified several binding partners of APP (Turner et al., 2003; Van Gassen et al., 2000) including Numb, Fe65, the JNK scaffolding protein JIP1-b and Dab. Fe65 is an adaptor protein containing phosphotyrosine interaction (PID) and phosphotyrosine binding (PTB) domains, mainly expressed in the nervous system. Fe65 binds to the YENPTY motif of the AICD domain, independent of the tyrosine phosphorylation via its PTB domain (Borg et al., 1996; Fiore et al., 1995). Binding of Fe65 to APP has been reported to modulate APP trafficking and processing to generate Aβ peptides (Ando et al., 2001). Furthermore APP-Fe65 interaction has been shown to have a role in the growth cone and synapses and in regulation of cell movement (Sabo et al., 2001; Sabo et al., 2003).

JIP1 is a member of the JNK (c-Jun N-terminal kinase) family proteins that has also been described to interact with AICD (Scheinfeld et al., 2003). Interestingly, APP-mediated neurite outgrowth has been shown through a JIP1-dependent pathway *in vitro* (Muresan and Muresan, 2005) and this interaction appears important to mediate anterograde axonal transport of APP (Fu and Holzbaur, 2013). JIP-APP interaction modulates the APP metabolism; it suppresses the secretion of the sAPP ectodomain and $A\beta_{40/42}$, as well as the intracellular release of CTFs (Taru et al., 2002), suggesting a role in the regulation of amyloidogenic pathways.

Numb proteins contain N-terminal phosphotyrosine binding domains and proline-rich regions, suggesting the role of Numb in protein-protein interactions (Pawson and Scott, 1997). Numb also interacts with APP via its phosphotyrosine binding domains and inhibits Notch signaling (Roncarati et al., 2002b). Similarly, Notch can bind to Numb to interact with APP (Fassa et al., 2005), suggesting that Numb forms independent complexes with APP and Notch and acts as a molecular link between them. Also, in AD patients and AD mouse models, perturbation of the levels of different Numb isoforms was observed, suggesting a role of Numb in the disease process (Chigurupati et al., 2011; Ntelios et al., 2012).

Another adaptor protein that binds to the YENPTY motif of APP is Dab1. During embryogenesis, Dab1 is involved in regulating the position of neurons in the brain laminar structure (Parisiadou and Efthimiopoulos, 2007). Modulation in the Dab1-APP interaction alters the proteolytic processing of APP and reduces Aβ production (Hoe et al., 2006; Morris and Cooper, 2001).

All together, these findings emphasize the importance of APP interactions with numerous adaptor proteins to convey and modulate the function of APP.

## 1.3 Zebrafish as a model organism

Zebrafish (*Danio rerio*) is a tropical freshwater fish belonging to the family *Cyprinidae* that is native to Southeast Asia. Scientists have used zebrafish as a model system to understand the developmental basis of vertebrate embryology since the 1930s (Clark and Ekker, 2015; Oppenheimer, 1936). Zebrafish is a small vertebrate that is more closely related evolutionarily to humans than the commonly used *Drosophila* or *C. elegans*. Zebrafish are easy to maintain and

breed and the costs are substantially lower compared to rodents. On a regular basis, a large number of progeny can be obtained per female, ≈ 200-300 embryos. The fertilization being external provides a number of technical advantages and makes it easier to microinject substances and perform cell transplantations or ablations. Embryonic development is rapid with all major organs developed by 5 days post-fertilization (dpf). The embryos are nearly transparent, which allows for detailed studies of normal development, as well as developmental changes in response to genetic perturbation or other exposures. Also, because of their optical clarity, the expression of genes can be monitored in real-time by using transgenic animals (Zhang and Gong, 2013). Because of these advantages, zebrafish has emerged as an alternative model to explore neurodegenerative diseases (Best and Alderton, 2008; Flinn et al., 2008; Newman et al., 2014; Xi et al., 2011). The modern era of zebrafish genetics was introduced by George Streisinger and colleagues and zebrafish has since then gained increasing popularity as a vertebrate model combining developmental biology and molecular genetics (Kimmel et al., 1995). A significant step in the zebrafish field was a large mutagenesis screen carried out in the 1990s (Driever et al., 1996; Haffter et al., 1996) from which many mutants were derived that increased our understanding of human diseases. Today, when the whole genome of the zebrafish is sequenced we know that approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe et al., 2013). With the recent advancements in genome editing, such as TALENs and CRISPR/Cas9, zebrafish has become even more amenable to address gene function.

### 1.3.1 The zebrafish amyloid precursor proteins

APP homologues in zebrafish were first identified in 2001 (Musa et al., 2001). Due to the genome duplication in the teleosts linage (Glasauer and Neuhauss, 2014; Postlethwait et al., 1998), zebrafish possess two highly conserved APP homologues, Appa and Appb. They show a high degree of sequence identity and functional conservation with human APP (Joshi et al., 2009; Wilson and Lardelli, 2013). While Appb more closely resembles the human APP695 splice variant, Appa is similar to the longer APP770 isoform (Joshi et al., 2009; Lee and Cole, 2007). Also, the zebrafish has single orthologues of the APLP1 and APLP2; Aplp1 and Aplp2 (Jelen et al., 2007; Liao et al., 2012b). During embryogenesis, zebrafish *appa* and *appb* exhibit distinct expression patterns. While *appa* is predominantly expressed in mesodermal tissues, *appb* is more

abundant and widespread in nervous tissue (developing brain and spinal cord) (Lee and Cole, 2007; Musa et al., 2001). Although, at early stages both genes display common expression in telencephalon, ventral diencephalon and trigeminal ganglia; *appa* is uniquely expressed in somites, otic vesicles and lens and *appb* is abundant in the hindbrain, the ventral mesencephalon and the developing spinal cord (Lee and Cole, 2007; Musa et al., 2001). Similar to humans, α-, β- and γ-secretases are expressed in zebrafish and seem to have conserved functions (Brunet et al., 2015). The zebrafish *psen1* (Leimer et al., 1999) and *psen2* (Groth et al., 2002) genes are orthologues of human *PSEN1* and *PSEN2*. Moreover, orthologues genes for the other components of γ-secretase complex, *pen2*, *ncstn*, *aph1b* have been identified similar to human *PEN2*, *NCSTN* and *APH1b* (Campbell et al., 2006; Francis et al., 2002; Xia, 2010; Zetterberg et al., 2006). Zebrafish also holds the orthologues of β-secretase (*BACE1* and *BACE2*), *bace1* and *bace2* (Moussavi Nik et al., 2012; van Bebber et al., 2013), similar to humans.

Morpholinos (MOs) are synthetic oligonucleotides that target mRNA of interest and down regulate synthesis of the corresponding protein. Knockdown of zebrafish App by antisense morpholino technique provided insights into the functional role of this protein during development. While, down-regulation of Appa only has mild effects on development, knockdown of Appb leads to defects in the cellular movements during convergent extension (Joshi et al., 2009). Loss of Appb activity has also been connected with defects in neural development (Song and Pimplikar, 2012). The same authors showed that only full-length human APP could rescue the neuronal defects suggesting that both intracellular and extracellular domains of APP are required for the normal function (Song and Pimplikar, 2012). These studies highlight the usefulness of the zebrafish to address the role of APP during development and although further investigations are necessary, it makes zebrafish an attractive system to study APP and its related processes in the developing nervous system.

# 2 AIM

## 2.1 The general aim

The overall aim of this thesis was to increase our understanding on the physiological functions of the amyloid precursor protein (APP) by modulating zebrafish Appb, a highly conserved homologue of human APP, during development.

## 2.2 Specific aims

*Paper I*

- To investigate the function of Appb in the axogenesis of motor neurons in the spinal cord and their subsequent synapse formation.

*Paper II*

- To study the cell-specific role of Appb during the formation of hindbrain neurons.

*Paper III*

- To characterize the CRISPR/Cas9 generated zebrafish *appb* mutants with emphasis on phenotypes observed in morphants.

*Paper IV*

- To determine the proteomic consequence of decreased Appb in developing zebrafish larvae using a quantitative TMT proteomics approach.

# 3 EXPERIMENTAL METHODS

## 3.1 Animal care and ethics statement

Zebrafish (*Danio rerio*) were maintained in Aquatic Housing Systems (Aquaneering, San Diego, USA) at 28°C under a 14 hour light/ 10 hour dark cycle in the facility at the Institute of Neuroscience and Physiology, University of Gothenburg. Fish were fed twice daily a diet of live-hatched brine shrimp (*Artemia*) and dry fish feed. The fish used for various experiments were wild-type AB, Tg(*isl1*:GFP), Tg(*mnx*:GFP) and Tg(*gata2*: GFP) Tg(*appb*:GFP) and the $appb^{26\_2}$ mutants. Mutations in *appb* were generated using CRISPR/Cas9 method at the National Institute of Health, USA. Embryos were staged by hours post-fertilization (hpf) or days post-fertilization (dpf) as described previously (Kimmel et al., 1995) and were reared until the desired stage of development. Before fixation, embryos were anesthetized in 0.02% tricaine methanesulfonate (MS-222) (Sigma-Aldrich) for all the experiments. These studies were approved by the ethical committee in Gothenburg. All procedures for the experiments were performed under standard conditions (Westerfield, 2007) and in accordance with the animal welfare guidelines of the Swedish National Board for Laboratory Animals.

## 3.2 Morpholino and mRNA microinjections

The morpholino antisense oligonucleotide (MO) targeting zebrafish *appb* (splice acceptor site *appb* MO; 5′-CTCTTTTCTCTCTCATTACCTCTTG-3' and translations site *appb* MO: 5′-TGTGTTCCCAAGCGCAGCACGTCCT-3′), Notch1a UTR and ATG MOs (*notch1a* MO; 5′-GCCTCGGCGTTACAACTTCTTTAA-3′, 5′-TTCACCAAGAAACGGTTCATAACTC-3′) were purchased from GeneTools (Philomath, USA). Both *appb* MO gave the same phenotype, however the splice-blocking MO was used in most of the experiments. Borosilicate injection needles were prepared using a P-97 Flaming/Brown micropipette puller (Sutter Instrument, Novato, USA). MO injection of 1 nl/embryo was performed at the one cell stage (Figure 4) with a FemtoJet® microinjector (Eppendorf AG, Hamburg, Germany). As controls, embryos injected with either an equal concentrations of standard control MO (Control

MO; 5′-CCTCTTACCTCAGTTACAATTTATA-3′) or uninjected embryos were used. For mRNA rescue experiment, the plasmid containing the *appb* gene was linearized and full-length *appb* mRNA synthesized using the mMessage Machine *in vitro* transcription kit (Invitrogen) and purified using phenol/chloroform extraction. Embryos were injected with mRNA and/or *appb* MO and examined at desired stages.

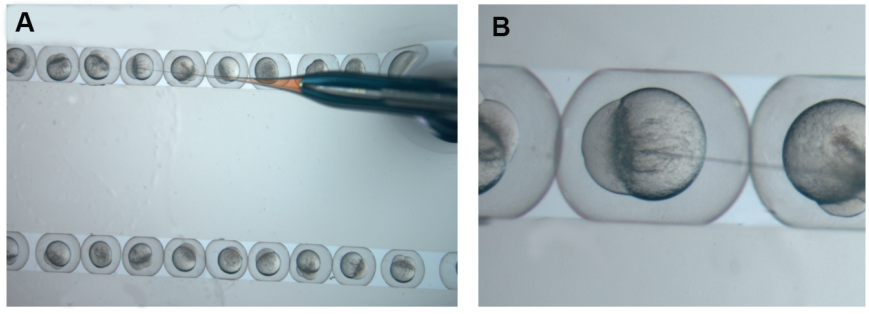


*Figure 4: (A) Fertilized zebrafish eggs are collected and aligned in trenches of an agarose microinjection chamber. (B) Morpholinos are injected into embryos at the one cell stage using borosilicate glass needle (higher magnification of A).*

## 3.3 Pharmacological treatment

γ-Secretase activity was chemically inhibited with DAPT (N-[N-(3,5-difluorophenacetyl-l-alanyl]-S-phenylglycine-t-butyl ester) (Geling et al., 2002; Song et al., 2010) in paper II. A stock solution of 10 mM DAPT (Calbiochem, La Jolla, CA) was reconstituted in 100% DMSO (dimethyl sulfoxide). Embryos were dechorionated at 6 hpf on an agarose-coated Petri dish with 0.5 mg/mL pronase (Sigma- Aldrich). To avoid precipitation of DAPT, four-well plates containing embryos in fish system water (60 mg Instant Ocean salt/L distilled water) were placed on a shaker (at maximum required speed) and DAPT added while pipetting, to achieve a final concentration of 50 mM in 1% DMSO. As a vehicle control, 1% DMSO was used. During drug exposure, embryos were incubated at 28.5 °C until 24 or 48 hpf.

## 3.4 Immunohistochemistry and confocal microscopy

Embryos between 22-24 hpf, were incubated in 0.003% PTU (1- phenyl-2-thiourea, Sigma-Aldrich) to prevent pigmentation. They were anesthetized using 0.02% MS-222 and fixed in 4% paraformaldehyde (PFA) at desired stages. For antibody staining in paper I, mouse znp1 (Developmental Studies Hybridoma Bank, DSHB) 1:500, mouse zn5 (DSHB) 1:1000, F59 (DSHB) 1:50, 4D9 (DSHB) 1:50, rabbit anti-GFP (Invitrogen) 1:1000, goat anti-mouse Alexa 488 (Invitrogen) and goat anti-rabbit Alexa 568 (Invitrogen) were used. Embryos were incubated in Phalloidin-Alexa568 (Invitrogen) 1:100, in PBS with 2% TritonX-100 for 90 min to stain muscle F-actin.

In papers II and III, for neurofilament RMO44 antibody staining, embryos were fixed in 2% trichloroacetic acid (Sigma Aldrich) at 48 hpf for 3 hrs at room temperature, washed in phosphate-buffered saline (PBS) and blocked in block solution (PBS, 0.5% Triton X-100, 10% normal goat serum, 0.1% BSA) for 1 h. Antibody labeling was performed using monoclonal mouse anti-neurofilament 160 RMO44 antibody (Sigma Aldrich) followed by goat anti-mouse Alexa 488 (Invitrogen) as secondary antibody at 1:1000 and 1:500 dilutions, respectively, and incubated overnight (ON) at 4°C. Brains were dissected out (Turner et al., 2014) and flat-mounted in a sandwich of large and small cover slips (Rath et al., 2012) with 80% glycerol. In paper II, rabbit anti-phospho-histone H3 (pH3, Millipore) 1:750 has been used for staining for the proliferation assay, and was performed on embryos fixed in 4% paraformaldehyde (PFA). Double-labeling of M-cell and Appb expressing cells was performed with the 3A10 antibody in Tg(*appb*:GFP) fish line. To examine the GFP expression in Tg(*appb*:GFP) embryos with respect to M-cell, fixed embryos were incubated in a mixture of mouse monoclonal 3A10 (DSHB) and polyclonal rabbit anti-GFP (Invitrogen). The procedure was followed as described previously (Hatta, 1992). Alexa Fluor 488 rabbit anti-GFP (Invitrogen) and Alexa 568-conjugated goat anti-mouse (Invitrogen) antibodies were used to visualize the signal.

Specimens mounted in a cover slip sandwich were imaged using Zeiss LSM710 confocal microscope (Carl-Zeiss, Jena, Germany) (Paper I and II) or Nikon A1 Confocal Microscope System (Nikon Instruments, Mellville, NY, USA) (Paper III). For all the experiments images were analyzed and produced

using ImageJ software (National Institute of Health, USA) and Adobe Photoshop CS6 (Adobe).

For cell death experiment in papers I and II, live embryos were stained with acridine orange (AO, Invitrogen). Dechorionated embryos were placed in 3-5 µg/ml AO diluted in embryo medium containing 0.02% MS-222 for 30 min at room temperature in dark. Embryos were then washed twice in embryo medium and embedded in low melting agarose and analyzed under confocal microscope for AO positive cells in the hindbrain.

For retrograde labeling in paper II, rhodamine dextran (10,000 MW, Invitrogen) was injected into the spinal cord (O'Malley et al., 1996) of anaesthetized animals at 3 dpf. Embryos were embedded in low melting agarose and imaged at 4 dpf under confocal microscope for dye labeling in the hindbrain.

## 3.5 *In situ* hybridization

In papers I, II, and III, whole-mount *in situ* hybridization (WISH) was performed on embryos fixed in 4% PFA in PBS at the desired time points, dehydrated in methanol and stored in -20 °C until analysis. Antisense digoxigenin-labeled RNA probes were synthesized from linearized DNA template against *appa, appb* (Musa et al., 2001), *aplp1, aplp2* (Liao et al., 2012), *neurog1* (neurogenin 1) (Korzh et al., 1998), *deltaA, deltaD* (Haddon et al., 1998), *her6* (hairy-related 6) (Pasini et al., 2001)), *notch1a, notch 1b* (Ke et al., 2008), *krox20* (Oxtoby and Jowett, 1993), *hoxb1a* (Prince et al., 1998) and *fgf3* (Kiefer et al., 1996). Probes were purified using Illustra MicroSpin G-50 Columns (GE Healthcare Life Sciences). Embryos were rehydrated gradually in PBSTw (0.1% Tween-20 in PBS), permeabilized in proteinase K (Sigma-Aldrich) and re-fixed in 4% formalin. Prehybridization/probe hybridization reactions were carried out at 70°C as optimal temperature and the color reaction was performed using NBT/BCIP substrate (Roche). Eventually, embryos were deyolked and mounted in glycerol as described above. Expression patterns of stained embryos were imaged using a Nikon stereomicroscope or Zeiss Axio Scope A1 and produced using Adobe Photoshop CS6 (Adobe).

## 3.6 Behavioral analysis

Swimming behavior was monitored using the Zebrabox tracking system (ViewPoint, Lyon, France). Embryos/larvae (as stated in the papers) were placed one per well in a cell culture plate containing embryo medium. Prior to testing, larvae were acclimatized for 2 h in the experimentation room and subsequently 20 min in the Zebrabox before tracking initiation. Locomotor activity was recorded at constant light and temperature. Behavior data was quantified using ZebraLab™ software and graphs were plotted in GraphPad Prism.

For electrical stimulation, control and *appb* MO-injected embryos were immobilized in 1.7% low melting agarose, in a custom-made wax chamber on a glass slide. Agarose was removed gently from the tail region to make the tail free to move as a response to the weak electrical stimulations applied to the head region. The electrode was coupled to a 2100 isolated pulse stimulator (A-M Systems Inc., Sequim, WA) eliciting pulses. Movies were recorded by a high-speed camera in paper I; the duration of tail flips was analyzed using VirtualDub 1.9.11 software. In paper II, ten electrical stimulations were supplied, one per second. Three trials were performed on each animal with 10 min time intervals. The number of tail responses were recorded with NIS Elements (Nikon) and counted manually.

## 3.7 Synapse quantification and muscle physiology

Pre- and post-synapses were quantified from confocal stacks in paper I using the Volocity® 3D Image Analysis Software 6.0.1 (Perkin Elmer, US). Data was analysed in Microsoft Excel and GraphPad Prism.

In paper I, control- and MO-injected larvae (4 dpf) were mounted for force-recording as described previously (Dou et al., 2008) to analyze the mechanical properties of the skeletal muscles. Briefly, larvae were euthanized and mounted on a micrometer screw using aluminum clips between a fixed hook and a force transducer for length adjustment. The preparations were held in bicarbonate buffered salt solution at 22°C. To give single twitches, stimulations were applied via two platinum electrodes using 0.5 ms pulses at optimal voltage with 2 min intervals. The optimal length for active force was

determined and the contraction at this length was considered as the maximal active force of the preparation. To determine the excitability of the neuromuscular activation pathway, muscles were also stimulated at optimal length using different pulse durations (0.01–0.5 ms).

## 3.8 Body length measurement

The microscopic images were acquired using Nikon stereomicroscope (Mellville, NY, USA). For one-cell stage, size of the embryo was measured across the yolk. The total body length of 24 hpf, 48 hpf and 3 dpf embryos was measured from head to the end of the tail fin. Scales at the same magnification of the images were employed and measured using NIS Elements (Nikon).

## 3.9 Western blotting

In paper III, embryos at 3 dpf were deyolked by pipetting in embryo medium. With a 23G syringe, sixty embryos were homogenized in lysis buffer (10 mM Tris-HCl pH 8.0, 2% sodium deoxycholate, 2 % SDS, 1 mM EDTA, 0.5 M NaCl, 15% glycerol) supplemented with protease inhibitor cocktail (Roche). In addition, samples were sonicated for 10 min and incubated on ice for 20 min. Supernatants were collected and concentration of the protein was determined using BCA Protein Assay Kit (ThermoFisher Scientific). Proteins were separated on a NuPAGE™ Novex™ Bis-Tris pre-cast gel (Invitrogen) and transferred on to 0.2 μM nitrocellulose membrane (GE Healthcare). After transfer, the membrane was blocked with 5% milk and immunoblotted with anti-APP (Y188) antibody (Abcam) at a dilution of 1:2000. Anti-rabbit HRP linked secondary antibody (Cell Signaling) at 1:5000 dilution was used to visualize the immunoreactivity. The signal was developed using SuperSignal West Dura Extended Duration Substrate kit (Thermo-Fisher) and imaged using ChemiDoc Imaging Systems (Bio-Rad). Thereafter, blots were re-probed with 1:20000 anti-GAPDH-HRP antibody (2D4A7) (Novus Biologicals) as a loading control. Western blot images were analyzed and produced using Image Lab™ Software (Bio-Rad).

## 3.10 Quantitative PCR

A total of 40 embryos were split into 10 embryos per sample and total RNA was extracted from each sample, by using TRI Reagent® (Sigma-Aldrich).

Briefly, the embryos were lysed in 500 µl TRI Reagent® via syringe, 25G 0.5-16mm needle and left 5 min at room temperature. Thereafter, the RNA isolation was performed according to the manufacturer's instructions with minor changes. Total RNA concentration was measured on a NanoDrop 2000/2000c spectrophotometer (ThermoScientific). Then, RNA samples were treated with RNase-free DNase (Promega) according to the manufacturer's instructions. After the treatment, total RNA was measured again and diluted in RNase-free water to a final concentration of 10 ng/µl. cDNA was synthesized from 200 ng of total RNA using a High Capacity cDNA kit with RNase inhibitor (Applied Biosystems) in a total reaction volume of 20 µl. Conversion was carried out in a single-cycle reaction on a 2720 Thermal Cycler as follows (Applied Biosystems): 25º C for 10 min, 37ºC for 120 min and 85ºC for 5 min.

Quantitative PCR was performed using inventoried TaqMan Gene Expression Assays with FAM reporter dye in TaqMan Universal PCR Master Mix with UNG according to the manufacturer's protocol, in a total reaction volume of 25 µl. qPCR reactions were carried out on Micro-Amp 96-well optical microtiter plates on a 7900HT Fast QPCR System (Applied Biosystems), using standard settings for Standard Curve qPCR. A concentration of 2.5 ng cDNA, from *appb*$^{26\_2}$ and wild-type sibling embryos (n=4) at 24 hrs of development, was used in the qPCR and all samples were run in duplicates. TaqMan® Gene Expression Assays (Applied Biosystems) were used for the following genes; Amyloid Beta (A4) Precursor Protein A (*appa*), Amyloid Beta (A4) Precursor Protein B (*appb*), Amyloid Beta Precursor Like Protein 1 (*aplp1*), Amyloid Beta Precursor Like Protein 2 (*aplp2*), Eukaryotic Translation Elongation Factor 1 Alpha 1, Like 1 (*eef1a1l1*) and Actin, Beta 1 (*actb1*).

qPCR results were analyzed with the SDS 2.3 software (Applied Biosystems). The relative quantity was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), with the sample of wild-type sibling embryos (24hpf) as the calibrator and average $C_T$:s of *actb1* and *eef1a1l1* as endogenous reference.

## 3.11 TMT labeling and LC-MS

In paper IV, zebrafish larvae at 3 dpf were anesthetized in 0.02% MS-222. Samples were placed on ice, deyolked and their heads and trunk were separated with a dissecting scalpel. Proteins were extracted from five samples containing heads or trunk from one hundred control or *appb* MO-injected

embryos using lysis buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris, pH 8.2 and protease inhibitor cocktail (Roche). Protein concentration was determined using a BCA Protein Assay Kit (ThermoFisher Scientific).

Proteins were reduced by incubating with 4.5 mM dithiothreitol (DTT), and then alkylated with 8.75 mM iodoacetamide (IAA). Samples were digested with 4 µg trypsin, acidified with trifluoracetic acid (TFA) and desalted using SepPak C18 SPE cartridges (Waters). Peptides were eluted with 50% anhydrous acetonitrile (ACN) and were dried in a vacuum centrifuge.

Peptide samples were re-solubilized in TEAB/ACN buffer (pH 8). 100 µL of TMT 10- plex was added to each sample to achieve a final concentration of 15mM TMT and incubated for 1 hour at room temperature. Samples were then quenched with hydroxylamine (final concentration of 0.25%) for 15 min and were then pooled and incubated for an additional 15 min.

Samples were reconstituted in 6 µl 2% acetonitrile and 0.1% TFA (Loading Buffer). Aliquots of 5 µl were loaded on a nanoflow-LC (RSLC nano, Thermo Scientific) equipped with a C18 trap column (PepMap Acclaim 75 µm *20 mm, Thermo Scientific), and a C18 separation column (PepMap Acclaim 75 µm * 500 mm, Thermo Scientific), coupled to a Q-Exactive electrospray ionization mass spectrometer (Thermo Scientific), fitted with a FlexiSpray ion source. The 2% acetonitrile containing 0.05% TFA was used as a loading buffer; 0.1% formic acid as buffer A; and 84% acetonitrile, 0.1% formic acid as buffer B. Gradient was applied as : t=0 min, B=3%; 140 min, B=30%; 160 min, B=45%; 165 min, B=80%. The mass spectrometer was operated in the positive ion mode. Data-dependent acquisition was used, acquiring one full MS scan (R 140k, AGC target 3e6, max IT 250 ms, scan range 400 to 1600 m/z) and up to 10 consecutive HCD MS/MS scans (R=70k, AGC target=1e6, max IT=250 ms, isolation window 1.2 m/z, NCE 32.0, charge exclusion: unassigned, >6). Samples were analyzed in triplicate.

Data was processed using Proteome Discoverer 2.1 software (ThermoFisher Scientific). Peptide identification was performed using Mascot (MatrixScience), searching the zebrafish subset of the UniProtKB-Swissprot database (release 13-10, www.uniprot.org). The search settings were as follows: precursor Δm tolerance: 15 ppm; fragment Δm tolerance: 0.05 Da; missed cleavage sites: 2; fixed modifications: carbamidomethylation.

Percolator [20] was used for scoring peptide specific matches and 1% false discovery rate (FDR) was set as threshold for identification. Reporter ion abundances were normalized based on the total abundance in each reporter ion channel and scaled on channel average so that for every protein in a file, the average intensity of all channels is 100.

## 3.12 Statistical analysis

Locomotor activity in continuous light (papers I and III) was assessed by two-way ANOVA. Statistical significance of differences in tail movement duration (paper I) between all groups was calculated using Kruskal–Wallis Test and Mann–Whitney U Test was used for comparison between two treatments. The number of tail flip, cell proliferation, apoptotic cells and rhombomere measurements were evaluated statistically using unpaired two-tailed *t*-test in paper II. For body length analysis, total locomotor activity over 60 min and qPCR (paper III), unpaired *t*-test was performed. Categorical data were presented as percentages in each group and compared using Chi-square test (papers II and III). In paper IV, comparisons of the protein abundances between controls and *appb* MO-injected samples were performed by two tailed unpaired *t*-tests. Protein abundance with a *p*-value $\leq 0.05$ and an average fold change (FC) $\geq$ 20% (log2 FC $\geq 0.263$, $\leq -0.263$) were considered as significantly differentially expressed proteins. Proteins identified accordingly were classified according to their subcellular distribution and molecular function using the bioinformatic tools DAVID v6.8 (Database for Annotation, Visualization and Integrated Discovery; http://david.abcc.ncifcrf.gov/) in order to identify both overrepresented Gene Ontology Biological Process terms and putative active biological pathways. The significance of gene-enrichment analysis was expressed by an EASE score corresponding to a modified Fisher's Exact P value. For all the experiments, statistical analysis was performed and graphs were made using GraphPad Prism software and significance was set at $p<0.05$.

# 4 RESULTS AND DISCUSSION

## 4.1 Paper I

The physiological function of APP has been hard to delineate because of potential redundancy between different APP-like proteins. Further, APP functions in early development have not been possible to study before. In contrast, the zebrafish model system allows for the analysis of cellular processes that have been hard to visualize in rodents and other mammals. In the present study, we addressed the function of zebrafish Appb, a highly conserved homologue of the human APP, in the development of motor neurons and in the formation of synapses.

Knocking down Appb is embryonic lethal and results in severe defects in the convergence-extension process during gastrulation (Joshi et al., 2009). The early requirement of Appb during development thus obscured studies of later processes during neurogenesis. However, by partial knockdown, creating a hypomorph, we found Appb to be involved in processes occurring after gastrulation. We showed undulating notochord and shorter body length as the first phenotypes observed by 24 hpf. In addition to wavy notochord, morphants showed affected locomotor behavior. During early development, zebrafish embryos exhibit sequential and stereotypic behaviors, including spontaneous coiling, rapid coiling by response to touch and ultimately organized swimming (Brustein et al., 2003). We measured the coiling activity by counting the number of tail beats at different time points during the early stages and found highly increased spontaneous coiling in morphants by 24 hpf compared to controls. Although the activity continued to be higher between 28-45 hpf as the spontaneous movements develop into swimming, in later stages the activity slowly decreased to that of controls. The observed rescue of behavioral and morphological phenotypes of *appb* knockdown by co-injections with mRNA coding for *appb* argues for a specific effect of the *appb* morpholino. Moreover, we observed extended tail-flip duration at 3 dpf in morphants induced by weak electrical stimulations. However, morpholino effects go down at later time points, restricting further locomotion studies.
Together, these behavioral tests highlight the importance of Appb for the formation of normal locomotion and suggest a function of Appb in the formation of a functional neural network in the CNS.

To examine which cells that might be affected by the loss of Appb we next analyzed the expression of *appb* during spinal cord development. Expression analysis with *in situ* hybridization showed pronounced expression of *appb* mRNA in post-mitotic motor neurons in the spinal cord. The subsequent down-regulation of Appb in the spinal cord is therefore likely to affect the initial development and maturation of these neurons. Thus, we imaged primary motor neurons (PMNs) in zebrafish embryos at 24 hpf, which revealed shorter axonal outgrowth of ventrally projecting CaP (caudal primary) combined with increased arborization in Appb hypomorph, whereas MiP (medial primary) axons were only slightly affected. It is already known that the PMNs elicit spontaneous activity as they navigate to their targets (Milner and Landmesser, 1999) and these activities decrease over time as the PMNs mature and establish NMJs. This coincides with our results where stalled axons might give rise to spontaneous bursts over a longer period of time resulting in the prolonged activity. The secondary motor neurons (SMNs), forming shortly after the PMNs, are more reminiscent of motor neurons in vertebrates including birds and mammals (Beattie et al., 1997). The SMNs were present in *appb* morphants but they displayed additional protrusions from the spinal cord and irregular patterning, suggesting an important role of Appb in the patterning of the projecting SMNs. Although both types of motor neurons follow the same path, SMNs do not require PMNs for their outgrowth. Therefore the mis-patterning of SMNs might not be the result of defects in the PMNs but possibly a direct effect of Appb reduction.

APP has been suggested to interact with guidance cues to act in neurite growth and axonal guidance (Lourenco et al., 2009; Magdesian et al., 2011). Our finding supports a role of APP in the outgrowth and patterning of motor neurons and it is possible that Appb interacts with cues in the surrounding somites to guide motor neurons on their path to reach the pre-patterned muscle targets. However, further studies are needed to provide evidence for such a mechanism.

Finally, we asked if the observed defect in motor neuron formation affects synapse formation. An appropriate relay of information from motor neurons to the muscle fibers is critical to establish normal swimming behavior in fish (Fetcho et al., 2008; Gabriel et al., 2011; Goulding, 2009). Formation of a functional muscular unit requires the appropriate contact between motor

neurons in the spinal cord and muscle fibers of the corresponding somite. Immunostaining of embryos and quantification of synapses revealed elevated densities of pre-synapses and less post-synapses in morphants. This is consistent with previous studies showing that APP is needed in both the pre- and post-synaptic neuron for the formation of normally patterned synapses (Wang et al., 2005a; Wang et al., 2009). The NMJs are established before the motor neurons reach them and are involved in guidance of the outgrowing motor neuron (Flanagan-Steet et al., 2005; Panzer et al., 2005). Loss of AChR (acetylcholine receptors) in these pre-formed junctions might possibly explain the abnormal motor neurons in *appb* morphants. Alternatively, the AChR clusters can disappear if the motor neurons do not target them. These changes did not seem to affect the overall morphology of the muscle fibers nor their contractile response. These findings suggest that muscle development and function is not affected upon partial knockdown of Appb.

All together, we show that Appb has a crucial function in the axogenesis of motor neurons in the spinal cord of the zebrafish. The neuronal defects are manifested as increased spontaneous activity during early development, followed by abnormal swimming behavior.

## 4.2 Paper II

The spinal cord motor circuitry obtains input from the hindbrain to accomplish touch response and locomotor behaviors, by conveying information from sensory neurons to muscle fibers. The locomotor abnormalities including defect in axonal branching and the seemingly strong expression of *appb* in the hindbrain as described in our previous paper, tempted us to further investigate the role of Appb in hindbrain neurogenesis.

We first performed *in situ* hybridization to analyze the expression of *appb* at different time-points during rhombomere formation in the hindbrain. The ladder-like expression pattern of *appb* emerged at 19.5 hpf, overlapping with the position of reticulospinal neurons. Next, we then decided to analyze reticulospinal neurons and found that the lack of Appb affects Mauthner cell (M-cell) development. The M-cell is the largest RS neuron positioned on each side of rhombomere 4 (r4) and sends its axon to the contralateral side of the spinal cord to make connections with motor neurons. We clearly observed that M-cells were absent either uni- or bilaterally in *appb* morphants (~88%)

although other RS neurons were grossly unaffected. Injection of full-length *appb* mRNA rescued the M-cell phenotype in morphants, supporting a specific effect of the morpholino on Appb expression. This result indicates that the Appb is critical for the development of the M-cell.

Sensory stimuli activate M-cells to fire, which further induces rapid muscle contractions to perform escape behavior. We therefore tested if the changed M-cell number altered the escape response. To mimic this behavior we used tactile stimulations, similar to those used in our previous paper, however here we provided ten continuous electrical stimulations, and counted the number of tail flips. A significant decreased in the response was observed in *appb* morphants whereas most controls responded 10 out of 10 stimuli. These results point towards an involvement of Appb in M-cell circuitry formation and that its loss of function leads to M-cell-mediated behavioral deficits.

The role of APP in neurogenesis during development and adult stages has been shown in mice (Lazarov and Demars, 2012) and since the expression of *appb* is high in axial structures at time points of M-cell formation, *i.e.*, around 7.5-10 hpf (Hanneman et al., 1988), we next asked if the reduced M-cell number is the result of lowered neurogenesis in the hindbrain. The mRNA expression of the proneural genes *neurog1* and *deltaA/D* was examined with *in situ* hybridization and a reduced expression of both of these genes was observed in the hindbrain of *appb* morphants compared to controls. Down-regulation of Delta expression follows on Notch activation and prevents neural differentiation (Nikolaou et al., 2009; Schweisguth, 2004). Furthermore, Notch signaling has an evolutionary conserved role in the developing brain in maintaining cell fate determination of NPCs (Lewis, 1998). Delta-Notch mediates the singling out of one M-cell on each side of the hindbrain through a mechanism called lateral inhibition (Haddon et al., 1998). We examined the expression of *her6* and *notch1a/b* to evaluate the effect on the Notch pathway. We found an increased expression of *her6* and *notch1a* in morphants, while *notch1b* was not clearly changed. The increased activity of Notch1a together with the decreased expression of proneural genes indicates that Appb might promote M-cell formation by modulating the Notch pathway. No changes observed by cell death assay further support this hypothesis.

M-cells are sensitive to the inhibition of Notch signaling resulting in supernumerary M-cells and this process is clearly shown in zebrafish mutants,

such as *deadly seven/Notch1a, mindbomb* and *slytherin* (Gray et al., 2001a; Itoh et al., 2003; Schier et al., 1996; Song et al., 2010; van Eeden et al., 1996). Most of these mutations lead to inhibition in Notch signaling which eventually decreases transcription of the downstream responsive *her* genes. As a consequence Delta expression is alleviated and allows for an increase expression in proneural genes and thereby increased neurogenesis. We found that decreased Appb levels give rise to the opposite situation with decreased expression of proneural genes by elevated Notch activity. Our results support previous studies in which NPC from *App* knockout mice showed decreased neural differentiation while mice overexpressing APP exhibit NPC with greater potential to differentiate into neurons (Hu et al., 2013). However, the decreased number of dividing cells found in the hindbrain of *appb* morphants is at odds with the general view that decreased differentiation allows for an increased proliferation. Interestingly, APP and its processed forms are involved in promoting proliferation of neuronal stem cells both *in vitro* and *in vivo* (Bolos et al., 2014; Caillé et al., 2004; Demars et al., 2011a; Ohsawa et al., 1999a), while increased Notch activity in progenitors inhibits proneural expression, which in turn prevents proliferation and differentiation (Lewis et al., 2009; Reynolds-Kenneally and Mlodzik, 2005). All together, these results suggest that reduction in Appb levels decreases neurogenesis and proliferation in the hindbrain and the reduction in M-cells is likely due to disturbed lateral inhibition.

Since our data suggest that elevated Notch activity suppresses M-cell development in *appb* morphants, we next aimed to inhibit Notch signaling to rescue the M-cell phenotype. DAPT is a γ-secretase inhibitor that has been used to inhibit Notch signaling in zebrafish (Song et al., 2010). DAPT treatment normalized the M-cell number in *appb* morphants in approximately 67% of the embryos as well as the expression of *ngn1, delta/D* and *her6.* Since inhibition of γ-secretase may affect cleavage of other proteins we specifically blocked the translation of the Notch1a, the main Notch receptor involved in M-cell development (Gray et al., 2001b). Similarly Notch1a knockdown rescued the M-cell number in ~61% embryos. Thus, these data show that the Notch activity in *appb* morphants can be balanced either by inhibiting cleavage of Notch by γ-secretase or by decreasing Notch1a levels. Although the exact mechanism behind this is not clear, it further supports the hypothesis that Appb modulates Notch activation to mediate M-cell formation.

A crosstalk between APP and Notch in the context of *in vivo* neurogenesis has been suggested before (Fischer et al., 2005; Kim et al., 2011; Merdes et al., 2004b). For example, both proteins may act as competitive substrates for γ-secretase cleavage (Berezovska et al., 2001; Lleo et al., 2003), implying that a changed expression level of one affects the cleavage of the other. According to this, decreased Appb levels would result in an augmented cleavage of Notch by γ-secretase and thus increased intracellular signaling. Our findings that M-cell formation was rescued after DAPT treatment or *notch1a* knockdown support this hypothesis. However, we cannot completely rule out other mechanisms by which APP could affect Notch activation such as physical interaction (Chen et al., 2006) or direct or indirect interaction of AICD (Roncarati et al., 2002a).

Several mutations with abnormalities in M-cell number have defects in segmentation where M-cells are missing either due to the lack of r4 or mislocalized as a result of changed identity of other rhombomeres (Maves et al., 2002; Moens et al., 1996; Prince et al., 1998; Sun and Hopkins, 2001; van Eeden et al., 1996). Therefore, we next examined hindbrain segmentation to confirm the identity of rhombomere 4. We used the r3/r5 marker *krox20* and r4-specific *hoxb1a* and *fgf3*. No change in the organization of r3-r5 but a significant decrease in the anterior-posterior length of r3 and r4 was observed. Similarly we did not observe any changes in the expression of *hoxb1a* and *fgf3* or specificity of either gene in morphants compared to control. Although *appb* morphants show changes in the size of rhombomere 4, maintained identity of rhombomeres 3-5 argues against a defect in segmentation. Therefore, it is unlikely that Appb plays a role in segmentation that would affect M-cell development. We also checked the organization of facial branchiomotor neurons using Tg(*isl:*GFP*)* fish line, since these neurons form early in r4 and migrate to r7 where they extend axons back to r4 and out to their targets (Chandrasekhar, 2004). The complex development of these neurons is sensitive to changes in hindbrain segmentation. We could not detect any changes in the spatial arrangement of these neurons. Thus, while the shorter lengths of r3 and r4 in *appb* MO might be a consequence of decreased neurogenesis, the loss of M-cells is likely not a result of changed r4 identity. In addition, the maintained patterning of facial motor neurons of *appb* morphants suggests that Appb not only acts to promote differentiation in general but supports a more restricted interaction between Appb and Notch1a.

We also analyzed if Appb is expressed in mature M-cells using Tg(*appb*:GFP) and performed double labeling with M-cell specific antibody 3A10 via immunostaining. Confocal imaging of the dissected hindbrains indicates that the M-cell expresses no or low levels of Appb as compared to the surrounding cells. However, a lack of markers for the early development of the M-cell restricts our ability to determine at which specific developmental stage Appb is expressed in the M-cell and when the interaction between Appb and Notch1a is required. The apparent lack of Appb expression in the M-cell at later stages indicates that such mechanisms might be mediated in a non-cell autonomous manner.

## 4.3 Paper III

In our previous papers, we have shown that *appb* is strongly expressed in the brain and spinal cord and required for motor neuron guidance and M-cell development. To explore the effect of a genetic mutation of *appb* we describe the characterization of zebrafish with a homozygous non-sense mutation generated with CRISPR/Cas9 technology.

We established two alleles with non-sense mutations in exon 2 of the *appb* gene. As they showed similar morphology, and we here use $appb^{26\_2}$ allele for all experiments. Sanger sequencing of mutant fish showed disrupted reading frame as the deletion site introduced a premature stop in exon 2. We next determined the protein expression by western blot. At present, the commercially available antibodies recognize both Appb and Appa. The band intensity was greatly reduced in $appb^{26\_2}$ mutants and *appb* MO-injected embryos and the remaining weak protein expression most likely represents Appa. This result shows that *appb* mutant has low or no Appb protein remaining.

Morphological analysis of *appb* mutants at early time points showed significantly smaller size at 1 cell stage and shorter body length until 48 hpf embryos compared to the wild-type. However, no significant change was observed at later stages, *i.e.*, 3 dpf. Additionally, at blastula stage we observed protruding cells, indicative of a perturbed cell adhesion in about 15% of $appb^{26\_2}$ embryos. Only half of these embryos survived to develop further, indicating compensatory mechanisms that might eventually work to overcome the very early morphological alterations. Together, $appb^{26\_2}$ mutants displayed

subtle morphological phenotypes at early stages but those that survived were grossly normal. These morphological data differ from the previously described *appb* morphants, showing severe morphological abnormalities (Joshi et al., 2009; Kaiser et al., 2012; Song and Pimplikar, 2012). However, *appb* mutants are similar to *App* knockout mice that are grossly normal with slightly smaller body (Müller et al., 1994).

Next, we analyzed the swimming behavior of *appb* mutant larvae under 60 min continuous light using video tracking system. Quantification of locomotor recording at 5 dpf shows *appb* mutants exhibiting hyperactive swimming behavior as analyzed by parameters; distance travelled, duration of movements and number of movements, confirming an important role of Appb in zebrafish swimming behavior. This data is consistent with the *appb* morphants where we have previously observed a significant increase in coiling by 21 to 26 hpf, and spontaneous swimming behavior at later stages (28-45 hpf). However this hyperactive behavior was slowly attenuated, which we believe may be due to the morpholino losing its effect beyond 3-4 days. In contrast, *App* knockout mice have reduced locomotor activity in adults (Guo et al., 2012; Zheng et al., 1995). It has to be emphasized here that the rodent behavioral studies are performed on adults and the zebrafish behavioral responses are monitored in early life. This difference would be significant because Appb might have different roles in behavior during development and in adult animals. Another possibility could be that the altered expression of App family members might have an impact on the hyperactive behavior observed which needs further investigation.

APP has functional redundancy with other family members (as reviewed in (Shariati and De Strooper, 2013)). Therefore, to investigate if the *appb* mutation affects other *app* family members, we performed whole mount *in situ* hybridization and qPCR on wild-type and $appb^{26\_2}$ mutants for *appa, appb, aplp1* and *aplp2* mRNA expression at 24 hpf embryos. Interestingly, expression analysis of *appb* mutants showed increased expression of *appa* and *aplp2* but nearly no change in *aplp1*. Moreover, *appb* expression drastically decreased in $appb^{26\_2}$ mutants, particularly no expression was observed specially in the hindbrain and spinal cord region. The APP family contains *App, Aplp1* and *Aplp2* genes that have partially overlapping functions (Jacobsen and Iverfeldt, 2009) where $Aplp2^{-/-}$ in combination with $App^{-/-}$ or $Aplp1^{-/-}$ are lethal, suggesting redundancy between *Aplp2* and the other family

members (Heber et al., 2000). It is therefore likely that redundancy between App family members compensate for the observed normal phenotypes in zebrafish *appb* mutants. Moreover, our data on the upregulation of mRNA expression of *appa*, the paralogue of *appb* and one of the other family members, *aplp2* in *appb* mutants supports the involvement of *appa* and *aplp2* genes in compensation for *appb* mutation resulting in embryos showing normal phenotype. Furthermore, if there are other proteins also involved in compensating the *appb* mutant phenotypes will need further investigation.

One of the major phenotypes in *appb* morphants is the M-cell defects described in paper II. However, M-cells of the genetic mutant were unaffected and presented bilaterally like in the wild-type embryos. To address this discrepancy, we injected *appb* morpholinos in $appb^{26\_2}$ mutants, since lack of Appb in mutants should not affect M-cell development. In contrast to the unilaterally or bilaterally absent M-cells found in most of the MO-injected wild-type embryos, no defects in the M-cell development was observed in any of the injected mutants. These results suggest that the *appb* knockout animals are less sensitive for injections of *appb* morpholino suggesting that there might be compensatory gene expression in the *appb* mutants that is not present in *appb* morphants. In our previous study, we have shown that Notch signaling is increased following *appb* knockdown, thereby decreasing the M-cell formation in zebrafish. Since APP and Notch both are competitive substrates for γ-secretase cleavage, it might be that the increase in *appa* and *aplp2* observed in *appb* mutants balance Notch signaling by acting as competitive substrates for γ-secretase and thereby allowing normal formation of M-cell in mutants. However, whether Notch is altered in *appb* mutants is yet to be verified. Finally, it is also possible that apart from *app* family members, other genes can compensate for *appb* loss of function, which remains a subject of study. A recent study has highlighted potential reasons for differences in results obtained in knockout vs. knockdown experiments. In knockout experiments, the cells that lack a certain gene may activate back-up genetic programs that may compensate for the deficit, whilst in acute knockdown experiments, there is no time for these back-up programs to get activated in a well-regulated manner (Rossi et al., 2015). If this turns out to be generally valid for the morpholino method, explaining many of the observed discrepancies between genetic mutants and morpholino knockdown phenotypes, we suggest that the two techniques (knockdown and knockout) should be used in parallel to

maximize our understanding of the function of certain genes, though both methods need critical comparison and solid interpretation of phenotypes.

## 4.4 Paper IV

In this manuscript, we address the effect of Appb down-regulation on the protein level in the head and trunk of zebrafish embryos using a quantitative LC-MS approach. Our previous studies (paper I and II) revealed hyperactivity, defect in motor neuron outgrowth and changes in synaptic density and M-cell formation in *appb* knockdown embryos. We therefore aimed at identifying proteins and pathways affected in *appb* morphants that may underlay the observed phenotypes.

To determine the molecular targets of Appb, we extracted proteins from five hundred heads and trunks of control and *appb* morphants at 3 dpf. We used a TMT 10-plex protein labeling approach and quantitatively analyzed ten samples at a time with mass spectrometry. The reproducibility of peptide identifications between the replicates shown as the coefficient of variation (CV), and was calculated as the peptide identification frequency that was similar between the head and trunk samples. Interestingly, we identified more than 8800 and 6800 proteins in the head and trunk respectively. Clear dysregulation of proteins in both head and trunk of *appb* MO-injected embryos were found and a total of 918 proteins in the head and 433 proteins in the trunk were selected as significantly differentially expressed (FC>20%, $p<0.05$) in *appb* morphants compared to the control. Gene ontology (GO) analysis, addressing major biological changes after Appb down-regulation, indicated similar distributions in differentially expressed proteins involved in biological processes in head and trunk, such as transport, lipid transport, cholesterol homeostasis and neuron projection regeneration. A large number of these proteins were localized to the mitochondria. Nevertheless, while proteins in the head were mainly annotated as cytoplasmic or from intracellular vesicles, proteins from the trunk were localized to the extracellular space, cytosol, lysosome and sarcolemma. Although a majority of the annotated molecular functions were similar between the head and trunk, functions such as nucleotide-binding, microtubule and motor activity showed to be specific for the head while heme-, iron and NAD-binding were specific to the trunk.

Moreover, in support of the previous finding showing binding of AICD to the promoter region to alter the transcription of various genes, we found the altered expression of CamKIIα (calmodulin-dependent protein kinase II), the ionotropic glutamate receptors (GluR) and the patch1 paralogue patch2 in morphants. This finding strengthens the involvement of Appb in the regulation of these genes.

From our previous studies, we found that *appb* morphants showed increased Notch activity, changes in axon guidance and synapse formation. Therefore, we searched for differential expression of proteins involved in these processes. Remarkably, we found several upstream and downstream proteins involved in the Notch pathway including the Notch2 receptor, lunatic fringe (Lnfg), Atp2a1l (ATPase sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ transporting 1-like), Atp2a1 (ATPase sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ transporting 1), and Crebbpb (CREB-binding protein-b) being upregulated. Although this data needs to be confirmed by other methods, it supports our previous finding that Notch pathway is altered in *appb* morphants.

APP is suggested to play a role in neurite outgrowth in *App* knockout mice and *in vitro*. In our previous report, we showed that, zebrafish with decreased levels of Appb have outgrowth defects and changes in patterning of their motor neurons. Within the list of significantly changed proteins, we find several proteins involved in axon guidance as down-regulated with the exceptions of Sdc2 (syndecan 2), Plxna3 (plexin3), Kif4 (kinesin family member 4), Fn1b (fibronectin 1b) and Epha2 (eph receptor A2) that were upregulated. These findings support a function of Appb in axon guidance and will serve as a valuable source for further studies.

The TMT 10-plex mass spectrometry method has not been used to identify whole organism changes in the zebrafish before. Using this cutting edge method, we not only show high reproducibility between our technical replicates but also the identification of a substantial number of proteins with significantly altered expression in *appb* knockdown fish corresponding to the effects observed. We believe that these results provide insights into the molecular processes and pathways orchestrated by Appb.

# 5 CONCLUSION

Attention towards the basic biological function of APP beyond its role in AD pathogenesis has increased. It is known by now that APP is essential in establishing and maintaining neuronal architecture. However, much remains to be elucidated especially with respect to the cellular mechanisms of APP function. This thesis aimed to increase our knowledge on the physiological role of App during development using zebrafish as a model system. A few studies on zebrafish Appb, a homologue of human APP, show its crucial roles during different stages of neural development. Our studies continue to address the central function of Appb and highlight its essential roles beyond disease biology.

The *ex utero* development of zebrafish in combination with their transparency allow unique *in vivo* studies of cellular changes, which has not been used to address APP function previously. We show the vital role of Appb in guiding spinal cord motor neurons during axogenesis and synapse formation at the NMJ. Also, the neuronal defect ultimately affects the locomotor behavior in the developing zebrafish larvae.

Further studies discovered a cell-specific utility of Appb in a subset of hindbrain neurons. Here we show that Appb-mediated competitive regulation of Notch1a activity is essential for hindbrain specific M-cell development and neuronal differentiation. This is the first study to our knowledge that confirms a cell-specific requirement of Appb-Notch interaction during development.

Contrary to our previous results using morpholinos, the initial characterization of the genetic *appb* mutants showed that these appeared morphologically normal. However, they were significantly smaller and showed cell adhesion defects during early development. They also exhibited behavioral changes and alterations in the expression of other *app* family members. We show that *appb* mutants do not exhibit M-cell phenotype as morphants but are not sensitive to the *appb* morpholinos injection. These data, together with the earlier generated morpholino data in zebrafish plus the data from mouse models suggest that Appb indeed plays important roles during development and that evolution has created a back-up system of APP-like protein-encoding genes and potentially other genes that may eventually compensate for any loss of its function.

Lastly, we used a quantitative TMT proteomics approach to discover proteins dysregulation after *appb* knockdown. In support to our previous studies we found upregulation of several proteins involved in the Notch pathway as well as changes in proteins involved in axon guidance and vesicle formation.

All together, these studies provide *in vivo* evidence of an essential role of Appb during neurogenesis in the spinal cord and hindbrain and together with the proteomics analysis, give new insights into the physiological function of App in vertebrates. We believe that the zebrafish model system used here is a valuable tool to improve our understanding of App function and that the above described studies will significantly contribute to advance knowledge on the physiological roles of this complex protein during neural development.

# 6 ACKNOWLEDGEMENTS

With this thesis, I would like to sincerely thank my supervisors, my colleagues, my friends and my family who have contributed to the completion of this thesis. I owe my gratitude to everyone and I will cherish this experience forever.

First, I would like to convey my deep gratitude to my supervisors, Henrik Zetterberg and Alexandra Abramsson, for giving me the opportunity to pursue my PhD. Your continuous support, encouragement and outstanding knowledge, helped me a lot for the progress of this thesis work and I feel lucky that I had you as my supervisors.

Henrik, you have been very stimulating during every meeting and discussion we had together, which assisted my scientific thinking and application. Your enthusiasm and energy towards science, passion towards life, deeply influenced me. I have been extremely fascinated by your swift responses when in need. I could not have asked for any better. My sincere thanks goes to Alexandra, for teaching me the base of this project, generating new ideas, long-critical discussions, for listening to and guiding me patiently through the problems and obstacles. I am also thankful to you for the fun time walks, for introducing me to climbing and for your tips for the life outside the lab. I have enjoyed working with you all the time and I have gained a lot of scientific knowledge from you. Thank you both for your patience and advices. I am extremely grateful for everything you have taught me.

I would like to extend my sincere thanks to my co-supervisor, Petronella Kettunen, for all the help in the lab and for other interesting project discussions, as well as the fun time outside the lab. I would like to thank you also for your valuable feedback and suggestions on the thesis writing.

I would like to express my gratitude to Malin Edling, Tuğçe Munise Şatır, Jasmine Chebli, Johan Gobom and Karl Hansson, for the essential contribution to the work presented in this thesis. My sincere thanks to Elisa Alexandersson, for the tremendous technical help in the fish facility and also for the great humor, which I enjoyed a lot.

I would like to thank our wonderful group members: Maria Holmström, Lotta Agholme, Petra Bergström, Faisal Nazir, Maria Olsson for the suggestions during the meetings and for always being available for my questions or for any help.

To all my colleagues at the Department of Clinical Chemistry, for helping me out whenever needed and for the great fika breaks.

I would like to thank my office mates, Susanne Nyström, Aida Muslimovic, Pegah Johansson (former) and Michaela Johansson for sharing the general knowledge and all the active discussions about health.

I would like to thank Inger Almgren, Liselotte Nilsson and Mari Klaesson from the administrative department for the time and efforts in helping out with all the official documents.

Carl-Henrik Andersson, my former lab mate and friend, for the nice exchange of knowledge, for being a great co-worker and a nice person.

I would like to thank Wojciech Michno, my dear friend, for the scientific discussions and coffee breaks during weekends.

I would like to thank Pradeep Kopparapu, for helping me whenever needed, for suggestions and for the nice food.

I deeply thank my parents for having faith in me and my dreams and supporting me to be as ambitious as I wanted. It is under your blessings that I gained so much drive and an ability to tackle challenges head on. Also, I would like to thank my parents-in-law, for treating me like a friend and for the great care. Finally and most importantly, I would like to express my deepest appreciation and endless thanks to my wife Tulasi. Tulasi, your care for me, unconditional support, your patience and unwavering love during this time, pushed me to reach here. You have brought out the best of me; I could not have done this without you.

# REFERENCES


Alzheimer, A. (1907). Über eine eigenartige Erkrankung der Hirnrinde. Allgemeine Zeitschrift fur Psychiatrie und Psychisch-gerichtliche Medizin 64, 8.

Abbott, A., Dolgin, E., 2016. Failed Alzheimer's trial does not kill leading theory of disease. Nature 540, 15-16.

Alexson, T.O., Hitoshi, S., Coles, B.L., Bernstein, A., van der Kooy, D., 2006. Notch signaling is required to maintain all neural stem cell populations--irrespective of spatial or temporal niche. Developmental neuroscience 28, 34-48.

Allinson, T.M.J., Parkin, E.T., Turner, A.J., Hooper, N.M., 2003. ADAMs family members as amyloid precursor protein α-secretases. Journal of neuroscience research 74, 342-352.

Alzheimer, A., Stelzmann, R.A., Schnitzlein, H.N., Murtagh, F.R., 1995. An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde". Clinical anatomy (New York, N.Y.) 8, 429-431.

Alzheimer'sAssociation, 2016. 2016 Alzheimer's disease facts and figures. Alzheimer's & dementia : the journal of the Alzheimer's Association 12, 459-509.

Ando, K., Iijima, K.I., Elliott, J.I., Kirino, Y., Suzuki, T., 2001. Phosphorylation-dependent regulation of the interaction of amyloid precursor protein with Fe65 affects the production of beta-amyloid. The Journal of biological chemistry 276, 40353-40361.

Andrew, R.J., Kellett, K.A.B., Thinakaran, G., Hooper, N.M., 2016. A Greek Tragedy: the Growing Complexity of Alzheimer Amyloid Precursor Protein Proteolysis. Journal of Biological Chemistry.

Bai, X.-c., Yan, C., Yang, G., Lu, P., Ma, D., Sun, L., Zhou, R., Scheres, S.H.W., Shi, Y., 2015. An atomic structure of human [ggr]-secretase. Nature 525, 212-217.

Barao, S., Moechars, D., Lichtenthaler, S.F., De Strooper, B., 2016. BACE1 Physiological Functions May Limit Its Use as Therapeutic Target for Alzheimer's Disease. Trends Neurosci 39, 158-169.

Baratchi, S., Evans, J., Tate, W.P., Abraham, W.C., Connor, B., 2012. Secreted amyloid precursor proteins promote proliferation and glial differentiation of adult hippocampal neural progenitor cells. Hippocampus 22, 1517-1527.

Baumgart, M., Snyder, H.M., Carrillo, M.C., Fazio, S., Kim, H., Johns, H., 2015. Summary of the evidence on modifiable risk factors for cognitive decline and dementia: A population-based perspective. Alzheimer's & Dementia: The Journal of the Alzheimer's Association 11, 718-726.

Bayer, T.A., Cappai, R., Masters, C.L., Beyreuther, K., Multhaup, G., 1999. It all sticks together--the APP-related family of proteins and Alzheimer's disease. Molecular psychiatry 4, 524-528.

Beattie, C.E., Hatta, K., Halpern, M.E., Liu, H., Eisen, J.S., Kimmel, C.B., 1997. Temporal Separation in the Specification of Primary and Secondary Motoneurons in Zebrafish. Developmental Biology 187, 171-182.

Berezovska, O., Jack, C., Deng, A., Gastineau, N., Rebeck, G.W., Hyman, B.T., 2001. Notch1 and amyloid precursor protein are competitive substrates for presenilin1-dependent gamma-secretase cleavage. The Journal of biological chemistry 276, 30018-30023.

Best, J.D., Alderton, W.K., 2008. Zebrafish: An in vivo model for the study of neurological diseases. Neuropsychiatric Disease and Treatment 4, 567-576.

Beyreuther, K., Pollwein, P., Multhaup, G., Monning, U., Konig, G., Dyrks, T., Schubert, W., Masters, C.L., 1993. Regulation and expression of the Alzheimer's beta/A4 amyloid protein precursor in health, disease, and Down's syndrome. Annals of the New York Academy of Sciences 695, 91-102.

Blennow, K., de Leon, M.J., Zetterberg, H., 2006. Alzheimer's disease. Lancet (London, England) 368, 387-403.

Bolos, M., Hu, Y., Young, K.M., Foa, L., Small, D.H., 2014. Neurogenin 2 mediates amyloid-beta precursor protein-stimulated neurogenesis. J Biol Chem 289, 31253-31261.

Borg, J.P., Ooi, J., Levy, E., Margolis, B., 1996. The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. Molecular and Cellular Biology 16, 6229-6241.

Botella-López, A., Cuchillo-Ibáñez, I., Cotrufo, T., Mok, S.S., Li, Q.-X., Barquero, M.-S., Dierssen, M., Soriano, E., Sáez-Valero, J., 2010. β-amyloid controls altered Reelin expression and processing in Alzheimer's disease. Neurobiology of Disease 37, 682-691.

Braak, H., Alafuzoff, I., Arzberger, T., Kretzschmar, H., Del Tredici, K., 2006. Staging of Alzheimer disease-associated neurofibrillary pathology using paraffin sections and immunocytochemistry. Acta neuropathologica 112, 389-404.

Braak, H., Braak, E., 1991. Neuropathological stageing of Alzheimer-related changes. Acta neuropathologica 82, 239-259.

Brunet, F.G., Fraser, F.W., Binder, M.J., Smith, A.D., Kintakas, C., Dancevic, C.M., Ward, A.C., McCulloch, D.R., 2015. The evolutionary conservation of the A Disintegrin-like and Metalloproteinase domain with Thrombospondin-1 motif metzincins across vertebrate species and their expression in teleost zebrafish. BMC Evolutionary Biology 15, 22.

Brustein, E., Saint-Amant, L., Buss, R.R., Chong, M., McDearmid, J.R., Drapeau, P., 2003. Steps during the development of the zebrafish locomotor network. Journal of physiology, Paris 97, 77-86.

Bush, A.I., Multhaup, G., Moir, R.D., Williamson, T.G., Small, D.H., Rumble, B., Pollwein, P., Beyreuther, K., Masters, C.L., 1993. A novel zinc(II) binding site modulates the function of the beta A4 amyloid protein precursor of Alzheimer's disease. The Journal of biological chemistry 268, 16109-16112.

Caille, I., Allinquant, B., Dupont, E., Bouillot, C., Langer, A., Muller, U., Prochiantz, A., 2004. Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. Development (Cambridge, England) 131, 2173-2181.

Caillé, I., Allinquant, B., Dupont, E., Bouillot, C., Langer, A., Müller, U., Prochiantz, A., 2004. Soluble form of amyloid precursor protein regulates proliferation of progenitors in the adult subventricular zone. Development 131, 2173-2181.

Campbell, W.A., Yang, H., Zetterberg, H., Baulac, S., Sears, J.A., Liu, T., Wong, S.T., Zhong, T.P., Xia, W., 2006. Zebrafish lacking Alzheimer presenilin enhancer 2 (Pen-2) demonstrate excessive p53-dependent apoptosis and neuronal loss. Journal of neurochemistry 96, 1423-1440.

Caporaso, G.L., Gandy, S.E., Buxbaum, J.D., Ramabhadran, T.V., Greengard, P., 1992. Protein phosphorylation regulates secretion of Alzheimer beta/A4 amyloid precursor protein. Proceedings of the National Academy of Sciences of the United States of America 89, 3055-3059.

Cappai, R., Elise Needham, B., Ciccotosto, G.D., 2007. The Function of the Amyloid Precursor Protein Family, in: Barrow, C.J., Small, D.H. (Eds.), Abeta Peptide and Alzheimer's Disease: Celebrating a Century of Research. Springer London, London, pp. 37-51.

CDC, 2003. From the Centers for Disease Control and Prevention. Public health and aging: trends in aging--United States and worldwide. Jama 289, 1371-1373.

Chandrasekhar, A., 2004. Turning heads: Development of vertebrate branchiomotor neurons. Developmental Dynamics 229, 143-161.

Chasseigneaux, S., Dinc, L., Rose, C., Chabret, C., Coulpier, F., Topilko, P., Mauger, G., Allinquant, B., 2011. Secreted Amyloid Precursor Protein β and Secreted Amyloid Precursor Protein α Induce Axon Outgrowth In Vitro through Egr1 Signaling Pathway. PLOS ONE 6, e16301.

Chen, C.D., Oh, S.Y., Hinman, J.D., Abraham, C.R., 2006. Visualization of APP dimerization and APP-Notch2 heterodimerization in living cells using bimolecular fluorescence complementation. Journal of neurochemistry 97, 30-43.

Cheng, N., Jiao, S., Gumaste, A., Bai, L., Belluscio, L., 2016. APP Overexpression Causes Aβ-Independent Neuronal Death through Intrinsic Apoptosis Pathway. eNeuro 3, ENEURO.0150-0116.2016.

Chigurupati, S., Madan, M., Okun, E., Wei, Z., Pattisapu, J.V., Mughal, M.R., Mattson, M.P., Chan, S.L., 2011. Evidence for altered Numb isoform levels in Alzheimer's disease patients and a triple transgenic mouse model. Journal of Alzheimer's disease : JAD 24, 349-361.

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A.Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., Selkoe, D.J., 1992. Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production. Nature 360, 672-674.

Clark, K., Ekker, S., 2015. How Zebrafish Genetics Informs Human Biology. Nature Education 8, 3.

Clarris, H.J., Key, B., Beyreuther, K., Masters, C.L., Small, D.H., 1995. Expression of the amyloid protein precursor of Alzheimer's disease in the developing rat olfactory system. Brain research. Developmental brain research 88, 87-95.

Cole, S.L., Vassar, R., 2007. The Basic Biology of BACE1: A Key Therapeutic Target for Alzheimer's Disease. Current Genomics 8, 509-530.

D'Arcangelo, G., Homayouni, R., Keshvara, L., Rice, D.S., Sheldon, M., Curran, T., 1999. Reelin is a ligand for lipoprotein receptors. Neuron 24, 471-479.

Dahms, S.O., Könnig, I., Roeser, D., Gührs, K.-H., Mayer, M.C., Kaden, D., Multhaup, G., Than, M.E., 2012. Metal Binding Dictates Conformation and Function of the Amyloid Precursor Protein (APP) E2 Domain. Journal of molecular biology 416, 438-452.

Daigle, I., Li, C., 1993. apl-1, a Caenorhabditis elegans gene encoding a protein related to the human beta-amyloid protein precursor. Proceedings of the National Academy of Sciences of the United States of America 90, 12045-12049.

Dawson, G.R., Seabrook, G.R., Zheng, H., Smith, D.W., Graham, S., O'Dowd, G., Bowery, B.J., Boyce, S., Trumbauer, M.E., Chen, H.Y., Van der Ploeg, L.H., Sirinathsinghji, D.J., 1999. Age-related cognitive deficits, impaired long-term potentiation and reduction in synaptic marker density in mice lacking the beta-amyloid precursor protein. Neuroscience 90, 1-13.

De Strooper, B., 2003. Aph-1, Pen-2, and Nicastrin with Presenilin generate an active gamma-Secretase complex. Neuron 38, 9-12.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A., Kopan, R., 1999. A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. Nature 398, 518-522.

De Strooper, B., Iwatsubo, T., Wolfe, M.S., 2012. Presenilins and γ-Secretase: Structure, Function, and Role in Alzheimer Disease. Cold Spring Harbor perspectives in medicine 2, a006304.
Dehmelt, L., Halpain, S., 2005. The MAP2/Tau family of microtubule-associated proteins. Genome biology 6, 204.
Demars, M.P., Bartholomew, A., Strakova, Z., Lazarov, O., 2011a. Soluble amyloid precursor protein: a novel proliferation factor of adult progenitor cells of ectodermal and mesodermal origin. Stem Cell Research & Therapy 2, 1-15.
Demars, M.P., Bartholomew, A., Strakova, Z., Lazarov, O., 2011b. Soluble amyloid precursor protein: a novel proliferation factor of adult progenitor cells of ectodermal and mesodermal origin. Stem cell research & therapy 2, 36.
Dickson, T.C., Vickers, J.C., 2001. The morphological phenotype of beta-amyloid plaques and associated neuritic changes in Alzheimer's disease. Neuroscience 105, 99-107.
Doehner, J., Knuesel, I., 2010. Reelin-mediated Signaling during Normal and Pathological Forms of Aging. Aging and disease 1, 12-29.
Dong, H., Goico, B., Martin, M., Csernansky, C.A., Bertchume, A., Csernansky, J.G., 2004. Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. Neuroscience 127, 601-609.
Donovan, M.H., Yazdani, U., Norris, R.D., Games, D., German, D.C., Eisch, A.J., 2006. Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. The Journal of comparative neurology 495, 70-83.
Dou, Y., Andersson-Lendahl, M., Arner, A., 2008. Structure and Function of Skeletal Muscle in Zebrafish Early Larvae. The Journal of General Physiology 131, 445-453.
Dries, D.R., Yu, G., 2008. Assembly, maturation, and trafficking of the gamma-secretase complex in Alzheimer's disease. Current Alzheimer research 5, 132-146.
Driever, W., Solnica-Krezel, L., Schier, A.F., Neuhauss, S.C., Malicki, J., Stemple, D.L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., Boggs, C., 1996. A genetic screen for mutations affecting embryogenesis in zebrafish. Development (Cambridge, England) 123, 37-46.
Duce, J.A., Tsatsanis, A., Cater, M.A., James, S.A., Robb, E., Wikhe, K., Leong, S.L., Perez, K., Johanssen, T., Greenough, M.A., Cho, H.-H., Galatis, D., Moir, R.D., Masters, C.L., McLean, C., Tanzi, R.E., Cappai, R., Barnham, K.J., Ciccotosto, G.D., Rogers, J.T., Bush, A.I., 2010. Iron-Export Ferroxidase Activity of β-Amyloid Precursor Protein Is Inhibited by Zinc in Alzheimer's Disease. Cell 142, 857-867.

Ebinu, J.O., Yankner, B.A., 2002. A RIP tide in neuronal signal transduction. Neuron 34, 499-502.

Eggert, S., Paliga, K., Soba, P., Evin, G., Masters, C.L., Weidemann, A., Beyreuther, K., 2004. The proteolytic processing of the amyloid precursor protein gene family members APLP-1 and APLP-2 involves alpha-, beta-, gamma-, and epsilon-like cleavages: modulation of APLP-1 processing by n-glycosylation. The Journal of biological chemistry 279, 18146-18156.

Fassa, A., Mehta, P., Efthimiopoulos, S., 2005. Notch 1 interacts with the amyloid precursor protein in a Numb-independent manner. Journal of neuroscience research 82, 214-224.

Fetcho, J.R., Higashijima, S., McLean, D.L., 2008. Zebrafish and motor control over the last decade. Brain research reviews 57, 86-93.

Fiore, F., Zambrano, N., Minopoli, G., Donini, V., Duilio, A., Russo, T., 1995. The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. The Journal of biological chemistry 270, 30853-30856.

Fischer, D.F., van Dijk, R., Sluijs, J.A., Nair, S.M., Racchi, M., Levelt, C.N., van Leeuwen, F.W., Hol, E.M., 2005. Activation of the Notch pathway in Down syndrome: cross-talk of Notch and APP. Faseb j 19, 1451-1458.

Flanagan-Steet, H., Fox, M.A., Meyer, D., Sanes, J.R., 2005. Neuromuscular synapses can form in vivo by incorporation of initially aneural postsynaptic specializations. Development (Cambridge, England) 132, 4471-4481.

Flinn, L., Bretaud, S., Lo, C., Ingham, P.W., Bandmann, O., 2008. Zebrafish as a new animal model for movement disorders. Journal of neurochemistry 106, 1991-1997.

Francis, R., McGrath, G., Zhang, J., Ruddy, D.A., Sym, M., Apfeld, J., Nicoll, M., Maxwell, M., Hai, B., Ellis, M.C., Parks, A.L., Xu, W., Li, J., Gurney, M., Myers, R.L., Himes, C.S., Hiebsch, R., Ruble, C., Nye, J.S., Curtis, D., 2002. aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell 3, 85-97.

Freude, K.K., Penjwini, M., Davis, J.L., LaFerla, F.M., Blurton-Jones, M., 2011. Soluble amyloid precursor protein induces rapid neural differentiation of human embryonic stem cells. The Journal of biological chemistry 286, 24264-24274.

Fu, M.M., Holzbaur, E.L., 2013. JIP1 regulates the directionality of APP axonal transport by coordinating kinesin and dynein motors. J Cell Biol 202, 495-508.

Gabriel, J.P., Ausborn, J., Ampatzis, K., Mahmood, R., Eklof-Ljunggren, E., El Manira, A., 2011. Principles governing recruitment of

motoneurons during swimming in zebrafish. Nature neuroscience 14, 93-99.
Geling, A., Plessy, C., Rastegar, S., Strahle, U., Bally-Cuif, L., 2004. Her5 acts as a prepattern factor that blocks neurogenin1 and coe2 expression upstream of Notch to inhibit neurogenesis at the midbrain-hindbrain boundary. Development (Cambridge, England) 131, 1993-2006.
Glasauer, S.M., Neuhauss, S.C., 2014. Whole-genome duplication in teleost fishes and its evolutionary consequences. Molecular genetics and genomics : MGG 289, 1045-1060.
Goate, A., Chartier-Harlin, M.C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., et al., 1991. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature 349, 704-706.
Golde, T.E., Koo, E.H., Felsenstein, K.M., Osborne, B.A., Miele, L., 2013. γ-Secretase inhibitors and modulators. Biochimica et Biophysica Acta (BBA) - Biomembranes 1828, 2898-2907.
Goldgaber, D., Lerman, M.I., McBride, O.W., Saffiotti, U., Gajdusek, D.C., 1987. Characterization and chromosomal localization of a cDNA encoding brain amyloid of Alzheimer's disease. Science (New York, N.Y.) 235, 877-880.
Goulding, M., 2009. Circuits controlling vertebrate locomotion: moving in a new direction. Nature reviews. Neuroscience 10, 507-518.
Gralle, M., Oliveira, C.L., Guerreiro, L.H., McKinstry, W.J., Galatis, D., Masters, C.L., Cappai, R., Parker, M.W., Ramos, C.H., Torriani, I., Ferreira, S.T., 2006. Solution conformation and heparin-induced dimerization of the full-length extracellular domain of the human amyloid precursor protein. Journal of molecular biology 357, 493-508.
Gray, M., Moens, C.B., Amacher, S.L., Eisen, J.S., Beattie, C.E., 2001a. Zebrafish deadly seven Functions in Neurogenesis. Developmental Biology 237, 306-323.
Gray, M., Moens, C.B., Amacher, S.L., Eisen, J.S., Beattie, C.E., 2001b. Zebrafish deadly seven functions in neurogenesis. Dev Biol 237, 306-323.
Groth, C., Nornes, S., McCarty, R., Tamme, R., Lardelli, M., 2002. Identification of a second presenilin gene in zebrafish with similarity to the human Alzheimer's disease gene presenilin2. Development genes and evolution 212, 486-490.
Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.C., Zaidi, M.S., Wisniewski, H.M., 1986a. Microtubule-associated protein tau. A component of Alzheimer paired helical filaments. The Journal of biological chemistry 261, 6084-6089.
Grundke-Iqbal, I., Iqbal, K., Tung, Y.C., Quinlan, M., Wisniewski, H.M., Binder, L.I., 1986b. Abnormal phosphorylation of the microtubule-

associated protein tau (tau) in Alzheimer cytoskeletal pathology. Proceedings of the National Academy of Sciences of the United States of America 83, 4913-4917.

Gu, Y., Misonou, H., Sato, T., Dohmae, N., Takio, K., Ihara, Y., 2001. Distinct Intramembrane Cleavage of the β-Amyloid Precursor Protein Family Resembling γ-Secretase-like Cleavage of Notch. Journal of Biological Chemistry 276, 35235-35238.

Guo, Q., Wang, Z., Li, H., Wiese, M., Zheng, H., 2012. APP physiological and pathophysiological functions: insights from animal models. Cell Res 22, 78-89.

Haapasalo, A., Kovacs, D.M., 2011. The Many Substrates of Presenilin/γ-Secretase. Journal of Alzheimer's Disease 25, 3-28.

Haass, C., Kaether, C., Thinakaran, G., Sisodia, S., 2012. Trafficking and Proteolytic Processing of APP. Cold Spring Harbor perspectives in medicine 2, a006270.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D., Lewis, J., 1998. Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. Development (Cambridge, England) 125, 359-370.

Haffter, P., Granato, M., Brand, M., Mullins, M.C., Hammerschmidt, M., Kane, D.A., Odenthal, J., van Eeden, F.J., Jiang, Y.J., Heisenberg, C.P., Kelsh, R.N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., Nusslein-Volhard, C., 1996. The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development (Cambridge, England) 123, 1-36.

Hanneman, E., Trevarrow, B., Metcalfe, W.K., Kimmel, C.B., Westerfield, M., 1988. Segmental pattern of development of the hindbrain and spinal cord of the zebrafish embryo. Development (Cambridge, England) 103, 49-58.

Hardy, J., 1997. Amyloid, the presenilins and Alzheimer's disease. Trends in Neurosciences 20, 154-159.

Hardy, J., Allsop, D., 1991. Amyloid deposition as the central event in the aetiology of Alzheimer's disease. Trends in pharmacological sciences 12, 383-388.

Hardy, J., Selkoe, D.J., 2002. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science (New York, N.Y.) 297, 353-356.

Hardy, J.A., Higgins, G.A., 1992. Alzheimer's disease: the amyloid cascade hypothesis. Science (New York, N.Y.) 256, 184-185.

Hartmann, D., Tournoy, J., Saftig, P., Annaert, W., De Strooper, B., 2001. Implication of APP secretases in notch signaling. Journal of molecular neuroscience : MN 17, 171-181.

Hayashi, Y., Kashiwagi, K., Ohta, J., Nakajima, M., Kawashima, T., Yoshikawa, K., 1994. Alzheimer Amyloid Protein Precursor

Enhances Proliferation of Neural Stem Cells from Fetal Rat Brain. Biochemical and biophysical research communications 205, 936-943.
Heber, S., Herms, J., Gajic, V., Hainfellner, J., Aguzzi, A., Rulicke, T., von Kretzschmar, H., von Koch, C., Sisodia, S., Tremml, P., Lipp, H.P., Wolfer, D.P., Muller, U., 2000. Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci 20, 7951-7963.
Hemming, M.L., Elias, J.E., Gygi, S.P., Selkoe, D.J., 2008. Proteomic Profiling of γ-Secretase Substrates and Mapping of Substrate Requirements. PLOS Biology 6, e257.
Henderson, A.S., Jorm, A.F., Korten, A.E., Creasey, H., McCusker, E., Broe, G.A., Longley, W., Anthony, J.C., 2009. Environmental risk factors for Alzheimer's disease: their relationship to age of onset and to familial or sporadic types. Psychological Medicine 22, 429-436.
Hoe, H.S., Lee, K.J., Carney, R.S., Lee, J., Markova, A., Lee, J.Y., Howell, B.W., Hyman, B.T., Pak, D.T., Bu, G., Rebeck, G.W., 2009. Interaction of reelin with amyloid precursor protein promotes neurite outgrowth. J Neurosci 29, 7459-7473.
Hoe, H.S., Tran, T.S., Matsuoka, Y., Howell, B.W., Rebeck, G.W., 2006. DAB1 and Reelin effects on amyloid precursor protein and ApoE receptor 2 trafficking and processing. The Journal of biological chemistry 281, 35176-35185.
Hoefgen, S., Coburger, I., Roeser, D., Schaub, Y., Dahms, S.O., Than, M.E., 2014. Heparin induced dimerization of APP is primarily mediated by E1 and regulated by its acidic domain. Journal of Structural Biology 187, 30-37.
Honda, T., Kobayashi, K., Mikoshiba, K., Nakajima, K., 2011. Regulation of cortical neuron migration by the Reelin signaling pathway. Neurochemical research 36, 1270-1279.
Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., Humphray, S., McLaren, K., Matthews, L., McLaren, S., Sealy, I., Caccamo, M., Churcher, C., Scott, C., Barrett, J.C., Koch, R., Rauch, G.J., White, S., Chow, W., Kilian, B., Quintais, L.T., Guerra-Assuncao, J.A., Zhou, Y., Gu, Y., Yen, J., Vogel, J.H., Eyre, T., Redmond, S., Banerjee, R., Chi, J., Fu, B., Langley, E., Maguire, S.F., Laird, G.K., Lloyd, D., Kenyon, E., Donaldson, S., Sehra, H., Almeida-King, J., Loveland, J., Trevanion, S., Jones, M., Quail, M., Willey, D., Hunt, A., Burton, J., Sims, S., McLay, K., Plumb, B., Davis, J., Clee, C., Oliver, K., Clark, R., Riddle, C., Elliot, D., Threadgold, G., Harden, G., Ware, D., Begum, S., Mortimore, B., Kerry, G., Heath, P., Phillimore, B., Tracey, A., Corby, N., Dunn, M., Johnson, C., Wood, J., Clark, S., Pelan, S., Griffiths, G., Smith, M., Glithero, R., Howden, P., Barker, N., Lloyd, C., Stevens, C., Harley, J., Holt, K., Panagiotidis, G., Lovell, J., Beasley, H., Henderson, C., Gordon, D., Auger, K., Wright, D.,

Collins, J., Raisen, C., Dyer, L., Leung, K., Robertson, L., Ambridge, K., Leongamornlert, D., McGuire, S., Gilderthorp, R., Griffiths, C., Manthravadi, D., Nichol, S., Barker, G., Whitehead, S., Kay, M., Brown, J., Murnane, C., Gray, E., Humphries, M., Sycamore, N., Barker, D., Saunders, D., Wallis, J., Babbage, A., Hammond, S., Mashreghi-Mohammadi, M., Barr, L., Martin, S., Wray, P., Ellington, A., Matthews, N., Ellwood, M., Woodmansey, R., Clark, G., Cooper, J., Tromans, A., Grafham, D., Skuce, C., Pandian, R., Andrews, R., Harrison, E., Kimberley, A., Garnett, J., Fosker, N., Hall, R., Garner, P., Kelly, D., Bird, C., Palmer, S., Gehring, I., Berger, A., Dooley, C.M., Ersan-Urun, Z., Eser, C., Geiger, H., Geisler, M., Karotki, L., Kirn, A., Konantz, J., Konantz, M., Oberlander, M., Rudolph-Geiger, S., Teucke, M., Lanz, C., Raddatz, G., Osoegawa, K., Zhu, B., Rapp, A., Widaa, S., Langford, C., Yang, F., Schuster, S.C., Carter, N.P., Harrow, J., Ning, Z., Herrero, J., Searle, S.M., Enright, A., Geisler, R., Plasterk, R.H., Lee, C., Westerfield, M., de Jong, P.J., Zon, L.I., Postlethwait, J.H., Nusslein-Volhard, C., Hubbard, T.J., Roest Crollius, H., Rogers, J., Stemple, D.L., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498-503.

Howell, B.W., Hawkes, R., Soriano, P., Cooper, J.A., 1997. Neuronal position in the developing brain is regulated by mouse disabled-1. Nature 389, 733-737.

Hu, Y., Hung, A.C., Cui, H., Dawkins, E., Bolos, M., Foa, L., Young, K.M., Small, D.H., 2013. Role of cystatin C in amyloid precursor protein-induced proliferation of neural stem/progenitor cells. The Journal of biological chemistry 288, 18853-18862.

Huang, H.C., Jiang, Z.F., 2011. Amyloid-beta protein precursor family members: a review from homology to biological function. Journal of Alzheimer's disease : JAD 26, 607-626.

Hurd, M.D., Martorell, P., Delavande, A., Mullen, K.J., Langa, K.M., 2013. Monetary costs of dementia in the United States. The New England journal of medicine 368, 1326-1334.

Hurd, M.D., Martorell, P., Langa, K., 2015. Future Monetary Costs of Dementia in the United States under Alternative Dementia Prevalence Scenarios. Journal of population ageing 8, 101-112.

Itoh, M., Kim, C.H., Palardy, G., Oda, T., Jiang, Y.J., Maust, D., Yeo, S.Y., Lorick, K., Wright, G.J., Ariza-McNaughton, L., Weissman, A.M., Lewis, J., Chandrasekharappa, S.C., Chitnis, A.B., 2003. Mind bomb is a ubiquitin ligase that is essential for efficient activation of Notch signaling by Delta. Dev Cell 4, 67-82.

Jacobsen, K.T., Iverfeldt, K., 2009. Amyloid precursor protein and its homologues: a family of proteolysis-dependent receptors. Cellular and molecular life sciences : CMLS 66, 2299-2318.

Jelen, N., Ule, J., Živin, M., Darnell, R.B., 2007. Evolution of Nova-Dependent Splicing Regulation in the Brain. PLOS Genetics 3, e173.
Jin, K., Galvan, V., Xie, L., Mao, X.O., Gorostiza, O.F., Bredesen, D.E., Greenberg, D.A., 2004. Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPSw,Ind) mice. Proceedings of the National Academy of Sciences of the United States of America 101, 13363-13367.
Joshi, P., Liang, J.O., DiMonte, K., Sullivan, J., Pimplikar, S.W., 2009. Amyloid precursor protein is required for convergent-extension movements during Zebrafish development. Dev Biol 335, 1-11.
Kaden, D., Voigt, P., Munter, L.M., Bobowski, K.D., Schaefer, M., Multhaup, G., 2009. Subcellular localization and dimerization of APLP1 are strikingly different from APP and APLP2. Journal of cell science 122, 368-377.
Kaiser, D.M., Acharya, M., Leighton, P.L., Wang, H., Daude, N., Wohlgemuth, S., Shi, B., Allison, W.T., 2012. Amyloid beta precursor protein and prion protein have a conserved interaction affecting cell adhesion and CNS development. PLoS One 7, e51305.
Kang, J., Lemaire, H.G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K., Muller-Hill, B., 1987. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature 325, 733-736.
Kerr, M.L., Small, D.H., 2005. Cytoplasmic domain of the beta-amyloid protein precursor of Alzheimer's disease: function, regulation of proteolysis, and implications for drug development. Journal of neuroscience research 80, 151-159.
Kim, M.Y., Mo, J.S., Ann, E.J., Yoon, J.H., Jung, J., Choi, Y.H., Kim, S.M., Kim, H.Y., Ahn, J.S., Kim, H., Kim, K., Hoe, H.S., Park, H.S., 2011. Regulation of Notch1 signaling by the APP intracellular domain facilitates degradation of the Notch1 intracellular domain and RBP-Jk. Journal of cell science 124, 1831-1843.
Kim, S.-Y., Kim, M.-Y., Mo, J.-S., Park, H.-S., 2007. Notch1 intracellular domain suppresses APP intracellular domain—Tip60–Fe65 complex mediated signaling through physical interaction. Biochimica et Biophysica Acta (BBA) - Molecular Cell Research 1773, 736-746.
Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203, 253-310.
Kocherhans, S., Madhusudan, A., Doehner, J., Breu, K.S., Nitsch, R.M., Fritschy, J.M., Knuesel, I., 2010. Reduced Reelin expression accelerates amyloid-beta plaque formation and tau pathology in transgenic Alzheimer's disease mice. J Neurosci 30, 9228-9240.
Koike, H., Tomioka, S., Sorimachi, H., Saido, T.C., Maruyama, K., Okuyama, A., Fujisawa-Sehara, A., Ohno, S., Suzuki, K., Ishiura, S., 1999. Membrane-anchored metalloprotease MDC9 has an alpha-

secretase activity responsible for processing the amyloid precursor protein. Biochemical Journal 343, 371-375.

Konig, G., Monning, U., Czech, C., Prior, R., Banati, R., Schreiter-Gasser, U., Bauer, J., Masters, C.L., Beyreuther, K., 1992. Identification and differential expression of a novel alternative splice isoform of the beta A4 amyloid precursor protein (APP) mRNA in leukocytes and brain microglial cells. The Journal of biological chemistry 267, 10804-10809.

Kwak, Y.-D., Marutle, A., Dantuma, E., Merchant, S., Bushnev, S., Sugaya, K., 2011. Involvement of notch signaling pathway in amyloid precursor protein induced glial differentiation. European journal of pharmacology 650, 18-27.

Lammich, S., Kojro, E., Postina, R., Gilbert, S., Pfeiffer, R., Jasionowski, M., Haass, C., Fahrenholz, F., 1999. Constitutive and regulated alpha-secretase cleavage of Alzheimer's amyloid precursor protein by a disintegrin metalloprotease. Proceedings of the National Academy of Sciences of the United States of America 96, 3922-3927.

LaVoie, M.J., Selkoe, D.J., 2003. The Notch ligands, Jagged and Delta, are sequentially processed by alpha-secretase and presenilin/gamma-secretase and release signaling fragments. The Journal of biological chemistry 278, 34427-34437.

Lazarov, O., Demars, M., 2012. All in the Family: How the APPs Regulate Neurogenesis. Frontiers in Neuroscience 6.

Lee, J.A., Cole, G.J., 2007. Generation of transgenic zebrafish expressing green fluorescent protein under control of zebrafish amyloid precursor protein gene regulatory elements. Zebrafish 4, 277-286.

Lee, K.J., Moussa, C.E., Lee, Y., Sung, Y., Howell, B.W., Turner, R.S., Pak, D.T., Hoe, H.S., 2010. Beta amyloid-independent role of amyloid precursor protein in generation and maintenance of dendritic spines. Neuroscience 169, 344-356.

Leimer, U., Lun, K., Romig, H., Walter, J., Grünberg, J., Brand, M., Haass, C., 1999. Zebrafish (Danio rerio) Presenilin Promotes Aberrant Amyloid β-Peptide Production and Requires a Critical Aspartate Residue for Its Function in Amyloidogenesis. Biochemistry 38, 13602-13609.

Lewis, J., 1998. Notch signalling and the control of cell fate choices in vertebrates. Seminars in cell & developmental biology 9, 583-589.

Lewis, J., Hanisch, A., Holder, M., 2009. Notch signaling, the segmentation clock, and the patterning of vertebrate somites. J Biol 8, 44.

Liao, H.-K., Wang, Y., Noack Watt, K.E., Wen, Q.I.N., Breitback, J., Kemmet, C.K., Clark, K.J., Ekker, S.C., Essner, J.J., McGrail, M., 2012a. Tol2 Gene Trap Integrations in the Zebrafish Amyloid Precursor Protein Genes appa and aplp2 Reveal Accumulation of Secreted APP at the Embryonic Veins. Developmental dynamics : an

official publication of the American Association of Anatomists 241, 415-425.
Liao, H.K., Wang, Y., Noack Watt, K.E., Wen, Q., Breitbach, J., Kemmet, C.K., Clark, K.J., Ekker, S.C., Essner, J.J., McGrail, M., 2012b. Tol2 gene trap integrations in the zebrafish amyloid precursor protein genes appa and aplp2 reveal accumulation of secreted APP at the embryonic veins. Dev Dyn 241, 415-425.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408.
Lleo, A., Berezovska, O., Ramdya, P., Fukumoto, H., Raju, S., Shah, T., Hyman, B.T., 2003. Notch1 competes with the amyloid precursor protein for gamma-secretase and down-regulates presenilin-1 gene expression. J Biol Chem 278, 47370-47375.
Loffler, J., Huber, G., 1992. Beta-amyloid precursor protein isoforms in various rat brain regions and during brain development. Journal of neurochemistry 59, 1316-1324.
Lopez-Toledano, M.A., Shelanski, M.L., 2007. Increased neurogenesis in young transgenic mice overexpressing human APP(Sw, Ind). Journal of Alzheimer's disease : JAD 12, 229-240.
Lorent, K., Overbergh, L., Moechars, D., De Strooper, B., Van Leuven, F., Van den Berghe, H., 1995. Expression in mouse embryos and in adult mouse brain of three members of the amyloid precursor protein family, of the alpha-2-macroglobulin receptor/low density lipoprotein receptor-related protein and of its ligands apolipoprotein E, lipoprotein lipase, alpha-2-macroglobulin and the 40,000 molecular weight receptor-associated protein. Neuroscience 65, 1009-1025.
Lourenco, F.C., Galvan, V., Fombonne, J., Corset, V., Llambi, F., Muller, U., Bredesen, D.E., Mehlen, P., 2009. Netrin-1 interacts with amyloid precursor protein and regulates amyloid-beta production. Cell death and differentiation 16, 655-663.
Lu, P., Bai, X.-c., Ma, D., Xie, T., Yan, C., Sun, L., Yang, G., Zhao, Y., Zhou, R., Scheres, S.H.W., Shi, Y., 2014. Three-dimensional structure of human [ggr]-secretase. Nature 512, 166-170.
Luo, L., Tully, T., White, K., 1992. Human amyloid precursor protein ameliorates behavioral deficit of flies deleted for Appl gene. Neuron 9, 595-605.
Magdesian, M.H., Gralle, M., Guerreiro, L.H., Beltrão, P.J.I., Carvalho, M.M.V.F., Santos, L.E.d.S., de Mello, F.G., Reis, R.A.M., Ferreira, S.T., 2011. Secreted Human Amyloid Precursor Protein Binds Semaphorin 3a and Prevents Semaphorin-Induced Growth Cone Collapse. PLOS ONE 6, e22857.
Maves, L., Jackman, W., Kimmel, C.B., 2002. FGF3 and FGF8 mediate a rhombomere 4 signaling activity in the zebrafish hindbrain. Development 129, 3825-3837.

Merdes, G., Soba, P., Loewer, A., Bilic, M.V., Beyreuther, K., Paro, R., 2004a. Interference of human and Drosophila APP and APP–like proteins with PNS development in Drosophila. The EMBO journal 23, 4082-4095.

Merdes, G., Soba, P., Loewer, A., Bilic, M.V., Beyreuther, K., Paro, R., 2004b. Interference of human and Drosophila APP and APP-like proteins with PNS development in Drosophila. The EMBO journal 23, 4082-4095.

Milner, L.D., Landmesser, L.T., 1999. Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. J Neurosci 19, 3007-3022.

Moens, C.B., Yan, Y.L., Appel, B., Force, A.G., Kimmel, C.B., 1996. valentino: a zebrafish gene required for normal hindbrain segmentation. Development 122, 3981-3990.

Morris, S.M., Cooper, J.A., 2001. Disabled-2 Colocalizes with the LDLR in Clathrin-Coated Pits and Interacts with AP-2. Traffic 2, 111-123.

Moussavi Nik, S.H., Wilson, L., Newman, M., Croft, K., Mori, T.A., Musgrave, I., Lardelli, M., 2012. The BACE1-PSEN-AbetaPP regulatory axis has an ancient role in response to low oxygen/oxidative stress. Journal of Alzheimer's disease : JAD 28, 515-530.

Mullan, M., Crawford, F., Axelman, K., Houlden, H., Lilius, L., Winblad, B., Lannfelt, L., 1992. A pathogenic mutation for probable Alzheimer's disease in the APP gene at the N-terminus of beta-amyloid. Nature genetics 1, 345-347.

Mullard, A., 2017. BACE inhibitor bust in Alzheimer trial. Nat Rev Drug Discov 16, 155-155.

Müller, U., Cristina, N., Li, Z.-W., Wolfer, D.P., Lipp, H.-P., Rülicke, T., Brandner, S., Aguzzi, A., Weissmann, C., 1994. Behavioral and anatomical deficits in mice homozygous for a modified β-amyloid precursor protein gene. Cell 79, 755-765.

Multhaup, G., Schlicksupp, A., Hesse, L., Beher, D., Ruppert, T., Masters, C.L., Beyreuther, K., 1996. The amyloid precursor protein of Alzheimer's disease in the reduction of copper(II) to copper(I). Science (New York, N.Y.) 271, 1406-1409.

Muresan, Z., Muresan, V., 2005. Coordinated transport of phosphorylated amyloid-β precursor protein and c-Jun NH(2)-terminal kinase–interacting protein-1. The Journal of Cell Biology 171, 615-625.

Musa, A., Lehrach, H., Russo, V.A., 2001. Distinct expression patterns of two zebrafish homologues of the human APP gene during embryonic development. Development genes and evolution 211, 563-567.

Naumann, N., Alpár, A., Ueberham, U., Arendt, T., Gärtner, U., 2010. Transgenic expression of human wild-type amyloid precursor protein decreases neurogenesis in the adult hippocampus. Hippocampus 20, 971-979.

Nelson, P.T., Braak, H., Markesbery, W.R., 2009. Neuropathology and Cognitive Impairment in Alzheimer Disease: A Complex but Coherent Relationship. Journal of neuropathology and experimental neurology 68, 1-14.
Newman, M., Ebrahimie, E., Lardelli, M., 2014. Using the zebrafish model for Alzheimer's disease research. Frontiers in Genetics 5, 189.
Nhan, H.S., Chiang, K., Koo, E.H., 2015. The multifaceted nature of amyloid precursor protein and its proteolytic fragments: friends and foes. Acta neuropathologica 129, 1-19.
Nicolas, M., Hassan, B.A., 2014. Amyloid precursor protein and neural development. Development (Cambridge, England) 141, 2543-2548.
Nikolaou, N., Watanabe-Asaka, T., Gerety, S., Distel, M., Koster, R.W., Wilkinson, D.G., 2009. Lunatic fringe promotes the lateral inhibition of neurogenesis. Development (Cambridge, England) 136, 2523-2533.
Nitsch, R.M., Slack, B.E., Wurtman, R.J., Growdon, J.H., 1992. Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. Science (New York, N.Y.) 258, 304-307.
Ntelios, D., Berninger, B., Tzimagiorgis, G., 2012. Numb and Alzheimer's Disease: The Current Picture. Frontiers in Neuroscience 6, 145.
O'Brien, R.J., Wong, P.C., 2011. Amyloid Precursor Protein Processing and Alzheimer's Disease. Annual review of neuroscience 34, 185-204.
Oh, S.Y., Ellenstein, A., Chen, C.D., Hinman, J.D., Berg, E.A., Costello, C.E., Yamin, R., Neve, R.L., Abraham, C.R., 2005. Amyloid precursor protein interacts with notch receptors. Journal of neuroscience research 82, 32-42.
Ohsawa, I., Takamura, C., Morimoto, T., Ishiguro, M., Kohsaka, S., 1999a. Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. European Journal of Neuroscience 11, 1907-1913.
Ohsawa, I., Takamura, C., Morimoto, T., Ishiguro, M., Kohsaka, S., 1999b. Amino-terminal region of secreted form of amyloid precursor protein stimulates proliferation of neural stem cells. Eur J Neurosci 11, 1907-1913.
Ohyagi, Y., Takahashi, K., Kamegai, M., Tabira, T., 1990. Developmental and differential expression of beta amyloid protein precursor mRNAs in mouse brain. Biochemical and biophysical research communications 167, 54-60.
Oppenheimer, J.M., 1936. Structures Developed in Amphibians by Implantation of Living Fish Organizer. Proceedings of the Society for Experimental Biology and Medicine 34, 461-463.
Panzer, J.A., Gibbs, S.M., Dosch, R., Wagner, D., Mullins, M.C., Granato, M., Balice-Gordon, R.J., 2005. Neuromuscular synaptogenesis in wild-type and mutant zebrafish. Dev Biol 285, 340-357.

Parisiadou, L., Efthimiopoulos, S., 2007. Expression of mDab1 promotes the stability and processing of amyloid precursor protein and this effect is counteracted by X11α. Neurobiology of aging 28, 377-388.

Parvathy, S., Hussain, I., Karran, E.H., Turner, A.J., Hooper, N.M., 1999. Cleavage of Alzheimer's Amyloid Precursor Protein by α-Secretase Occurs at the Surface of Neuronal Cells. Biochemistry 38, 9728-9734.

Pawson, T., Scott, J.D., 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science (New York, N.Y.) 278, 2075-2080.

Perl, D.P., 2010. Neuropathology of Alzheimer's Disease. Mount Sinai Journal of Medicine: A Journal of Translational and Personalized Medicine 77, 32-42.

Pimplikar, S.W., Nixon, R.A., Robakis, N.K., Shen, J., Tsai, L.-H., 2010. Amyloid-Independent Mechanisms in Alzheimer's Disease Pathogenesis. The Journal of Neuroscience 30, 14946-14954.

Polvikoski , T., Sulkava , R., Haltia , M., Kainulainen , K., Vuorio , A., Verkkoniemi , A., Niinistö , L., Halonen , P., Kontula , K., 1995. Apolipoprotein E, Dementia, and Cortical Deposition of β-Amyloid Protein. New England Journal of Medicine 333, 1242-1248.

Postlethwait, J.H., Yan, Y.L., Gates, M.A., Horne, S., Amores, A., Brownlie, A., Donovan, A., Egan, E.S., Force, A., Gong, Z., Goutel, C., Fritz, A., Kelsh, R., Knapik, E., Liao, E., Paw, B., Ransom, D., Singer, A., Thomson, M., Abduljabbar, T.S., Yelick, P., Beier, D., Joly, J.S., Larhammar, D., Rosa, F., Westerfield, M., Zon, L.I., Johnson, S.L., Talbot, W.S., 1998. Vertebrate genome evolution and the zebrafish gene map. Nature genetics 18, 345-349.

Prince, M., Guerchet, M., Prina, M., 2013. Policy brief for heads of government: the global impact of dementia 2013–2050, 2013. Last accessed 15, 14.

Prince, M., Guerchet, M., Prina, M., 2015. The epidemiology and impact of dementia: current state and future trends. Geneva: World Health Organization.

Prince, V.E., Joly, L., Ekker, M., Ho, R.K., 1998. Zebrafish hox genes: genomic organization and modified colinear expression patterns in the trunk. Development 125, 407-420.

Prüßing, K., Voigt, A., Schulz, J.B., 2013. Drosophila melanogaster as a model organism for Alzheimer's disease. Molecular Neurodegeneration 8, 35.

Puig, K.L., Combs, C.K., 2013. Expression and function of APP and its metabolites outside the central nervous system. Experimental gerontology 48, 608-611.

Qiu, C., Kivipelto, M., von Strauss, E., 2009. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. Dialogues in clinical neuroscience 11, 111-128.

Rajput, A.H., Uitti, R.J., Sudhakar, S., Rozdilsky, B., 1989. Parkinsonism and neurofibrillary tangle pathology in pigmented nuclei. Annals of neurology 25, 602-606.
Reinhard, C., Hebert, S.S., De Strooper, B., 2005. The amyloid-beta precursor protein: integrating structure with biological function. The EMBO journal 24, 3996-4006.
Reitz, C., Mayeux, R., 2014. Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. Biochemical pharmacology 88, 640-651.
Reynolds-Kenneally, J., Mlodzik, M., 2005. Notch signaling controls proliferation through cell-autonomous and non-autonomous mechanisms in the Drosophila eye. Dev Biol 285, 38-48.
Ring, S., Weyer, S.W., Kilian, S.B., Waldron, E., Pietrzik, C.U., Filippov, M.A., Herms, J., Buchholz, C., Eckman, C.B., Korte, M., Wolfer, D.P., Muller, U.C., 2007. The secreted beta-amyloid precursor protein ectodomain APPs alpha is sufficient to rescue the anatomical, behavioral, and electrophysiological abnormalities of APP-deficient mice. J Neurosci 27, 7817-7826.
Robakis, N.K., 2011. Mechanisms of AD neurodegeneration may be independent of Aβ and its derivatives. Neurobiology of aging 32, 372-379.
Rohan de Silva, H.A., Jen, A., Wickenden, C., Jen, L.S., Wilkinson, S.L., Patel, A.J., 1997. Cell-specific expression of beta-amyloid precursor protein isoform mRNAs and proteins in neurons and astrocytes. Brain research. Molecular brain research 47, 147-156.
Roncarati, R., Sestan, N., Scheinfeld, M.H., Berechid, B.E., Lopez, P.A., Meucci, O., McGlade, J.C., Rakic, P., D'Adamio, L., 2002a. The gamma-secretase-generated intracellular domain of beta-amyloid precursor protein binds Numb and inhibits Notch signaling. Proceedings of the National Academy of Sciences of the United States of America 99, 7102-7107.
Roncarati, R., Šestan, N., Scheinfeld, M.H., Berechid, B.E., Lopez, P.A., Meucci, O., McGlade, J.C., Rakic, P., D'Adamio, L., 2002b. The γ-secretase-generated intracellular domain of β-amyloid precursor protein binds Numb and inhibits Notch signaling. Proceedings of the National Academy of Sciences 99, 7102-7107.
Rosen, D.R., Martin-Morris, L., Luo, L.Q., White, K., 1989. A Drosophila gene encoding a protein resembling the human beta-amyloid protein precursor. Proceedings of the National Academy of Sciences of the United States of America 86, 2478-2482.
Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Holper, S., Kruger, M., Stainier, D.Y., 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature 524, 230-233.
Rossjohn, J., Cappai, R., Feil, S.C., Henry, A., McKinstry, W.J., Galatis, D., Hesse, L., Multhaup, G., Beyreuther, K., Masters, C.L., Parker,

M.W., 1999. Crystal structure of the N-terminal, growth factor-like domain of Alzheimer amyloid precursor protein. Nature structural biology 6, 327-331.

Sabo, S.L., Ikin, A.F., Buxbaum, J.D., Greengard, P., 2001. The Alzheimer Amyloid Precursor Protein (APP) and Fe65, an APP-Binding Protein, Regulate Cell Movement. The Journal of Cell Biology 153, 1403-1414.

Sabo, S.L., Ikin, A.F., Buxbaum, J.D., Greengard, P., 2003. The amyloid precursor protein and its regulatory protein, FE65, in growth cones and synapses in vitro and in vivo. J Neurosci 23, 5407-5415.

Sanders, C.R., 2016. How gamma-secretase hits a moving target. eLife 5.

Sarasa, M., Sorribas, V., amp,́ctor, Terrado, J., Climent, S., Palacios, J.M., Mengod, G., 2000. Alzheimer β-amyloid precursor proteins display specific patterns of expression during embryogenesis. Mechanisms of Development 94, 233-236.

Scheinfeld, M.H., Ghersi, E., Davies, P., D'Adamio, L., 2003. Amyloid beta protein precursor is phosphorylated by JNK-1 independent of, yet facilitated by, JNK-interacting protein (JIP)-1. The Journal of biological chemistry 278, 42058-42063.

Schettini, G., Govoni, S., Racchi, M., Rodriguez, G., 2010. Phosphorylation of APP-CTF-AICD domains and interaction with adaptor proteins: signal transduction and/or transcriptional role – relevance for Alzheimer pathology. Journal of neurochemistry 115, 1299-1308.

Scheuner, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T.D., Hardy, J., Hutton, M., Kukull, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wasco, W., Lannfelt, L., Selkoe, D., Younkin, S., 1996. Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin 1 and 2 and APP mutations linked to familial Alzheimer's disease. Nature medicine 2, 864-870.

Schier, A.F., Neuhauss, S.C., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D.Y., Zwartkruis, F., Abdelilah, S., Stemple, D.L., Rangini, Z., Yang, H., Driever, W., 1996. Mutations affecting the development of the embryonic zebrafish brain. Development 123, 165-178.

Schweisguth, F., 2004. Regulation of Notch Signaling Activity. Current Biology 14, R129-R138.

Selkoe, D., Kopan, R., 2003. Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. Annu Rev Neurosci 26, 565-597.

Selkoe, D.J., 1991. The molecular pathology of Alzheimer's disease. Neuron 6, 487-498.

Selkoe, D.J., 2011. Resolving controversies on the path to Alzheimer's therapeutics. Nature medicine 17, 1060-1065.

Selkoe, D.J., Hardy, J., 2016. The amyloid hypothesis of Alzheimer's disease at 25 years. EMBO molecular medicine 8, 595-608.
Selkoe, D.J., Podlisny, M.B., 2002. Deciphering the genetic basis of Alzheimer's disease. Annual review of genomics and human genetics 3, 67-99.
Selkoe, D.J., Wolfe, M.S., 2007. Presenilin: Running with Scissors in the Membrane. Cell 131, 215-221.
Serrano-Pozo, A., Frosch, M.P., Masliah, E., Hyman, B.T., 2011a. Neuropathological Alterations in Alzheimer Disease. Cold Spring Harbor Perspectives in Medicine: 1, a006189.
Serrano-Pozo, A., Frosch, M.P., Masliah, E., Hyman, B.T., 2011b. Neuropathological alterations in Alzheimer disease. Cold Spring Harbor perspectives in medicine 1, a006189.
Shah, S., Lee, S.F., Tabuchi, K., Hao, Y.H., Yu, C., LaPlant, Q., Ball, H., Dann, C.E., 3rd, Sudhof, T., Yu, G., 2005. Nicastrin functions as a gamma-secretase-substrate receptor. Cell 122, 435-447.
Shariati, S.A., De Strooper, B., 2013. Redundancy and divergence in the amyloid precursor protein family. FEBS letters 587, 2036-2045.
Shimojo, H., Ohtsuka, T., Kageyama, R., 2011. Dynamic Expression of Notch Signaling Genes in Neural Stem/Progenitor Cells. Frontiers in Neuroscience 5, 78.
Shu, R., Wong, W., Ma, Q.H., Yang, Z.Z., Zhu, H., Liu, F.J., Wang, P., Ma, J., Yan, S., Polo, J.M., Bernard, C.C.A., Stanton, L.W., Dawe, G.S., Xiao, Z.C., 2015. APP intracellular domain acts as a transcriptional regulator of miR-663 suppressing neuronal differentiation. Cell Death Dis 6, e1651.
Sisodia, S.S., Koo, E.H., Hoffman, P.N., Perry, G., Price, D.L., 1993. Identification and transport of full-length amyloid precursor proteins in rat peripheral nervous system. J Neurosci 13, 3136-3142.
Skovronsky, D.M., Moore, D.B., Milla, M.E., Doms, R.W., Lee, V.M., 2000. Protein kinase C-dependent alpha-secretase competes with beta-secretase for cleavage of amyloid-beta precursor protein in the trans-golgi network. The Journal of biological chemistry 275, 2568-2575.
Slunt, H.H., Thinakaran, G., Von Koch, C., Lo, A.C., Tanzi, R.E., Sisodia, S.S., 1994. Expression of a ubiquitous, cross-reactive homologue of the mouse beta-amyloid precursor protein (APP). The Journal of biological chemistry 269, 2637-2644.
Soba, P., Eggert, S., Wagner, K., Zentgraf, H., Siehl, K., Kreger, S., Löwer, A., Langer, A., Merdes, G., Paro, R., Masters, C.L., Müller, U., Kins, S., Beyreuther, K., 2005. Homo- and heterodimerization of APP family members promotes intercellular adhesion. The EMBO journal 24, 3624-3634.
Solans, A., Estivill, X., de La Luna, S., 2000. A new aspartyl protease on 21q22.3, BACE2, is highly similar to Alzheimer's amyloid precursor protein beta-secretase. Cytogenetics and cell genetics 89, 177-184.

Song, P., Pimplikar, S.W., 2012. Knockdown of amyloid precursor protein in zebrafish causes defects in motor axon outgrowth. PLoS One 7, e34209.

Song, Y., Willer, J.R., Scherer, P.C., Panzer, J.A., Kugath, A., Skordalakes, E., Gregg, R.G., Willer, G.B., Balice-Gordon, R.J., 2010. Neural and Synaptic Defects in slytherin, a Zebrafish Model for Human Congenital Disorders of Glycosylation. PLOS ONE 5, e13743.

Sun, Z., Hopkins, N., 2001. vhnf1, the MODY5 and familial GCKD-associated gene, regulates regional specification of the zebrafish gut, pronephros, and hindbrain. Genes Dev 15, 3217-3229.

Tamaoka, A., Odaka, A., Ishibashi, Y., Usami, M., Sahara, N., Suzuki, N., Nukina, N., Mizusawa, H., Shoji, S., Kanazawa, I., et al., 1994. APP717 missense mutation affects the ratio of amyloid beta protein species (A beta 1-42/43 and a beta 1-40) in familial Alzheimer's disease brain. The Journal of biological chemistry 269, 32721-32724.

Tang, K., Wang, C., Shen, C., Sheng, S., Ravid, R., Jing, N., 2003. Identification of a novel alternative splicing isoform of human amyloid precursor protein gene, APP639. Eur J Neurosci 18, 102-108.

Tanzi, R.E., 2012. The genetics of Alzheimer disease. Cold Spring Harbor perspectives in medicine 2.

Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A., St George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M., Neve, R.L., 1987. Amyloid beta protein gene: cDNA, mRNA distribution, and genetic linkage near the Alzheimer locus. Science (New York, N.Y.) 235, 880-884.

Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa-Komaroff, L., Gusella, J.F., Neve, R.L., 1988. Protease inhibitor domain encoded by an amyloid protein precursor mRNA associated with Alzheimer's disease. Nature 331, 528-530.

Taru, H., Kirino, Y., Suzuki, T., 2002. Differential roles of JIP scaffold proteins in the modulation of amyloid precursor protein metabolism. The Journal of biological chemistry 277, 27567-27574.

Terry, R.D., Masliah, E., Salmon, D.P., Butters, N., DeTeresa, R., Hill, R., Hansen, L.A., Katzman, R., 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. Annals of neurology 30, 572-580.

Thal, D.R., Rub, U., Orantes, M., Braak, H., 2002. Phases of A beta-deposition in the human brain and its relevance for the development of AD. Neurology 58, 1791-1800.

Thinakaran, G., Koo, E.H., 2008. Amyloid precursor protein trafficking, processing, and function. The Journal of biological chemistry 283, 29615-29619.

Trapp, B.D., Hauer, P.E., 1994. Amyloid precursor protein is enriched in radial glia: implications for neuronal development. Journal of neuroscience research 37, 538-550.

Trommsdorff, M., Gotthardt, M., Hiesberger, T., Shelton, J., Stockinger, W., Nimpf, J., Hammer, R.E., Richardson, J.A., Herz, J., 1999. Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking the VLDL receptor and ApoE receptor 2. Cell 97, 689-701.

Turner, P.R., O'Connor, K., Tate, W.P., Abraham, W.C., 2003. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Progress in neurobiology 70, 1-32.

Tyan, S.H., Shih, A.Y., Walsh, J.J., Maruyama, H., Sarsoza, F., Ku, L., Eggert, S., Hof, P.R., Koo, E.H., Dickstein, D.L., 2012. Amyloid precursor protein (APP) regulates synaptic structure and function. Molecular and cellular neurosciences 51, 43-52.

Urbanc, B., Cruz, L., Le, R., Sanders, J., Ashe, K.H., Duff, K., Stanley, H.E., Irizarry, M.C., Hyman, B.T., 2002. Neurotoxic effects of thioflavin S-positive amyloid deposits in transgenic mice and Alzheimer's disease. Proceedings of the National Academy of Sciences of the United States of America 99, 13990-13995.

van Bebber, F., Hruscha, A., Willem, M., Schmid, B., Haass, C., 2013. Loss of Bace2 in zebrafish affects melanocyte migration and is distinct from Bace1 knock out phenotypes. Journal of neurochemistry 127, 471-481.

van der Kant, R., Goldstein, Lawrence S.B., 2015. Cellular Functions of the Amyloid Precursor Protein from Development to Dementia. Developmental Cell 32, 502-515.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., Kelsh, R.N., Mullins, M.C., Odenthal, J., Warga, R.M., Allende, M.L., Weinberg, E.S., Nusslein-Volhard, C., 1996. Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development (Cambridge, England) 123, 153-164.

Van Gassen, G., Annaert, W., Van Broeckhoven, C., 2000. Binding Partners of Alzheimer's Disease Proteins: Are They Physiologically Relevant? Neurobiology of Disease 7, 135-151.

Van Nostrand, W.E., Schmaier, A.H., Farrow, J.S., Cunningham, D.D., 1990. Protease nexin-II (amyloid beta-protein precursor): a platelet alpha-granule protein. Science (New York, N.Y.) 248, 745-748.

Van Nostrand, W.E., Wagner, S.L., Suzuki, M., Choi, B.H., Farrow, J.S., Geddes, J.W., Cotman, C.W., Cunningham, D.D., 1989. Protease nexin-II, a potent antichymotrypsin, shows identity to amyloid beta-protein precursor. Nature 341, 546-549.

van Tetering, G., van Diest, P., Verlaan, I., van der Wall, E., Kopan, R., Vooijs, M., 2009. Metalloprotease ADAM10 is required for Notch1

site 2 cleavage. The Journal of biological chemistry 284, 31018-31027.

Vassar, R., 2005. β-Secretase, APP and Aβ in Alzheimer's disease. Subcell Biochem 38.

Vassar, R., Kandalepas, P.C., 2011. The β-secretase enzyme BACE1 as a therapeutic target for Alzheimer's disease. Alzheimer's Research & Therapy 3, 20-20.

Vehmas, A.K., Kawas, C.H., Stewart, W.F., Troncoso, J.C., 2003. Immune reactive cells in senile plaques and cognitive decline in Alzheimer's disease. Neurobiology of aging 24, 321-331.

Walsh, D.M., Minogue, A.M., Sala Frigerio, C., Fadeeva, J.V., Wasco, W., Selkoe, D.J., 2007. The APP family of proteins: similarities and differences. Biochemical Society transactions 35, 416-420.

Wang, P., Yang, G., Mosier, D.R., Chang, P., Zaidi, T., Gong, Y.D., Zhao, N.M., Dominguez, B., Lee, K.F., Gan, W.B., 2005a. Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-like protein 2. J Neurosci 25.

Wang, P., Yang, G., Mosier, D.R., Chang, P., Zaidi, T., Gong, Y.D., Zhao, N.M., Dominguez, B., Lee, K.F., Gan, W.B., Zheng, H., 2005b. Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-Like protein 2. J Neurosci 25, 1219-1225.

Wang, Z., Wang, B., Yang, L., Guo, Q., Aithmitti, N., Songyang, Z., Zheng, H., 2009. Presynaptic and postsynaptic interaction of the amyloid precursor protein promotes peripheral and central synaptogenesis. J Neurosci 29, 10788-10801.

Wasco, W., Gurubhagavatula, S., Paradis, M.D., Romano, D.M., Sisodia, S.S., Hyman, B.T., Neve, R.L., Tanzi, R.E., 1993. Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor. Nature genetics 5, 95-100.

Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L., Beyreuther, K., 1989. Identification, biogenesis, and localization of precursors of Alzheimer's disease A4 amyloid protein. Cell 57, 115-126.

Wilson, L., Lardelli, M., 2013. The development of an in vivo gamma-secretase assay using zebrafish embryos. Journal of Alzheimer's disease : JAD 36, 521-534.

Wimo, A., Jonsson, L., Bond, J., Prince, M., Winblad, B., 2013. The worldwide economic impact of dementia 2010. Alzheimer's & dementia : the journal of the Alzheimer's Association 9, 1-11.e13.

Wisniewski, K., Jervis, G.A., Moretz, R.C., Wisniewski, H.M., 1979. Alzheimer neurofibrillary tangles in diseases other than senile and presenile dementia. Annals of neurology 5, 288-294.

Wolfe, M.S., Guenette, S.Y., 2007. APP at a glance. Journal of cell science 120, 3157-3161.

Xi, Y., Noble, S., Ekker, M., 2011. Modeling Neurodegeneration in Zebrafish. Current Neurology and Neuroscience Reports 11, 274-282.

Xia, D., Watanabe, H., Wu, B., Lee, S.H., Li, Y., Tsvetkov, E., Bolshakov, V.Y., Shen, J., Kelleher, R.J., 3rd, 2015. Presenilin-1 knockin mice reveal loss-of-function mechanism for familial Alzheimer's disease. Neuron 85, 967-981.

Xia, W., 2010. Exploring Alzheimer's disease in zebrafish. Journal of Alzheimer's disease : JAD 20, 981-990.

Yan, R., Munzner, J.B., Shuck, M.E., Bienkowski, M.J., 2001. BACE2 functions as an alternative alpha-secretase in cells. The Journal of biological chemistry 276, 34019-34027.

Yasuoka, K., Hirata, K., Kuraoka, A., He, J.-w., Kawabuchi, M., 2004. Expression of amyloid precursor protein-like molecule in astroglial cells of the subventricular zone and rostral migratory stream of the adult rat forebrain. Journal of Anatomy 205, 135-146.

Yoshikai, S., Sasaki, H., Doh-ura, K., Furuya, H., Sakaki, Y., 1990. Genomic organization of the human amyloid beta-protein precursor gene. Gene 87, 257-263.

Zetterberg, H., Campbell, W.A., Yang, H.W., Xia, W., 2006. The cytosolic loop of the gamma-secretase component presenilin enhancer 2 protects zebrafish embryos from apoptosis. The Journal of biological chemistry 281, 11933-11939.

Zhang, X., Gong, Z., 2013. Fluorescent Transgenic Zebrafish Tg(nkx2.2a:mEGFP) Provides a Highly Sensitive Monitoring Tool for Neurotoxins. PLoS ONE 8, e55474.

Zhang, X., Li, Y., Xu, H., Zhang, Y.-w., 2014. The γ-secretase complex: from structure to function. Frontiers in Cellular Neuroscience 8, 427.

Zhao, G., Cui, M.Z., Mao, G., Dong, Y., Tan, J., Sun, L., Xu, X., 2005. gamma-Cleavage is dependent on zeta-cleavage during the proteolytic processing of amyloid precursor protein within its transmembrane domain. The Journal of biological chemistry 280, 37689-37697.

Zhao, G., Mao, G., Tan, J., Dong, Y., Cui, M.Z., Kim, S.H., Xu, X., 2004. Identification of a new presenilin-dependent zeta-cleavage site within the transmembrane domain of amyloid precursor protein. The Journal of biological chemistry 279, 50647-50650.

Zheng, H., Jiang, M., Trumbauer, M.E., Sirinathsinghji, D.J., Hopkins, R., Smith, D.W., Heavens, R.P., Dawson, G.R., Boyce, S., Conner, M.W., Stevens, K.A., Slunt, H.H., Sisoda, S.S., Chen, H.Y., Van der Ploeg, L.H., 1995. beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81, 525-531.

Zhu, G., Wang, D., Lin, Y.H., McMahon, T., Koo, E.H., Messing, R.O., 2001. Protein kinase C epsilon suppresses Abeta production and

promotes activation of alpha-secretase. Biochemical and biophysical research communications 285, 997-1006.