THE EFFECT OF FAD-ASSOCIATED MUTATIONS IN AMYLOID-BETA PRECURSOR PROTEIN AND PRESENILIN-1 GENES ON ALZHEIMER'S DISEASE PATHOGENESIS

by

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Abstract

Pathogenic mutations in amyloid-β precursor protein (APP) and presenilins (PS) genes cause familial Alzheimer's disease (FAD). FAD is an uncommon form of Alzheimer's disease (AD) with early onset (before age 65) and a rapid progression but its neuropathology is indistinguishable from the sporadic AD. Amyloid plaque is the unique hallmark of AD, which consists primarily of 40- and 42-residue amyloid β protein (Aβ40 and Aβ42) with the more hydrophobic Aβ42 as its major component. Aβ is derived from APP through sequential cleavages by β-secretase and γ-secretase. According to the "Amyloid hypothesis", Aβ accumulation initiates the pathogenic cascades leading to AD, including the formation of neurofibrillary tangles, activation of astrocytes and neuronal loss. It has been well established that pathogenic mutations in both APP and PS genes contribute to AD pathogenesis via impaired generation of Aβ. This powerful genetic discovery lends great credence to the "Amyloid hypothesis", given that APP is the precursor of Aβ and PS acts as the enzyme to generate Aβ. The thorough understanding of the mechanism of these pathogenic mutations could lead to decipher the AD conundrum. Until now, all pathogenic APP mutations are autosomal dominant mutations except for APP_{A673V}. We discovered that APP_{A673V} structurally facilitates β-cleavage at Asp-1 site while inhibited the general APP processing including all α-/β-/γ-cleavages possibly due to the intensified lysosome-dependent degradation. The overall effect of APP_{A673V} on the production of Aβ makes the homozygous state necessary for APP_{A673V} to produce enough Aβ to initiate AD pathogenesis. Mutations in PS genes are another major cause of FAD. As another substrate of γ-secretase apart from APP, Notch plays a fundamental role in neurodevelopment and neurodegeneration. It has been well established that

pathogenic PS mutations impaired Notch signaling. $PS1_{\Delta S169}$ is a recently discovered PS1 mutation in a Chinese FAD family. We extensively characterized the function of $PS1_{\Delta S169}$ in mammalian cells and transgenic mice and found that $PS1_{\Delta S169}$ promoted AD pathogenesis via altering γ-cleavage of APP without impairing Notch processing, excluding the contribution of Notch signaling to AD pathogenesis. Our study highlights the possibility of developing specific γ-secretase inhibitors, which may spare Notch signaling in AD therapy.

Preface

Immediately after completing a postgraduate training with Master degree in Neurology, I joined Dr. Weihong Song's research team to study the molecular pathogenesis of Alzheimer's disease. I was fortunate to be introduced to a project to study a novel pathological Presenilin 1 (PS1) mutation — $PS1_{\Delta S169}$ identified in a Chinese AD family. From January of 2008, Dr. Fang Cai, a post doctorate and lab manager, and I worked on reexamining all the sequences of gDNA samples from this family and confirmed the segregation of $PS1_{\Delta S169}$ with the early-onset of AD in this FAD family with FAD. In chapter 3, with the assistance of Dr. Cai, I generated a construct harboring $PS1_{\Delta S169}$ and started the characterization of this novel mutation *in vitro.* I generated the stable cell lines expressing different PS1 variants, including $PS1_{\Delta S169}$. The cell lines 20E2 and HAW, the plasmids pcDNA4-$PS1_{WT}$, pcDNA4-$PS1_{Y115H}$, and pcDNA4-$PS1_{C410Y}$ as well as Notch constructs were previously generated in Dr. Song's laboratories. Shortly after the confirmation of the effect of $PS1_{\Delta S169}$ on APP processing *in vitro*, Dr. Cai and I started establishing transgenic mice that overexpressed $PS1_{\Delta S169}$ in 2008. Working together with Dr. Cai, we generated a construct expressing $PS1_{\Delta S169}$ under the mouse neuron specific promoter Thy1. The construct was sent to British Columbia Preclinical Research Consortium for pronuclear microinjection to generate transgenic mice. Dr. Cai designed the primers to genotype the first generation of founder of the heterozygous $PS1_{\Delta S169}$ transgenic founder mice. Then, $PS1_{\Delta S169}$ transgenic mice were bred and crossed with APP23 mice and Ms. Haiyan Zou, a former technician in our laboratory, did most of the genotyping work. Dr. Cai did most work in copy number determination. In chapter 4, I

assisted the work of genotyping and copy number determination and did more than 70% of the behavior tests. Dr. Cai, Daochao Huang and Yi Yang performed the remaining 30% behavior tests. Daochao Huang was a former visiting scholar and Yi Yang is the graduate student in our laboratory. Dr. Cai and I also carried out all the mice tissue collection work. Dr. Cai trained me on how to perform a histopathological analysis of mice brain under standardized procedures for the detection of neuritic plaques in brain sections (Ly PPT, Cai F., Song W. (2011) Detection of Neuritic plaques in Alzheimer's disease mouse model. Journal of Visual Experiment. E2831).

Since my project was mainly focusing on FAD-associated mutations, in the middle of my PhD training (in April of 2011) , Dr. Song gave me another project on the recessive APP mutation – APP_{A673V} (Chapter 2). Under the supervision of Dr. Song I worked on this project, which included the generation and characterization of constructs and stable cell lines. Dr. Zhe Wang, a postdoctorate in our laboratory, and I worked together in studying the maturation and degradation of APP in chapter 2. Mingming Zhang, a graduate student in our research team, and I performed toxicity assays of $A\beta_{A2V}$ in primary neurons.

In Chapter 1, a large proportion of Section 1.4 concerning γ-secretase has been submitted for review for publication. I wrote the first draft and am the first-author on the manuscript. Mingming Zhang and Dr. Cai provided the assistance and co-write the final version of manuscript. All procedures in Chapter 4 were approved by the University of British Columbia Animal Care Committee (Protocols A05-1888, A10-0040, and A06-0007).

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Aβ Amyloid-β

ABC avidin:biotinylated enzyme complex

AEBSF 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride; serine protease inhibitor AD Alzheimer's disease

Aph-1 Anterior pharynx factor-1

APLP amyloid precursor like protein

AICD APP intracellular domain

ANOVA analysis of variance

APP amyloid-β precursor protein

APP23 single transgenic mice carrying Swedish $APP^{KM670/671NL}$ mutation

APP23/PS45 double transgenic mice for Swedish $APP^{KM670/671NL}$ and $PS1^{G384A}$

APPSwe Swedish APP mutation

ARU Animal Research Unit

BACE1 β-site APP Cleaving Enzyme 1

BACE2 β-site APP Cleaving Enzyme 2

BBB blood brain barrier

bp base pair

BSA bovine serum albumin

CDK cyclin dependent protein kinase

Complete DMEM DMEM with 10% fetal bovine serum, 1% L-glutamine, 1% Penicillin/Streptomycin, and 1% sodium pyruvate.

CTFα C-terminal fragment α (C83)

CTFβ C-terminal fragment β (C99 and C89) DAB 3,3'-Diaminobenzidine

DEPC diethylpyrocarbonate

DMEM Dulbecco's modified eagles' medium DMSO dimethyl sulfoximine

ELISA enzyme-linked immunosorbent assay

FBS fetal bovine serum

GSI γ-secretase inhibitor; L685,458

HEK293 human embryonic kidney 293 cell line

hC99-myc HEK293 cells stably expressing human C99 CTF with myc tag

KD knockdown

KDa kilodalton

KO knockout

LTD long term depression

LTP long term potentiation

MEF mouse embryonic fibroblasts

mRNA messenger RNA

MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N2a mouse neuroblastoma

Nct nicastrin

NFκB nuclear factor kappa B

NFT neurofibrillary tangles

NSAID nonsteroidal anti-inflammatory drug Pen-2 presenilin enhancer 2

PBS phosphate buffered saline

PBS-Tx PBS with 0.1% Triton X-100

PBS-T PBS with 0.1% Tween-20

PCR polymerase chain reaction

PFA paraformaldehyde

PHF paired helical filaments

PI3K phosphoinositide 3 kinase

PKB protein kinase B, a.k.a Akt

PS1 Presenilin 1

PS2 Presenilin 2

PVDF-FL Immobilon®-FL polyvinylidene difluoride

RIPA DOC radio-immunoprecipitation assay deoxycholate

RT-PCR reverse transcription polymerase chain reaction sAPPα secretory APPα

sAPPβ secretory APPβ

SDS sodium dodecyl sulfate

SDS-PAGE SDS-polyacrylamide gel electrophoresis

WT wildtype

TNFα tumor necrosis factor α

20E2 HEK293 cells stably expressing human APP695 with Swedish mutation

Acknowledgements

The first person I would like to thank during my PhD training is Dr. Weihong Song, who is my PhD supervisor and introduced me to the field of neuroscience. Research was new and foreign to me, but Dr. Song has always patiently guided me and provided me with thoughtful questions to investigate. I am blessed to have Dr.Song's support during graduate school and it has definitely made my journey in research much more enjoyable. Through the opportunities that Dr. Song provided me, I have come to meet many wonderful people.

I also would like to thank all my committee-meeting members: Drs. Paul Pavlidis, Shernaz Bamji, Catherine Rankin and Yutian Wang. You have given me lots of enlightening suggestion on my PhD project in the annual committee meeting. Special thanks to Drs. Catherine Rankin and Yutian Wang, who send me their own CIHR proposal drafts as reference and even taught me in person how to effectively write good proposals. It was such a wonderful experience to learn from all of you. Many thanks.

To the current and past colleagues in the Song lab, your support and therapeutic talks were highly appreciated. In particular, Dr. Fang Cai and Dr. Weihui Zhou, they were my first teachers in the Song lab to help a novice like me to quickly pick up good laboratory techniques. Your experiences saved me a lot of time and set a good example for me. And also thanks so much Haiyan Zhou, Yi Yang and Daochao Huang, for the heavy workload in the transgenic mice experiment. During my pregnancy, all the lab members helped me a lot, especially Fiona Zhang, Mingming Zhang and Zhe Wang.

The PhD journey was long and tedious sometimes; however, because of that I had chance to know some wonderful people. We were not just colleagues but also friends. It was you that make my lonely study life more colorful and studying abroad more bearable. Mingming, Fiona, Phillip and Derek, thanks for the listening and the accompaniment. Words cannot express my gratitude and it was my honor to have such friends in my life.

Last but not the least, I would like to thank my family. First and foremost are my parents, who were always by my side no matter what happened. Thanks Mama for coming to Vancouver for 3 months to take care of your pregnant and grumpy daughter, please forgive my ill temper due to stress and hormones. At last, I would like to apologize to my husband Wenxiao Yang; I am so sorry for my absence of more than 5 years; I thank you for all your patience and understanding; I thank you for your forever forgiveness for my ill temper in times of stress and I love you.

To my daughter, Anna Yang, even you are too small to understand, mama would like to say sorry to you. In order to finish my PhD in Canada, I am sorry to leave you when you were just one month old and I am sorry to have breast-fed you for only one month. You are and will always be the most precious gift to me and I love you, sweetheart.

To my family.

Chapter 1

General introduction

1.1 Dementia and Alzheimer's disease.

The world's population has been ageing rapidly in the past century due to significant improvements on health care and nutrition. Dementia is a global cognitive dysfunction syndrome due to brain disease, with the aging as its well-established risk factor. In 2010, the worldwide population with dementia was estimated at 35.6 million and is projected to double every 20 years, to 65.7 million in 2030 and 115.4 million in 2050, equivalent to one new case every four seconds (International, 2012). Worldwide dementia cost in 2010 was estimated to have been US$604 billion and its burden was not only on the economy but also had dramatic impact on the patients and their families—medically, psychologically and emotionally (London, 2010). The behavioral and psychological symptoms of dementia profoundly affect the quality of life of dementia patients and of their families.

The commonest subtypes of dementia in the order of frequency are Alzheimer's disease (AD), vascular dementia (VaD), dementia with Levy bodies (DLB), dementia in Parkinson's disease and frontotemporal dementia (FTD) (London, 2009). The population prevalence of different subtypes of dementia differs among studies, given the different recruitment criteria. A recent community-based study provided information on the relative prevalence of a wider range of

subtypes: AD (41%), VaD (32%), DLB (8%), dementia in Parkinson's Disease (3%) and FTD (3%) (Stevens et al., 2002). The boundaries between these dementia subtypes have been promiscuous and the mixed forms often co-exist, although each subtype is characterized by their own pathological features: the amyloid plaques and neurofibrillary tangles and brain atrophy in AD; the multi-infarct evidence in VaD, Lewy bodies in DLB and the early involvement of the frontal temporal lobe in FTD. Apart from pathological features, different dementia subtypes are also characterized by their own set of clinical symptoms: AD is featured by difficulty in remembering names and recent events clinically; DLB is featured by initial symptoms such as sleep disturbances, visual hallucinations and muscle rigidity; FTD is featured by changes in personality, behavior and language problems; and dementia in Parkinson's Disease (PD) is featured by typical PD symptoms meeting the diagnosis of PD (Alzheimer's, 2012).

Alzheimer's disease (AD) is the most common type of dementia. On November 4, 1906, Dr. Alois Alzheimer described Alzheimer's disease publicly for the first time using the case of his patient, Auguste Deter, who had extensive atrophy of cortex and two unique brain deposits – one located in the nerve cells ("neurofilbrillary tangles" in modern terminology) and the other was located extracellularly (now known as "amyloid plaque"). Emil Kraepelin, a German psychiatrist, suggested naming the syndrome of "presenile dementia" as Alzheimer Disease. In 1911, Dr. Alzheimer published an article that was more detailed, describing cases of Johann F. and a number of other patients with "Alzheimer's disease". The patients usually began with difficulty in forming new memory, accompanied by apathy or depression; in later stages,

disorientation, behavior change, impairment on judgment and language, and deficits in motor system were observed.

Alzheimer's disease (AD) is a heterogeneous clinico-pathogogical entity. The diagnosis of AD is classified into four categories: definite AD, probably AD, possible AD and unlikely AD (McKhann et al., 2011). Because of the limited application of autopsy and biopsy, most AD patients actually are possible AD patients, if typical, progressive cognitive impairment and the absence of other dementia diseases are established clinically. Definite AD requires meeting the probably AD criteria while having neuropathological evidence via autopsy or biopsy. The presence of both neuritic plaques and neurofibrillary tangles in the neocortex is the typical neuropathological evidence, suggesting high likelihood of Alzheimer's disease. In addition to plaques and tangles, neuron degeneration and synaptic loss are also characteristic neuropathology of AD.

1.1.1 Pathological features of AD.

Amyloid plaques, neurofibrillary tangles, neuronal loss and brain atrophy are the main pathological features of AD. Among them, amyloid plaques are unique for AD, whereas neuronal loss or synaptic loss is best correlated with the severity of clinical symptoms. According to the classical "amyloid hypothesis", the temporal sequence of these pathological features is the deposition of Aβ, and then the increased tau phosphorylation and tangles formation, both of which are toxic and induce synaptic loss and neuron death (Hardy and Selkoe, 2002). However, the temporal sequence of these pathological features is still controversial. Synaptic pathologies were reported in AD transgenic mice before plaque formation, indicating that the Aβ oligomers or the intracellular Aβ might have already induced synaptic toxicity before the plaque formation. Back in

early 1990s, Braak et al. defined the progression stages of plaques and tangles according to their spatiotemporal pattern in AD patients. Compared with plaques, the spatiotemporal progression of tangles is stereotypical and predictable: tangles appear firstly in the transentorhinal region (stage I); then CA1 region of the hippocampus (stage II); then limbic structure like subiculum of the hippocampal formation (stage III); next the amygdala, thalamus and claustrum (stage IV); finally spread to all isocortical areas (stage V). Despite being less predictable, the spatiotemporal progression of plaques still could be divided into 3 stages: the isocortical stage, the limbic stage and the subcortical stage (Braak and Braak, 1991). Clinicopathological studies indicated that the amount and distribution of tangles correlate better with the severity and the duration of AD than the plaques (Arriagada et al., 1992, Bierer et al., 1995, Giannakopoulos et al., 2003).

Amyloid plaque is the unique pathological feature for AD, which is defined as the extracellular deposits of amyloid β. It should be of note that amyloid plaques are heterogeneous in terms of either the microscopic morphology or the composition. The amyloid plaques meeting the diagnosis criteria of AD are dense-core plaques or neuritic plaques. The dense-core plaques are amyloid deposits with compact core positive for Thioflavin-S or Congo-Red staining, which are typically surrounded by dystrophic neuritis as well as the activated astrocytes and microglial cells; whereas the so-called “diffuse plaques” are amyloid deposits with ill-defined contours and negative for Thioflavin-S or Congo-Red staining (Serrano-Pozo et al., 2011). Diffuse plaques are often found in the general elderly population, while the dense-core plaques are usually exclusively present in AD population. The composition of amyloid plaques is also heterogeneous. Apart from various other non-amyloid

components in amyloid plaques (proteoglycans, inflammatory molecules, metal ions, APOE, low density lipoprotein receptor-related protein, see review (Atwood et al., 2002), a spectrum of Aβ peptides has been isolated from the brains of AD patients. This large spectrum of Aβ peptides can be resulted from heterogeneous γ-cleavages as well as the multiple modification and proteolysis during the many years when Aβ peptides reside in these extracellular amyloid plaques. The neuropathologies are undistinguishable between FAD and sporadic AD. Of note, cotton wool plaques (CWP) are more frequently observed in presenilin mutations-associated FAD patients and firstly reported in FAD-associated PS1 deletion mutation of exon 9 (Crook et al., 1998a). CWPs are characterized by large size (up to 100μm), high immunoactivity for Aβ42, eosinophilic, lack of thioflavin-S positive dense cores, poor neuritic response and the displace of surrounding tissues; however, CWPs are not diffuse plaques and the underlying mechanism is not clear.

1.1.2 Mutations in APP, PS1&PS2 genes and FAD.

Although more than 95% AD are sporadic with onset after age 65, dramatic progress in the field has come from a number of genetic studies on familial Alzheimer's disease (FAD) (Campion et al., 1999). FAD is defined as an uncommon form of Alzheimer's disease that usually strikes before the age of 65 and is inherited in an autosomal dominant manner. Mutations in three genes account for the early onset of FAD — Amyloid-β precursor protein (APP), Prensenilin 1 (PS1) and Presenilin 2 (PS2) (Table 1.1). (http://www.molgen.ua.ac.be/admutations/Default.cfm?MT=1&ML=1&Page=MutByQuery&Query=tblContexts.ID=3&Selection=Gene%20=%20APP).

Gene	Chromosome	Defect	Onset Age	Proposed function
APP	21	Missense mutations Trisomy 21	40-50	Aβ production
Presenilin 1	14	Point mutations (Substitution/ Deletion/Frame shift mutation)	30-50	Aβ production
Presenilin 2	1	Missense mutations	50	Aβ production
APOE4e	19	Polymorphism	>65	Aβ clearance and Aβ aggregation
SORL1	11	Polymorphism	>65	APP Endocytosis
CLU	8	Polymorphism	>65	Aβ clearance, deposition and toxicity
CR1	1	Polymorphism	>65	Aβ clearance
PICALM	11	Polymorphism	>65	Clathrin-mediated endocytosis
BIN1	2	Polymorphism	>65	Synaptic vesicle endocytosis
ABCA7	19	Polymorphism	>65	Protein transportation
CD33	19	Polymorphism	>65	Clathrin-mediated Endocytosis
CD2AP	6	Polymorphism	>65	Receptor-mediated Endocytosis
TREM2	6	Polymorphism	>65	Inflammation

APP: amyloid precursor protein; APOE4e: apolipoprotein E4e isoform; SORL1, Sortilin-related receptor; CLU, clusterin; CR1, complement component (3b/4b) receptor 1 (Knops blood group); PLCALM, phosphatidylinositol binding clathrin assembly protein; BIN1, bridging integrator 1; ABCA7, ATP-binding cassette, sub-family A (ABC1), member 7; CD2AP, CD2-associated protein; TREM2, Triggering receptor expressed on myeloid cells 2. For more information, see Alzgene meta-analysis (http://www. alzgene/org.

Table 1.1 Genetic factors contributing to Alzheimer's disease.

1.1.3 Genetic risk factors and sporadic AD.

The rare mutations in APP and PS genes are causing the early-onset of FAD. Is there any established genetic factors contributing to the high risk of late-onset of sporadic AD? Genes involved in the formation of plaques and tangles (APP,

PS1/PS2 and MAPT), have therefore long been considered as putative candidates for higher susceptibility of sporadic AD. The contribution of APP, PS1/PS2 and MAPT in sporadic AD remains questionable, although MAPT is established as a risk factor in Parkinson's disease (PD) (Simon-Sanchez et al., 2009), progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Houlden et al., 2001a, Pittman et al., 2005). In PD, SNCA and LRRK2 genes not only contribute to dominant familial PD, but also impose higher risk to sporadic PD (Ross and Farrer, 2010). However, it lacks strong evidence to support that common variations in APP and PS1/PS2 genes contribute to higher risk in sporadic AD (Guyant-Marechal et al., 2007, Reiman et al., 2007, Gerrish et al., 2012). One possible explanation is that the function of APP and Presenilins is so essential for the normal processing of APP that single nucleotides polymorphism (SNP) has dramatic effect on their functional performance.

Until recently, apolipoprotein E4ε isoform (APOE4ε) is the only established genetic risk factor for the sporadic form of AD (Corder et al., 1993, Saunders et al., 1993, Strittmatter et al., 1993). APOE is the major apolipoprotein and cholesterol carrier in the brain and synthesized predominantly by astrocytes and to some extend by microglia (Pitas et al., 1987, Uchihara et al., 1995). The frequency of APOE4ε is about 15% in general population but is about 40% in sporadic AD patients. Compared with those without APOE4ε alleles, individual with one APOE4ε allele are 3 to 4 times more likely to develop AD (Corder et al., 1993, Strittmatter et al., 1993). With the advances in microarray technology, numerous genome-wild association studies (GWAS) are undergoing in a non-hypothesis-biased manner, aiming at identifying additional AD susceptibility loci that are common in the general population. It has been implicated that the

following loci are low-risk factors in sporadic AD: CLU, PICALM, CR1, BIN1, MS4A, CD2AP, CD33, EPHA1, and ABCA7 (Harold et al., 2009, Lambert et al., 2009, Hollingworth et al., 2011, Naj et al., 2011) (Table 1.1). Recently, two groups independently report heterozygous rare variants in TREM2 associated with high risk of sporadic AD (Guerreiro et al., 2013, Jonsson et al., 2013). The frequency of the variants in TREM2 is 1~2% in sporadic AD patients but only 0.05% in general population. TREM2 encodes a single-pass type I membrane Ig super family protein, which is involved in activating immune responses in macrophages and dendritic cells in central nervous system. The increased predisposition of AD in TREM2 variants implicates the inflammation does play an essential role in AD pathogenesis, probably via influencing the clearance of Aβ and its deposits or modulating the neuronal toxicity of Aβ.

1.2 Amyloid hypothesis.

Neuritic plaques and neurofibrillary tangles (NTFs) are two hallmarks of AD (Katzman, 1986). Neuritic plaques are also known as senile plaques or amyloid plaques, which are mainly extracellular deposits of amyloid beta protein (Aβ). Neuritic plaques consist primarily of 42-residue amyloid proteins (Aβ42), which are derived from proteolytic processing of beta amyloid precursor protein (APP) (Glenner and Wong, 1984b, Iwatsubo et al., 1994). Neurofibrillary tangles (NFTs) are intraneuronal inclusions composed of hyperphosphorylated forms of the microtubule-associated protein – Tau (Grundke-Iqbal et al., 1986, Kosik et al., 1986, Goedert et al., 1988, Iqbal et al., 1989). Unlike neuritic plaques, which is the unique pathological feature of AD, NFTs are detected in a whole spectrum of neurodegenerative tauopathies including Pick's disease, progressive supranuclear palsy, corticobasal degeneration, Parkinson's disease, frontotemporal dementia and frontotemporal dementia with Parkinsonism on

chromosome 17 (FTDP-17) (De Strooper, 2010). For instance, FTDP-17 is caused by mutations in the MAPT gene, which encodes a microtubule-binding protein — Tau (Hutton et al., 1998). The formation of NTFs is promoted by the hyper-phosphorylation of tau, which could be influenced by multiple factors such as the Aβ-induced hyper-phosphorylation of tau (Ittner and Gotz, 2011). Several parallel studies have suggested that neurofibrillary tangles of wild-type tau found in AD brains are likely to form after changes in Aβ metabolism and initial extracellular amyloid plaque formation (Hardy et al., 1998, Lewis et al., 2001), indicating the primary role of Aβ in AD.

The amyloid hypothesis was first proposed since it was discovered that patients with Trisomy 21 (Down Syndrome) have inevitably developed AD neuropathology (Olson and Shaw, 1969). Subsequent identification of AD-associated mutations in APP gene further bolsters the notion that APP plays a central role in AD pathogenesis (Goate et al., 1991, Hardy, 1992, Hendriks et al., 1992, Mullan et al., 1992a, Prasher et al., 1998). In addition to the effect of APP mutations on the production of Aβ, mutations in Presenilin 1/Presenilin 2 genes and the polymorphism on *APOE4* also contribute to abnormal Aβ metabolism. The mutual target of all established AD genetic factors strongly suggest that the abnormal accumulation of Aβ, particularly the more hydrophobic and aggregation-prone Aβ42, initiates neuronal dysfunction and death, neurofibrillary degeneration and microglial activation, leading to neurodegeneration in AD (Fig 1.1) (Hardy and Selkoe, 2002).

Although the amyloid hypothesis offered a comprehensive framework to elucidate AD pathogenesis, the exact underlying mechanism is still lacking and

several research results argue against this theory. For instance, amyloid plaques are also present in normal seniors and the severity of AD symptoms is correlated better with neurofibrillary tangles but not the amyloid burden (Terry et al., 1991, Arriagada et al., 1992, Gomez-Isla et al., 1997). Recent studies also indicate that AD symptoms have a correlation with soluble Aβ oligomers and the classical amyloid plaques appear to be reservoirs for the toxic, smaller, metastable Aβ species (Lue et al., 1999, McLean et al., 1999, Wang et al., 1999, Naslund et al., 2000).

The causative role of Aβ and amyloid plaques in AD pathogenesis is arguable. Given the large number of pathogenic mutations in presenilin and its abundant substrates, the "presenilin hypothesis" was proposed (Shen and Kelleher, 2007). This theory emphasized the importance of presenilins in AD pathogenesis, based on the obvious neurodegeneration deficit in presenilins conditional-knockout mice. Nevertheless, as Aβ is the sole target of all genetic factors (Table 1.1), the amyloid hypothesis is still dominating the studies on AD pathogenesis (Fig 1.1). This dissertation explores how amyloid cascades have been affected by mutations in APP or Presenilin 1 genes.

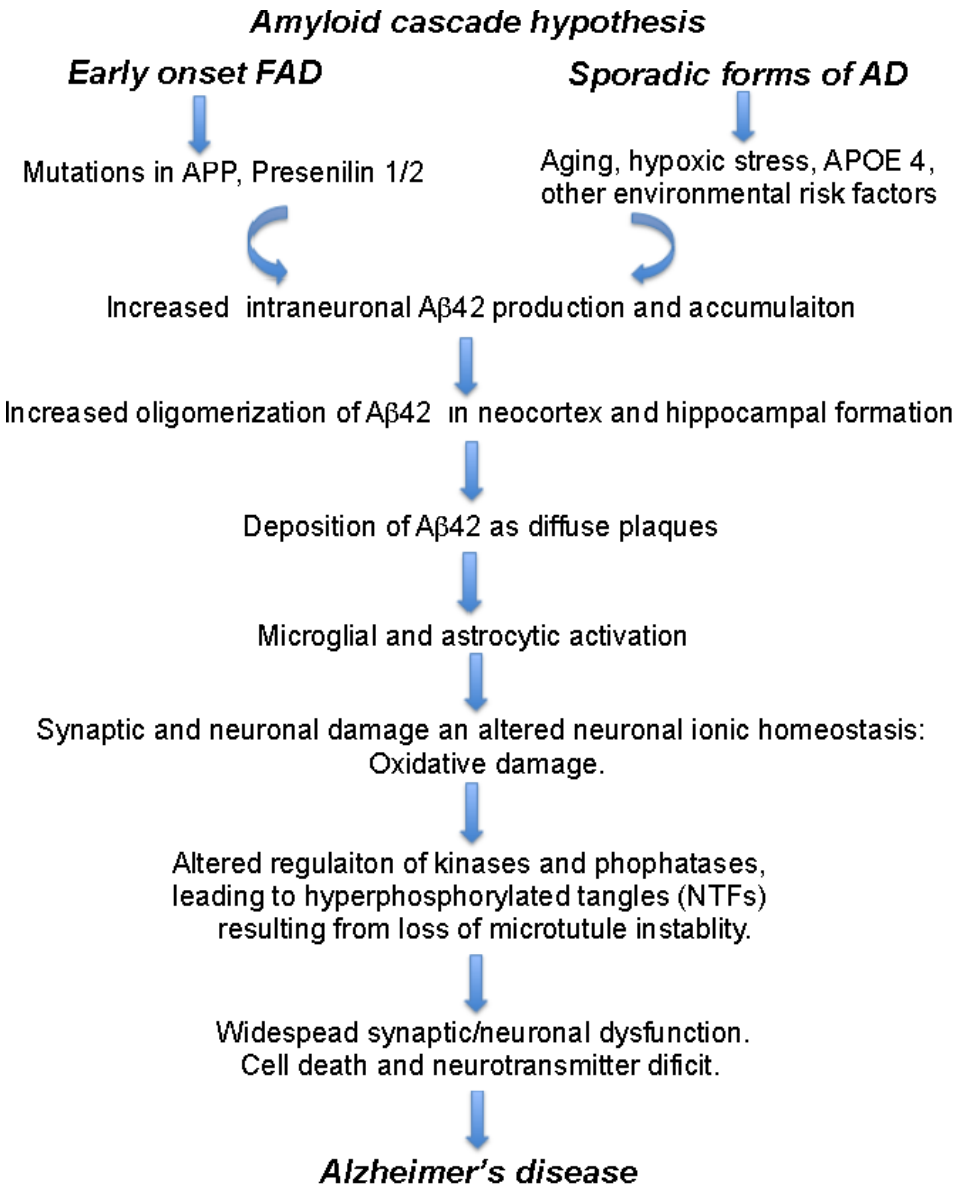
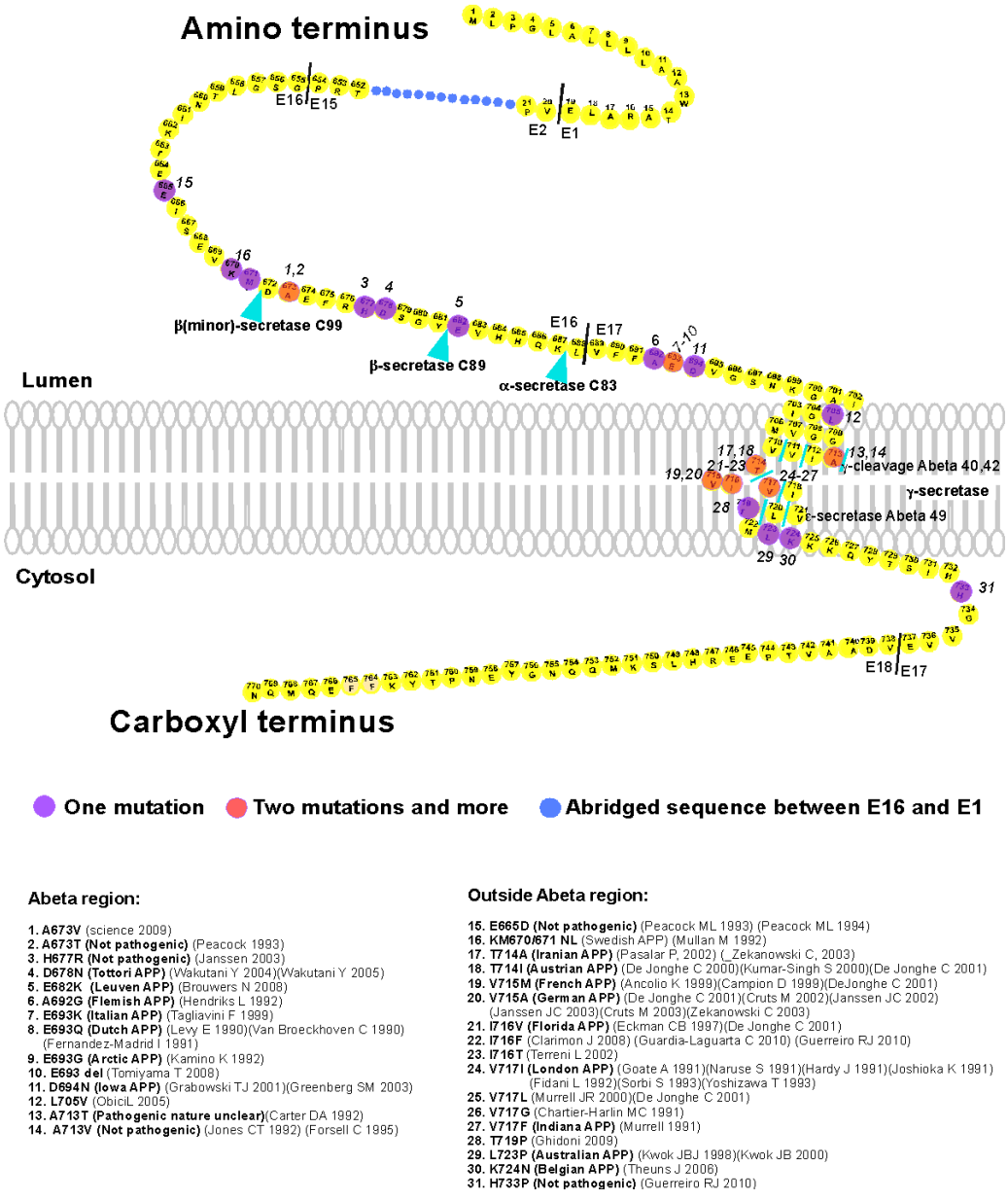


Figure 1.1 The amyloid hypothesis of Alzheimer's disease.
The genetic factors of early onset FAD and risk factors of sporadic AD converge to an increase in Aβ42 production, which induces sequential pathogenic events leading to AD. Aβ oligomers could directly initiate a toxic cascade rather than being deposited as plaques. Adapted from Hardy and Selkoe, 2002.

1.2.1 Amyloid-beta precursor protein (APP).

Amyloid beta protein (Aβ), the core component of neuritic plaque, is derived from amyloid-beta precursor protein (APP) by sequential cleavages of β- and γ-secretase (Glenner and Wong, 1984b, a, Goldgaber et al., 1987, Kang et al., 1987, Robakis et al., 1987, Tanzi et al., 1987, Weidemann et al., 1989, Sun et al., 2012). The APP gene is located on Chromosome 21, coding for a ubiquitously expressed type I transmembrane protein, for which several isoforms can be derived from alternative splicing of mRNA (Fig 1.2). APP770/751 (containing 770/751 amino acids) are mainly expressed in non-neuronal cells, whereas APP695, lacking the Kunitz protease inhibitor (KPI) region, is dominant in neurons (Kitaguchi et al., 1988, de Sauvage and Octave, 1989) (Bendotti et al., 1988, Arai et al., 1991, Konig et al., 1992, Sandbrink et al., 1994). APP and APP-like proteins (APL1 and APL2) belong to the same protein family (Wasco et al., 1992, Sprecher et al., 1993, Wasco et al., 1993) and conserved homologs of APP have been found in *C. elegans* (Daigle and Li, 1993) and *Drosophila* (Luo et al., 1992).



Abeta region:

1. **A673V** (science 2009)
2. **A673T (Not pathogenic)** (Peacock 1993)
3. **H677R (Not pathogenic)** (Janssen 2003)
4. **D678N (Tottori APP)** (Wakutani Y 2004)(Wakutani Y 2005)
5. **E682K (Leuven APP)** (Brouwers N 2008)
6. **A692G (Flemish APP)** (Hendriks L 1992)
7. **E693K (Italian APP)** (Tagliavini F 1999)
8. **E693Q (Dutch APP)** (Levy E 1990)(Van Broeckhoven C 1990) (Fernandez-Madrid I 1991)
9. **E693G (Arctic APP)** (Kamino K 1992)
10. **E693 del** (Tomiyama T 2008)
11. **D694N (Iowa APP)** (Grabowski TJ 2001)(Greenberg SM 2003)
12. **L705V** (ObiciL 2005)
13. **A713T (Pathogenic nature unclear)**(Carter DA 1992)
14. **A713V (Not pathogenic)** (Jones CT 1992) (Forsell C 1995)

Outside Abeta region:

15. **E665D (Not pathogenic)** (Peacock ML 1993) (Peacock ML 1994)
16. **KM670/671 NL** (Swedish APP) (Mullan M 1992)
17. **T714A (Iranian APP)** (Pasalar P, 2002) (_Zekanowski C, 2003)
18. **T714I (Austrian APP)** (De Jonghe C 2000)(Kumar-Singh S 2000)(De Jonghe C 2001)
19. **V715M (French APP)** (Ancolio K 1999)(Campion D 1999)(DeJonghe C 2001)
20. **V715A (German APP)** (De Jonghe C 2001)(Cruts M 2002)(Janssen JC 2002) (Janssen JC 2003)(Cruts M 2003)(Zekanowski C 2003)
21. **I716V (Florida APP)** (Eckman CB 1997)(De Jonghe C 2001)
22. **I716F** (Clarimon J 2008) (Guardia-Laguarta C 2010) (Guerreiro RJ 2010)
23. **I716T** (Terreni L 2002)
24. **V717I (London APP)** (Goate A 1991)(Naruse S 1991)(Hardy J 1991)(Joshioka K 1991) (Fidani L 1992)(Sorbi S 1993)(Yoshizawa T 1993)
25. **V717L** (Murrell JR 2000)(De Jonghe C 2001)
26. **V717G** (Chartier-Harlin MC 1991)
27. **V717F (Indiana APP)** (Murrell 1991)
28. **T719P** (Ghidoni 2009)
29. **L723P (Australian APP)** (Kwok JBJ 1998)(Kwok JB 2000)
30. **K724N (Belgian APP)** (Theuns J 2006)
31. **H733P (Not pathogenic)** (Guerreiro RJ 2010)

Figure 1.2 The structure of amyloid-beta precursor protein (APP).
This diagram shows the amino acid sequence of APP and the distribution of FAD-associated mutations. The smaller blue circles represent abridged sequence between E1 (exon1) and E16 (exon16). All bigger colored circles not yellow represent the FAD-associated mutations and purple circles represent only one mutation identified at this locus and orange circles represent more than one mutations identified at this locus. Green triangles and green bars represent secretase cleavage sites as indicated in this diagram. The following legends elaborate all APP mutations with their names and the original papers.

APP is a type I transmembrane protein with a relatively large extracellular/luminal domain at the NH2 terminus and a short cytoplasmic domain at the COOH terminus (Fig 1.2). During its transport from ER to plasma membrane, APP undergoes N- and O- glycosylation (Weidemann et al., 1989). Further sulfation and phosphorylation take place at the late Golgi apparatus and at the plasma membrane (Hung and Selkoe, 1994, Suzuki et al., 1994, Walter et al., 1997). The majority of APP is detected in the Golgi, whereas the plasma membrane associated APP accounts for only 10% of the total APP protein (estimation based on overexpression experiment in cell culture); and from the plasma membrane, APP is rapidly undergoes internalized for following recycling and degradation (Sisodia, 1992) (Fig 1.3).

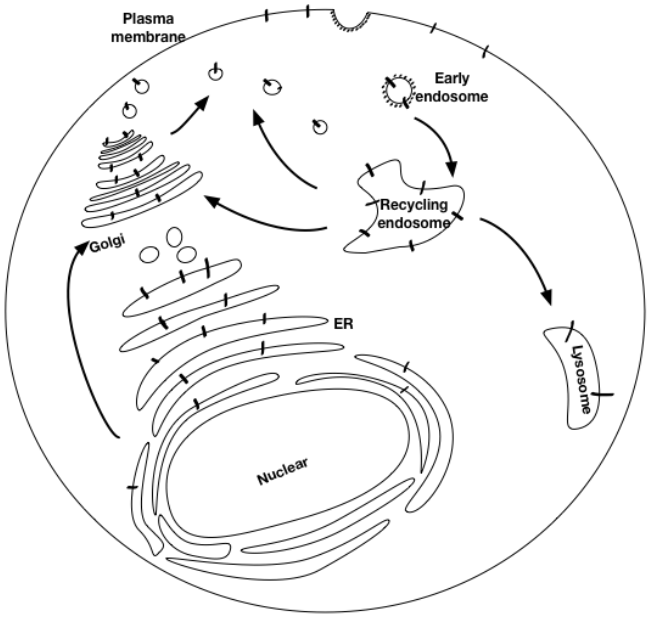


Figure 1. 3 The intracellular APP Trafficking.
Nascent wild type APP (black bars) undergoes multiple modifications in classical secretory pathway from ER to Golgi. Only a small proportion of APP is delivered to the plasma membrane to be processed by α-secretase. Some plasma membrane APP undergoes endocytosis where the β-cleavages occur. Aβ generation is believed to occur in endocytic organelles such as endosomes. Adapted from Thinakaran, 2006.

Since its discovery, multiple physiological functions have been linked to APP (Mattson, 1997); however, its specific role remains to be elucidated. We introduce three well-explored functions of APP here. Firstly, APP has been proposed to be a trophic molecule. Dating back to 1989, know-down of APP was linked to the slow growth of fibroblast induced by an antisense APP construct, which could be reversed by APP treatment (Saitoh et al., 1989). Later it was reported that APP promoted neuronal maturation (Hung et al., 1992) as the memory-enhancing effect of APP could be blocked by intracerebroventribular injection of anti-APP antibody in adult rats (Meziane et al., 1998). Subsequently, N-terminal residues 28-123 of APP were reported to display a similar structure as that of well-known cysteine-rich growth factors (Rossjohn et al., 1999). The following studies specified that the trophic effect of APP was due to one of its secreted derivative—sAPPα. sAPPα was reported to have the ability to induce neuronal stem cells into astrocytes *in vitro* (Kwak et al., 2006). In the subventricular zone of adult mice brains, sAPPα, together with EGF, promoted the proliferation of EGF-responsive neural stem cells (Caille et al., 2004). Secondly, APP has been proposed to be a cell adhesion factor, which is reasonable since substrate adhesion has been involved in neurotrophic proliferation. A RHDS motif within the C-terminal of sAPP was found to contribute to cell adhesion function via integrin-like receptors and APP was required for the migration of neuronal precursors to the cortical plate (Ghiso et al., 1992, Yamazaki et al., 1997, Young-Pearse et al., 2007). Last but not least, APP has been proposed to be a cell-surface receptor. Both APP and Notch undergo the same γ-cleavage at the transmembrane domain to generate the APP intracellular domain (AICD) and the Notch intracellular domain (NICD), respectively. Considering the role of NICD in fundamental cell signaling, the function of AICD in cell signaling pathways has been well

explored but still lacks convincing support. Since AICD was found to form a transcriptionally active complex with Fe65 and Tip60 (Cao and Sudhof, 2001), several downstream candidate genes have been proposed: KAI1 (a tumor suppressor gene), neprilysin (a neutral endopeptidase with Aβ-degrading activity), LRP1, and the EGF receptor (Baek et al., 2002, Pardossi-Piquard et al., 2005, Liu et al., 2007, Zhang et al., 2007b). Considering the various phenotypes of presenilin activity, it would be very interesting if AICD were really as transcriptionally active as NICD. However, all of these pathways still lack *in vivo* evidence.

APP-deficient animals lack typical phenotype, unlike presenilin-deficient or Notch-deficient animals. APP-deficient mice only exhibited reactive gliosis and decreased locomotor activity (Zheng et al., 1995). The mild phonotype might be due to an overlapping effect of other two APP protein family members – APLP1 and APLP2. $Aplp2^{-}/Aplp1^{-}$, $App^{-}/Aplp2^{-}$ double mutants and $App^{-}/Aplp1^{-}/Aplp2^{-}$ exhibited early postnatal lethality (Herms et al., 2004, Anliker and Muller, 2006). Further studies of these animals revealed that APP/APLP was specifically involved in synapse formation – reduction in apposition of pre- and postsynaptic elements of the junctional synapses; effect on active zone size, synaptic vesicle density, and number of docked vesicles per active zone; aberrant localization of the choline transporter at neuromuscular junctions (Wang et al., 2005, Yang et al., 2005, Wang et al., 2007).

1.2.2 APP processing and Aβ generation.

APP undergoes posttranslational proteolytic processing by α-, β- and γ-secretases (Fig 1.4). The majority of APP is constitutively processed in

the non-amyloidogenic pathway via α-secretase cleavage, which occurs inside the Aβ sequence to yield a secretory product, thereby precluding Aβ production (Esch et al., 1990). In the amyloidogenic pathway, APP undergoes sequential cleavage by β- and γ-secretases to generate Aβ.

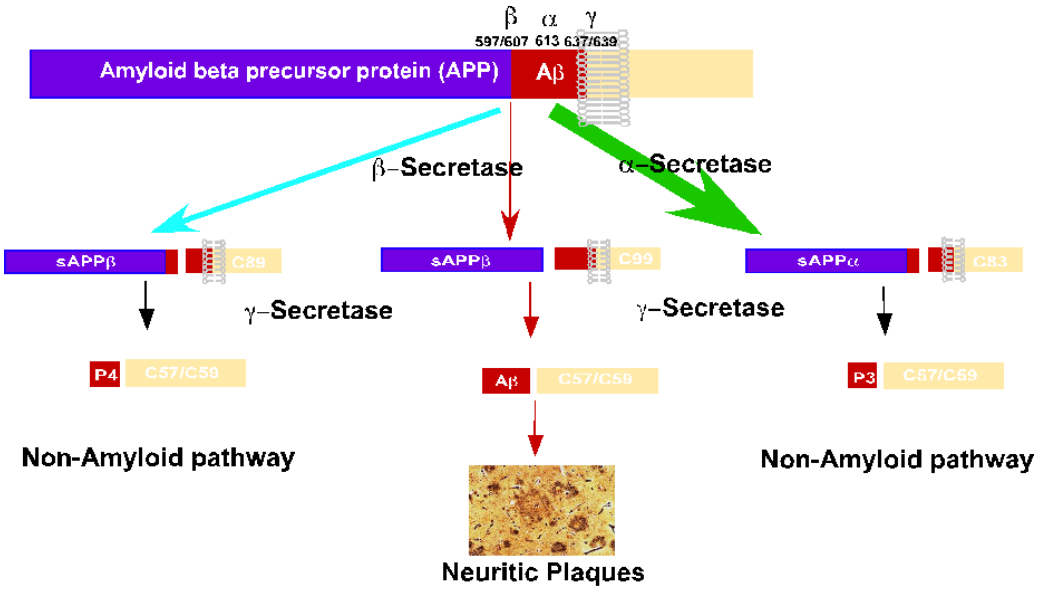


Figure 1.4 APP processing pathways.
Under physiological conditions, the majority of APP undergoes the non-amyloidogenic pathway. α-secretase processes APP within the Aβ domain. This cleavage abolishes Aβ generation and produces C83 fragments. Predominant β-scretase processes APP at the Glu-11 site to generate C89 fragments as the major product. In the amyloidogenic pathway, β-scretase processes APP at the Asp-1 site to generate C99 fragments, which become the substrate to γ-scretase for Aβ generation. The cleavage sites for each α-, β- and γ-secretase are indicated.

A transmembrane aspartic protease BACE1 was identified as β-secretase (Hussain et al., 1999, Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999), which processes APP at Asp+1 and Glu+11 of the Aβ domain, to generate a major product C89 and a minor product C99 fragment (Vassar et al., 1999, Li et al., 2006). The C99 fragment is further processed by γ-secretase at the

intramembrane Val-40 and Ala-42 sites to generate Aβ40 and Aβ42 (De Strooper et al., 1998, Yu et al., 2000b, Francis et al., 2002a, Goutte et al., 2002a, Edbauer et al., 2003b, Kimberly et al., 2003b, Takasugi et al., 2003) (Fig 1.5).

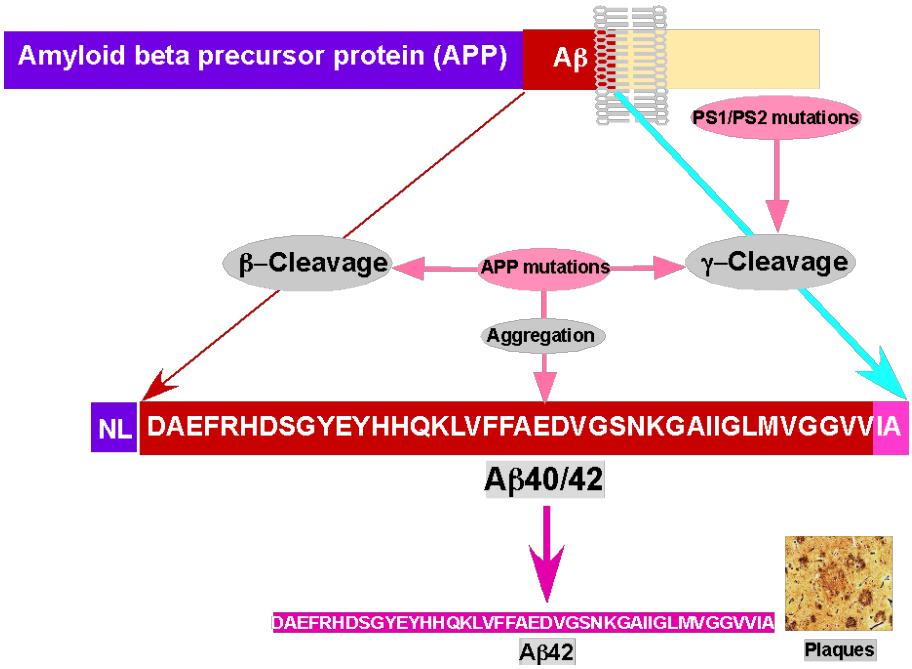


Figure 1.5 Amyloid beta protein (Aβ).
Amyloid beta protein (Aβ) is a peptide of 36–43 amino acids that has been processed from APP. The most common forms of Aβ are Aβ40 and Aβ42, both of which are the γ-cleavage product of APP C99 fragment. Aβ40 is the major γ-secretase product of C99, whereas Aβ42 is the predominant component of neuritic plaques. Numerous mutations in APP and PS genes contribute to AD pathogenesis by affecting the production of Aβ or the aggregation property of Aβ.

Amyloid plaques consist of insoluble fibrous Aβ aggregates characterized by a cross-beta sheet quaternary structure and typically identified histologically by a change in the fluorescence intensity of planar aromatic dyes such as thioflavin T or Congo red. Aβ was a typical amyloid protein and was first purified from

twisted beta-pleated sheet fibrils in cerebrovascular amyloidosis in AD-associated amyloid plaques (Glenner and Wong, 1984a,b); and this 4.2-kDa peptide, 40 or 42 amino acids in length, was speculated to have come from a larger precursor. In 1987, a full length APP with 695 residues was cloned, containing the amyloid β sequence, which encompassed 28 amino acids of the ectodomain and 11 to 14 amino acids of the transmembrane domain of APP (Kang et al., 1987, Podlisny et al., 1987, Robakis et al., 1987, Tanzi et al., 1987) Aβ is the direct product of C99 fragment of APP derived from a sequential proteolytic cleavage of APP in the amyloidogenic pathway (Fig 1.4 and Fig 1.5). Aβ can exist in various assembly states, including monomers, dimers, trimers, tetramers, dodecamers, higher-order oligomers and protofibrils, as well as mature fibrils, which can form microscopically-visible amyloid plaques in brain tissues (Glabe, 2008). Recent studies suggest that it is Aβ oligomers rather than fibrils or deposits that initiate the abnormalities in synaptic functions and neural network activity (Cleary et al., 2005a, Lesne et al., 2006, Cheng et al., 2007, Shankar et al., 2007, Selkoe, 2008, Shankar et al., 2008b, Tomiyama et al., 2010). Even though the preparations of Aβ in these studies were not strictly comparable, the essential role of Aβ oligomers in the amyloid cascade hypothesis could be implicated.

1.2.3 Aβ — A two-edged sword: physiological and pathological roles.

Aβ could be detected in the CSF of normal individuals and in neuron culture media (Haass et al., 1992, Tamaoka et al., 1997), indicating a physiological role of Aβ in the central nervous system. Studies on primary neurons suggested that the inhibition of endogenous Aβ production increases neuronal cell death, which could be mitigated by the addition of physiological level (*p*M) of Aβ40

but not other forms of Aβ (Plant et al., 2003). Moreover, the production of Aβ and its extracellular secretion are strictly regulated by neuronal activity both *in vitro* and *in vivo* (Kamenetz et al., 2003, Cirrito et al., 2005). Increased neuronal activity promotes Aβ generation and Aβ in turn negatively regulates neuronal activity to stabilize hyperactive neurons (Kamenetz et al., 2003). This synaptic activity-dependent modulation of Aβ production was mediated by clathrin-dependent endocytosis of surface APP at presynaptic terminals, endosomal proteolytic cleavage of APP, and Aβ release at synaptic terminals (Cirrito et al., 2005). These findings supported the notion that Aβ physiologically takes part in the neuronal function.

Another edge of the "Aβ sword" is the contribution of Aβ to AD pathogenesis. As discussed in the last section, Aβ oligomers, but not amyloid plaques, are believed to be the culprit in AD. Excessive Aβ production promotes LTD-induced postsynaptic depression and loss of dendritic spines (Snyder et al., 2005, Shankar et al., 2007, Li et al., 2009), induces glutamatergic synaptic transmission and causes synaptic loss (Hsia et al., 1999, Mucke et al., 2000, Walsh et al., 2002). Considering that synaptic loss is one of pathological features of AD and is well correlated with cognitive decline (DeKosky and Scheff, 1990), the toxic effect of Aβ oligomers on synapse could be the fundamental basis of AD pathogenesis. A working model to fit both physiological and pathological roles of Aβ was proposed by Palop &Mucke (Palop and Mucke, 2010) (Fig 1.6). This model elaborated on how Aβ directly elicited synaptic damage downstream of the amyloid cascade hypothesis, and emphasized that maintaining a delicate balance of Aβ is crucial in having

functional neural network.

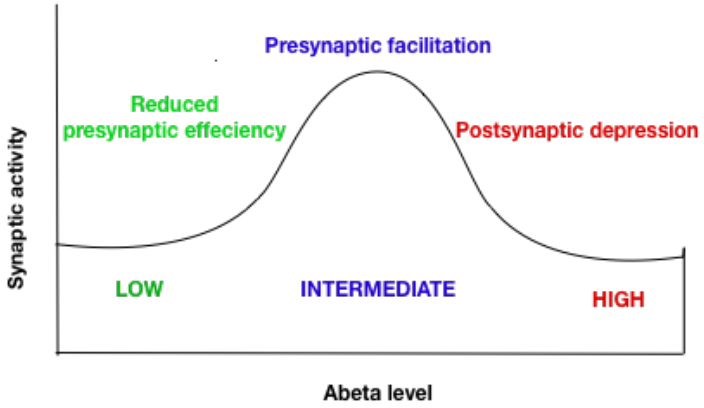


Figure 1. 6 Aβ in Synaptic modulation.
Hypothetical relationship between Aβ level and synaptic activity. Intermediate levels of Aβ enhance synaptic activity presynaptically, whereas abnormally high or low levels of Aβ impair synaptic activity by inducing postsynaptic depression or reducing presynaptic efficacy, respectively. Adapted from Palop and Mucke, 2010.

1.2.4 Aβ oligomer and the toxicity.

The central role of Aβ in AD pathogenesis is unequivocal, however, the mechanism underlying the toxicity of Aβ is still in debate. First and foremost, what is the real toxic substance: the plaques or other substance undiscovered? Given the poor correlation of plaques and the cognitive impairment in AD patients, researchers propose that the Aβ oligomers may be the long-term-searching toxic substance of Aβ. Back in 1991, it had been found that *in vitro* Aβ monomer could be converted into toxic oligomers in buffer (Pike et al., 1991). However, until now, the terminology of the toxic Aβ oligomer has been still ambiguous and lack of accurate definition. The Aβ oligomers are prepared either in *in vitro* buffer or derived directly from brain extract via various

methods. The various procedures in the preparation or extraction result in various forms of Aβ oligomers: Aβ-derived diffusible ligands (ADDL), Aβ*56, AβO, annular protofibrils (APF), SDS-stable dimers and trimers, SDS-stable amylospheroid (ASPD) (Lambert et al., 1998, Walsh et al., 2002, Kayed et al., 2003, Cleary et al., 2005b, Lesne et al., 2006, Shankar et al., 2008a, Noguchi et al., 2009, Lasagna-Reeves et al., 2011). The biological effects of these oligomers include LTP impairment, NMDAR-dependent synaptotoxicity, increased tau phosphorylation, disturbance in Ca2+ hemostasis, NMDAR-independent cytotoxicity in primary neurons and cognitive impairment in mice (Walsh et al., 2002, Hoshi et al., 2003, Cleary et al., 2005b, Demuro et al., 2005, Lesne et al., 2006, Shankar et al., 2008a, Zempel et al., 2010). It should be clarified that the so-named SDS-resistant Aβ oligomers, which are frequently used in most of related literature, are quite confusing. It has been proven that SDS itself could induce the formation of oligomers, thus SDS-involved method is not reliable to distinguish and analyze different forms of oligomers (Bitan et al., 2005, Hepler et al., 2006). Last but not the least, it should be bear in mind that a dynamic equilibrium is eternal between the amyloid plaques and Aβ oligomers. With the deep exploration, it would be more clear about the relationship between these two and which is the real toxic substance or the toxic group in AD pathogenesis (Benilova et al., 2012).

1.3 BACE1 and β-secretase in Alzheimer's disease.

Beta-site APP-cleaving enzyme 1 (BACE1) has been identified as the β-secretase that produces C99, the direct precursor of Aβ (Hussain et al., 1999, Sinha et al., 1999, Vassar et al., 1999, Yan et al., 1999). BACE2 is the homolog of BACE1 (Saunders et al., 1999, Yan et al., 1999, Sun et al., 2005). Despite

about 45% homologous sequence, BACE2 has distinct transcriptional regulation and functions compared with BACE1. BACE2 is not β-secretase; rather, it is a novel θ-secretase that cleaves APP within the Aβ sequence at Phe^{20} θ site, further downstream of the Leu^{17} α-secretase cleavage site and thus precludes the amyloid pathway (Sun et al., 2006b). BACE1 gene is located on chromosome 11q23.3, and codes for a type I membrane-associated aspartyl protease with 501 amino acids. As a low abundance protein, BACE1 is expressed in relatively high levels in the brain and pancreas (Vassar et al., 1999, Marcinkiewicz and Seidah, 2000). BACE1 pre-mRNA undergoes complex alternative splicing, resulting in various isoforms that contribute to the different BACE1 activities in the brain and pancreas (Mowrer and Wolfe, 2008). BACE1-501 is the major isoform with the highest β-secretase activity and mainly localizes in the endosome and the Golgi network (Vassar et al., 1999, Bodendorf et al., 2001, Tanahashi and Tabira, 2001, Ehehalt et al., 2002). Post-translational modifications are necessary for BACE1 protein maturation and activity. After removal of the N-terminal pro-peptide in the trans-Golgi network, which is the hallmark of BACE1 maturation (Bennett et al., 2000, Capell et al., 2000, Creemers et al., 2001), BACE1 is further glycosylated at Asn -153, -172, -223 and -354 (Capell et al., 2000, Haniu et al., 2000, Huse and Doms, 2000).

1.3.1 Tight regulation of BACE1 gene expression.

Tightly controlled BACE1 gene expression plays an essential role in regulating APP processing pathways. Under physiological conditions, only a minority of APP undergoes β-secretase cleavage and a very small amount of Aβ is produced. Unlike the robust expression of APP and Presenilin in neuronal and

non-neuronal cells driven by a strong housekeeping gene-like promoter (Song and Lahiri, 1998a, b), BACE1 gene transcription was markedly lower, resulting from weak BACE1 promoter activity (Li et al., 2006). In addition, at the translation initiation level, multiple ATGs in the 5'untranslated region (UTR) of BACE1 gene are shown to suppress its translation initiation (Rogers et al., 2004). As well leaky scanning and reinitiating mechanism are found to be involved in the inhibition of the physiological fourth AUG-initiated BACE1 translation (Zhou and Song, 2006). Taken together, a weak promoter and suppression of translation initiation synergistically result in weak BACE1 protein expression in normal conditions. Considering the relatively robust APP and Presenilin expression, BACE1 could be viewed as a rate-limiting enzyme in the APP amyloidogenic pathway and limited BACE gene expression is responsible for the small amount of Aβ production under physiological conditions.

BACE1 gene expression is up-regulated in AD. The human BACE1 gene promoter region contains a set of putative transcription factor-binding sites, including GC box, HSF-1, PU-box, AP1, AP2, lymphokine response element, nuclear factor-kappa B (NF-κB) p65 and hypoxia-inducible factor 1 (Hif-1) (Christensen et al., 2004, Ge et al., 2004, Sun et al., 2006a, Chen et al., 2011). Later *in vitro* and *in vivo* studies validate that hypoxia could up-regulate BACE1 expression by acting on the Hif-1 response element in its promoter region (Sun et al., 2006a, Xue et al., 2006, Zhang et al., 2007a), and hypoxia treatment significantly promoted neuritic plaque formation as well as memory impairment in APP transgenic mice (Sun et al., 2006a). In summary, mounting evidence points to BACE1 being the rate-limiting factor in the Aβ production

pathway, including the tight control of BACE1 gene expression on different levels and the existence of multiple influencing factors like Hif-1 in hypoxia. As a β-secretase product and the immediate substrate of Aβ, the yield of C99 was strictly regulated to prevent Aβ generation and the resulting AD pathogenesis under normal conditions.

1.3.2 BACE1 processes APP at major Glu-11 and minor Asp-1 sites.

BACE1 processes APP at two sites – Glu-11 and Asp-1 (numbering according to the Aβ sequence), to generate C89 and C99 respectively (Vassar et al., 1999, Li et al., 2006). Further processing of C89 in the transmembrane region by γ-secretase leads to a truncated Aβ with 30-32 amino acids; whereas C99 is the immediate substrate for Aβ generation. Any factors, facilitating C99 production, will promote Aβ generation and promote amyloidogenic pathogenesis, according to the "Amyloid hypothesis" (Fig 1.5).

Any factor that increases general BACE1 activity or shifts cleavage from Glu-11 to Asp-1 site could contribute to Aβ generation and amyloid pathogenesis (Deng et al., 2013). As discussed in the last section, the expression of BACE1 is under strict regulation at both the transcriptional and translational levels, which results in the majority of APP being processed by α-secretase in the non-amyloidogenic pathway under normal conditions (Esch et al., 1990, Oltersdorf et al., 1990, Sisodia et al., 1990). Under pathogenic conditions where the expression of BACE1 is up-regulated even slightly, a significant proportion of APP processing will be shifted to the amyloidogenic pathway. It was

documented that the activity of BACE1 increased with aging (Russo et al., 2000, Fukumoto et al., 2002, Holsinger et al., 2002, Yang et al., 2003, Fukumoto et al., 2004). Previous studies of our laboratories demonstrated that BACE1 expression could be modulated by hypoxia, consistent with the observation of the increased BACE1 levels in sporadic AD (Sun et al., 2006a). In the case of Down syndrome, BACE1 level is up-regulated by abnormal trafficking and maturation (Sun et al., 2006c). In another scenario, any factors shifting the β-cleavage site from Glu-11 to Asp-1 would also contribute to AD pathogenesis. Recently our laboratories demonstrated that Glu-11 was the major cleavage site of BACE1 in wild type APP with C89 as the predominant product, whereas the major cleavage site of BACE1 in Swedish APP mutant was Asp-1 with C99 as the predominant product (Deng et al., 2013). Clearly, Swedish APP contributed to AD pathogenesis by shifting the β-cleavage site from Glu-11 to Asp-1. The underlying mechanism of the shift in cleavage sites was unknown.

1.3.3 BACE1 and AD therapy.

BACE1 has been the therapeutic target of AD since its discovery. Unlike the embryonic lethal phenotype of Presenilin $^{-/-}$ mice, BACE1$^{-/-}$ mice were viable and fertile (Luo et al., 2001, Roberds et al., 2001); however, knocking out BACE1 induces hypomyelination in both of the central and peripheral nervous systems (Willem et al., 2006, Hu et al., 2008). Apart from APP, BACE1 has other substrates with important functions, including Neuregulin1/3(Willem et al., 2006, Hu et al., 2008), sodium channel Nav1.1 b2 subunit (Wong et al., 2005, Kim et al., 2007), low density lipoprotein receptor related protein (LRP) (von Arnim et al., 2005), interleukin-1 receptor II (Kuhn et al., 2007) and APP-like proteins 1 and 2 (APLP1/APLP2) (Eggert et al., 2004, Li and Sudhof, 2004,

Pastorino et al., 2004). Aβ is produced under normal conditions at low amounts (Haass et al., 1992, Tamaoka et al., 1997), indicating a physiological role of Aβ in the central nervous system. Thereby, the application of BACE1 inhibitor to treat AD could have side effects, including hypomyelination, behavioral abnormalities and others. Another alternative for BACE1 manipulation is to regulate its preferential cleavage site. Since the Swedish APP mutation changes the major β-cleavage site in APP from Glu-11 to Asp-1 with predominant C99 fragment generation, it is possible to regulate the major cleavage site of BACE1. In chapter 2, we will determine the effect of a recessive APP mutation on the preferential cleavage of β-secretase and explore the underlying mechanism.

1.4 γ-secretase complex.

γ-secretase originally is known as the enzyme responsible for the last step of Aβ generation (Selkoe et al., 1996). γ-cleavage takes place within the hydrophobic transmembrane domain (TMD), and this process is termed regulated intramembrane proteolysis (RIP) (Brown et al., 2000). The first molecule, which was discovered to possess γ-secretase activity, was presenilin (PS1 and PS2), first cloned in 1995 through positional cloning strategies in FAD kindred (Levy-Lahad et al., 1995a, Rogaev et al., 1995, Sherrington et al., 1995). In 1998, De Strooper and colleagues discovered that PS1 knockout mice showed a markedly reduced activity in γ-secretase cleavage of APP (De Strooper et al., 1998) and parallel studies confirmed a complete elimination of γ-secretase activity in PS1/PS2 knockout cell (Herreman et al., 2000, Zhang et al., 2000a), establishing the γ-secretase activity of presenilins.

1.4.1 Presenilins.

Presenilins have two homologs, PS1 and PS2, with 67% identical sequences (Levy-Lahad et al., 1995a). mRNA of both presenilins is ubiquitously detected in many human and mouse tissues, including brain, heart, kidney and muscle (Lee et al., 1996). PS1 and PS2 are highly conserved and functionally redundant with SEL-12 as their homolog in *Caenorhabditis elegans* (Levitan and Greenwald, 1995).

PS1 is a multi-transmembrane protein with a nine-transmembrane topology (Fig.1.7) (Laudon et al., 2005, Spasic et al., 2006), and is abundantly present in the ER and trans-Golgi network (Walter et al., 1996, Culvenor et al., 1997, Annaert et al., 1999, Kim et al., 2000). Under physiological conditions, the majority of PS1 undergoes endoproteolysis within a large hydrophobic loop on the cytoplasmic side to generate a N-terminal fragment (NTF) and a C-terminal fragment (CTF) (Thinakaran et al., 1996a). The endoproteolytic cleavage takes place at heterogeneous sites from amino acids 292 to 299 (Podlisny et al., 1997, Shirotani et al., 1997, Jacobsen et al., 1999). While some studies reported that the cleavage is performed by an independent protease known as the "presenilinase" (Campbell et al., 2003, Nyabi et al., 2003), growing evidence supports the hypothesis that presenilin undergoes autoendoproteolysis (Wolfe et al., 1999b, Edbauer et al., 2003a, Xia, 2003, 2008, Ahn et al., 2010b, Fukumori et al., 2010). Endoproteolysis might be important to activate γ-secretase by converting it to a presenilin NTF/CTF heterodimer via removing the auto-inhibitory effect of the large hydrophobic loop (Knappenberger et al., 2004, Fukumori et al., 2010). However, it is not clear whether endoproteolysis is an absolute requirement for presenilins maturation since some presenilin mutants

were enzymatically active in the absence of endoproteolysis, as are the cases in FAD-associated PS1ΔE9 and PS2 M292D (Jacobsen et al., 1999, Steiner et al., 1999).

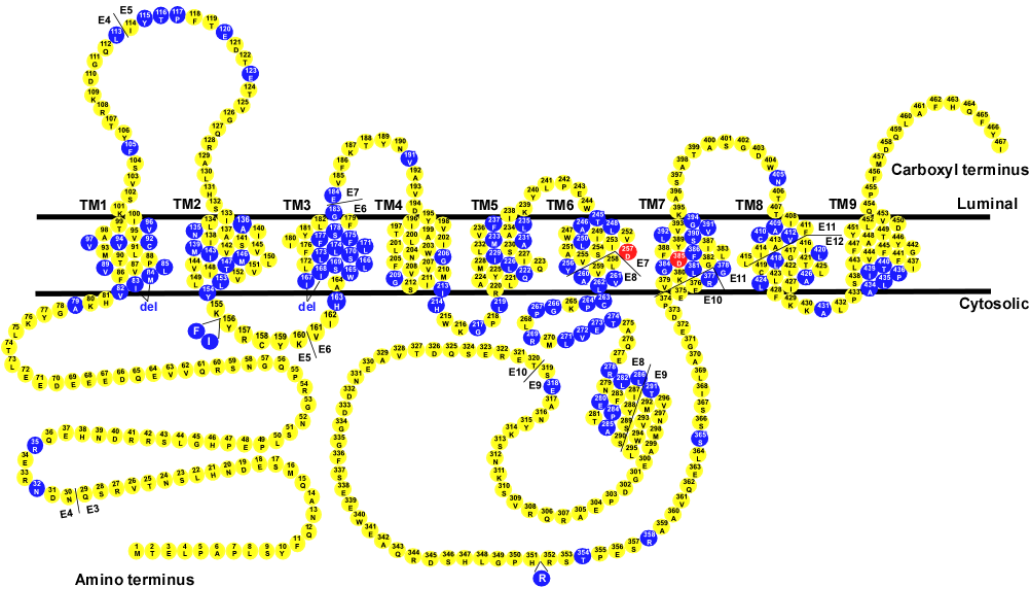


Figure 1.7 Presenilin 1 structure.
This diagram shows the amino acid sequence of PS1 and the distribution of FAD-associated mutations. Blue circles represent the FAD-associated mutations and red circles represent the two catalytic active aspartates.

The most outstanding function of presenilins is the processing of numerous type I transmembrane proteins within the intramembrane domain as a component of γ-secretase (Wakabayashi and De Strooper, 2008). Corresponding to its γ-secretase function, most FAD-associated PS mutations have been reported to result in increased level of the more hydrophobic Aβ species — Aβ 42 either in patients' plasma samples or the fibroblasts derived from FAD patients (Scheuner et al., 1996). Besides, substantiating evidence suggested that presenilins were involved in multiple functions independent of its γ-secretase

activity: presenilins were involved in β-catenin regulation via interacting with and stabilizing β-catenin (Zhang et al., 1998, Yu et al., 2000a, Meredith et al., 2002); presenilins regulated protein trafficking and turnover by targeting proteins such as β1-integrins (Zou et al., 2008), telencephalin (Esselens et al., 2004), epidermal growth factor receptor (Repetto et al., 2007), via defective protein-degradation organelles like endosomes or via the lysosome-autophagy pathway(Lee et al., 2010, Jang et al., 2011, Zhang et al., 2012); presenilins took part in calcium homeostasis of ER via debatable mechanisms such as regulating the ER calcium release channels like inositol 1,4,5-trisphosphate receptor (IP_3R) (Kasri et al., 2006, Cheung et al., 2008) and ryanodine receptor (RyR) (Chan et al., 2000), stimulating the sarcoendoplasmic reticulum calcium APTase (SERCA) and acting as ER low-conductance calcium leaking channels for ER to maintain the normal function of ER (Tu et al., 2006, Nelson et al., 2007); last but not least, presenilins were recently found to regulate neurotransmitter release via a proposed effect on calcium homeostasis (Zhang et al., 2009, Pratt et al., 2011).

1.4.2 γ-Secretase complex assembly.

γ-secretase is essential for the cleavage of APP C99 to generate Aβ (Scheuner et al., 1996). γ-secretase is a multi-unit enzymatic complex, including presenilin NTF/CTF heterodimer, nicastrin, Aph-1 and Pen-2 (Yu et al., 2000b, Francis et al., 2002b, Goutte et al., 2002b, Edbauer et al., 2003a, Kimberly et al., 2003a, Takasugi et al., 2003). Presenilin was the first molecule identified to be associated with γ-secretase *in vivo* and *in vitro*. PS1 knockout-out mice displayed a markedly reduced amount of γ-cleavage of APP (De Strooper et al., 1998) and double knockout of PS1 and PS2 in mice completely abolished γ-

secretase activity (Herreman et al., 2000, Zhang et al., 2000a). Using anti-PS antibody, Yu et al identified nicastrin, an integral transmembrane protein with a large N-terminal domain, as the second γ-secretase component (Yu et al., 2000b). However, the expression of both presenilins and nicastrin does not suffice to restore γ-secretase activity, indicating the existence of other components. Further gene-screening studies on the *glp*-1 (Notch homolog) deficient phenotype of *C.elegans* discovered Aph-1 and Pen-2 as the third and fourth components of γ-secretase (Francis et al., 2002a, Goutte et al., 2002a). Mammalian Aph-1 is a 30kDa multi-transmembrane protein similar to presenilin, whereas mammalian Pen-2 is a 12kDa hairpin-like transmembrane protein. Co-expression of presenilin, Aph-1, Pen-2 and nicastrin increases γ-secretase activity in transfected cells and the four proteins together are sufficient to reconstitute γ-secretase activity in yeast (Kimberly et al., 2003b, Luo et al., 2003).

Previous studies demonstrated that the minimal molecular weight of the γ-secretase complex was around 200-250 kDa, implying that a 1:1:1:1 stoichiometry of PS/Nicastrin/Aph-1/Pen-2 is present in the γ-secretase complex (Kimberly et al., 2003b). Though it is widely accepted that all four molecules form the minimal γ-secretase complex assembly, a recent report suggested that a PS/Pen-2/Aph-1 complex is sufficient for the catalytic activity in the absence of Nicastrin (Zhao et al., 2010). Another study demonstrated that PS1ΔE9 alone had partial γ-secretase activity and PS1ΔE9/Pen-2 was sufficient to restore full γ-secretase activity (Ahn et al., 2010a). These studies highlight the complexity of γ-secretase complex assembly. Given the stoichiometry of the

γ-secretase complex and the existence of PS and Aph-1 homologs, there are at least six different forms of the γ-secretase complex that could be assembled (Shirotani et al., 2004, Shirotani et al., 2007). PS1-involved complex or PS2-involved complex processed APP C99 differentially and showed distinct susceptibility to certain γ-secretase inhibitors (Mastrangelo et al., 2005, Bentahir et al., 2006), indicating that different γ-secretase complexes could possibly have very distinct functions.

1.4.3 Structure of γ-Secretase complex.

Presenilins form the catalytic core of the γ-secretase complex. Presenilins and signal peptides peptidases (SPPs), both belong to aspartyl intramembrane cleaving proteases (I-CLiPs) (Wolfe and Kopan, 2004). The two catalytic aspartate residues (Asp^{257} in transmembrane 6 (TM6) and Asp^{385} in TM7) are located at NTF and CTF of presenilins, respectively. Mutations in either two aspartates could abolish the enzymatic activity of the γ-secretase complex (Wolfe et al., 1999b). With a large and highly glycosylated ectodomain, nicastrin has been implicated as a site for the initial recognition of substrates (Shah et al., 2005). Electronic microscopic analysis and single particle imaging of γ-secretase revealed the existence of an intramembrane water-accessible cylindrical chamber in γ-secretase with a low-density cavity on the extracellular side (Lazarov et al., 2006, Osenkowski et al., 2009). Parallel substituted cysteine accessible method (SCAM) and cross-link experiments confirmed that TM6, TM7 and TM9 of PS formed the intramembrane chamber with two catalytic aspartates residing oppositely on TM6 and TM7, respectively (Fig.1.8) (Sato et al., 2006b, Tolia et al., 2006b, Sato et al., 2008b, Takagi et al., 2010, Watanabe et al., 2010). Constitutive autoendoproteolysis of PS removes the

inhibitory allosteric effect of the large hydrophobic loop in the catalytic chamber of PS, thereby eliminating the loop's inhibitory allosteric effect (Knappenberger et al., 2004, Fukumori et al., 2010). γ-secretase components directly interacted with one another (Kaether et al., 2004, Steiner et al., 2008), where Nct/Aph-1 subunits and Pen-2 tightened the relatively loose PS TM6/TM7/TM9 intramembrane cavity, and rearrange the PALP motif of TM9 such that it is closer to the catalytic center to render the enzyme activity to the γ-secretase (Takeo et al., 2012) (Fig.1.8).

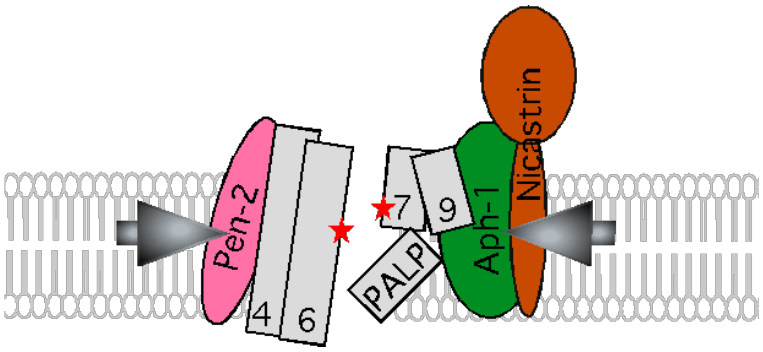


Figure 1.8 γ-secretase complex and formation of the catalytic pore of PS1.
PS1 TMDs were shown as columns with numbers. Without any subunits, PS forms a relatively open pore structure within the membrane. Upon the binding of other subunits, the catalytic structure is activated by the structural changes of PS TMDs, and the PALP motif moves to the proximity to the catalytic center. Two stars represent the two catalytic aspartate residues: Asp^{257} and Asp^{385}.

Recently, Shi and coworkers reported the crystal structure of a presenilin/SPP homologue (PSH) from the archaeon Methanoculleus marisnigri JR1 and predicted the structure of presenilin based on the conserved sequence between the two homologues (Li et al., 2013). They confirmed the existence of the water permissible cavity but also revealed some differences in TM7 and TM9 compared with the NMR structure of PS1 CTF. The elegant work by Shi and

his team has shed light on the elucidation of the crystal structure of presenilins.

1.4.4 APP and Notch as classical substrates.

γ-secretase preferentially processes the type I integral membrane protein after the shedding of the ectodomain (Struhl and Adachi, 2000). It is intriguing that many of γ-secretase's classical substrates also participate in multiple signaling pathways, such as cell adhesion and migration, neuritis outgrowth and synapse formation, and many of these events are often disrupted during AD pathogenesis (Bossy-Wetzel et al., 2004). Over 80 substrates have been linked to γ-secretase, including APP, Notch, neuregulin, ErbB4, E-cadherins and N-cadherins, CD44 and growth hormone receptor (Song et al., 1999b, Zhang et al., 2000b, Ni et al., 2001, Kim et al., 2002, Lammich et al., 2002, Marambaud et al., 2002, May et al., 2002, Marambaud et al., 2003, Haapasalo and Kovacs, 2011).

APP and Notch are the two most well known γ-secretase substrates. γ-secretase was named for its ability to process APP at the γ-cleavage site to generate Aβ, which is currently believed to play an essential role in the "amyloid cascades" during AD pathogenesis. Notch is a type I transmembrane cell surface receptor that mediates cell fate decisions in both vertebrates and invertebrates (Artavanis-Tsakonas et al., 1995, Kopan et al., 1996). After being cleaved by furin, cell surface Notch receptors bind to the DSL (Delta/Serrate/LAG-2) family ligands on the surface of neighboring cells, and the transmembrane-intracellular fragment of Notch undergoes further proteolysis to release the

Notch intracellular domain (NICD) from the membrane to the nucleus to activate target genes (Struhl et al., 1993, Kidd et al., 1998). Presenilin has been shown to play an important role in Notch signaling. PS-deficient mice with a disruption of PS genes exhibit Notch-knockout phenotype (Shen et al., 1997, Wong et al., 1997). Knocking-out of PS abolishes intramembrane γ-secretase cleavage of Notch to release NICD (De Strooper et al., 1999, Song et al., 1999a, Zhang et al., 2000a) and FAD-associated PS mutations impair NICD generation (Song et al., 1999a). Although impaired Notch-signaling is involved in synaptic plasticity and late-onset cognitive decline (Sestan et al., 1999, Presente et al., 2001, Presente et al., 2002, Wang et al., 2004, Salama-Cohen et al., 2006), the contribution of Notch signaling to AD pathogenesis remain to be elucidated.

1.4.5 γ-secretase cleavages at ε-site and γ-site.

γ-secretase can process substrates at multiple cleavage sites. γ-secretase cleaves the transmembrane domain of APP at two positions: the γ-site to generate Aβ and the downstream ε-site to produce the APP intracellular domain (AICD) (Weidemann et al., 2002) . Cleavage at the γ-site is heterogeneous, producing Aβ of 39-43 residues, whereas cutting at the ε-site produces almost exclusively a 50-residue AICD. The same phenomenon occurs in Notch processing, involving heterogeneous cleavages at the S4 site (γ-site) to generate Nβ, and homogeneous cleavage at the S3 site (ε-site) to generate NICD (Okochi et al., 2002). Recent studies supported that ε-cleavage occurs prior to γ-cleavage (Qi-Takahara et al., 2005a, Sato et al., 2005, Kakuda et al., 2006). Qi-Takahara and colleagues were the first to detect the Aβ49, the proteolytic counterpart to $AICD_{50-99}$ (Qi-Takahara et al., 2005a). Subsequently, the same group demonstrated that ε-cleavage occurs first to produce Aβ48 and Aβ49 for γ-

cleavage later, based on the presence of the induction period for the generation of tripeptides/tetrapeptides detected by liquid chromatography tandem mass spectrometry (LC-MS/MS) in a cell-free γ-secretase system (Takami et al., 2009). Meanwhile, various Aβ species (ranging from 49- to 40-amino acids) and corresponding tripeptides released from the trimming procedure have been identified using LC-MS/MS, confirming the sequential cleavage by γ-secretase at the ε-site then the γ-site (Funamoto et al., 2004, Qi-Takahara et al., 2005a, Takami et al., 2009).

1.4.6 γ-secretase inhibitors (GSIs) and modulators (GSMs).

As γ-secretase is the essential enzyme to generate Aβ, γ-secretase inhibitors has always been a popular research target for the potential treatment of AD. The development of γ-secretase inhibitor had started before the discovery of γ-secretase, and the first inhibitor turned out to be the aspartyl protease inhibitor (Higaki et al., 1995, Wolfe et al., 1998, Wolfe et al., 1999a). Since then several classes of γ-secretase inhibitors have been developed: the transition-stage analog inhibitor of aspartyl proteases with L-685,458 as representative (Li et al., 2000, Shearman et al., 2000); a substrate-based helical peptide GSI with D-peptide and L-peptide as representative (Das et al., 2003, Bihel et al., 2004); helical peptide type γ-secretase inhibitors with DAPT and Compound E as representatives (Seiffert et al., 2000, Dovey et al., 2001). The major challenge faced in the development of γ-secretase inhibitors is how to circumvent its inhibitory effect on the Notch signaling pathway. *In vivo* studies have demonstrated that GSIs lead to toxicity due to impairment in Notch signaling, including gastrointestinal bleeding and immunosuppression (Searfoss et al., 2003, Wong et al., 2004). Semigacestat (LY-450,139), which was a compound

modified from compound E, had failed in Phase III clinical trials because of causing severe gastrointestinal toxicity, immunnomodulation and skin cancer.

Thus, γ-secretase modulators (GSMs) have attracted interest due to its Notch-sparing ability. One typical category is a subset of NSAIDs (non-steroid anti-inflammatory drug), including ibuprofen, sulindac sulfide and indomethacin. They selectively lower Aβ42 without affecting the ε-cleavage, in other words, sparing the NICD generation (Weggen et al., 2001). However, not all NSAIDs could be pharmaceutically categorized as GSMs because their GSM functions do not appear to be relevant to cyclooxygenase property (Peretto et al., 2005) and the underlying mechanism remains unclear. Another category of potential GSMs, specifically regulating APP processing but not Notch (Gleeve), is ATP and other nucleotides and γ-secretase activating protein (GSAP) (Netzer et al., 2003, Fraering et al., 2005, He et al., 2010). These studies point to the possible existence of an allosteric site in γ-secretase that allows small molecules to regulate substrate selectivity. However, no γ-secretase inhibitors have been proven safe for clinical use so far. The essential issue is the lack of a precise structure of the γ-secretase complex and a precise working model that can determine substrate selectivity.

1.5 Familial Alzheimer's disease (FAD)-associated mutations.

Genetically, there are four known genes associated with AD pathogenesis: APP, PS1, PS2 and APOE. Apart from APOE, whose polymorphism (APOE ε4) is established as a genetic risk factor for sporadic late-onset AD, mutations in the

other three genes are linked to early-onset FAD with an autosomal-dominant inheritance pattern. Although more than 95% of AD cases are sporadic form, progress on AD etiology has mainly come from the genetic studies of FAD-associated mutations.

1.5.1 FAD-associated APP mutations.

APP was first linked to familial Alzheimer's disease (FAD) via Down Syndrome (DS), because APP was located in chromosome 21 and the genetic deficit of Down syndrome, also known as Trisomy 21, was due to the presence of an extra copy of chromosome 21(Glenner and Wong, 1984a). DS patients often develop characteristic AD symptoms and neuropathological features later in life. Although changes such as enhanced BACE1 maturation (Podlisny et al., 1987, Sun et al., 2006c) have been found in DS and may have contributed to amyloid pathogenesis in Down syndrome, the presence of an extra copy of APP gene *per se* may be the biggest risk factor for DS. Later studies verified that APP gene locus duplication and missense mutation resulted in an autosomal-dominant form of FAD (Podlisny et al., 1987, Goate et al., 1991, Rovelet-Lecrux et al., 2006b, Sleegers et al., 2006b). To date, there are more than 30 APP point mutations discovered on 17 amino acid sites (Fig 1.2). APP mutations promote AD pathogenesis via affecting APP processing or altering the aggregation property of Aβ (AD Mutations Database, Table 1.2).

Gene	Mutation Type	Mutation Locus	Mutation Effect	Examples
APP	Missense	Aβ sequence	↑Aβ aggregation	E693
APP	Missense	C-terminal of Aβ sequence	↑Aβ42/40	L717I
APP	Missense	N-terminal of Aβ sequence	↑C99 production	KM670/671NL
APP	Locus duplication	Whole gene	Dosage effect	

Table 1.2 Summary of FAD-associated mutations in APP gene.

The age of onset of FAD-associated APP mutations varies from early 40s to early 60s (Roks et al., 2000, Grabowski et al., 2001). It was reported that the polymorphism on the APOE gene contributed to the variation of age of onset in FAD (St George-Hyslop et al., 1994). Cerebral amyloid angiopathy and seizure are the two most varied features of FAD-associated APP mutations. The most remarkable clinical feature of APP mutations is the high incidence of cerebral amyloid angiopathy (CAA) (Van Broeckhoven et al., 1990, Rovelet-Lecrux et al., 2006a). CAA is often correlated with cerebral hemorrhage, stroke-like events, seizure and leukoencephalopathy, which are the cardinal clinical phenotypes of FAD caused by APP mutation (Van Broeckhoven et al., 1990, Grabowski et al., 2001, Sleegers et al., 2006a, Basun et al., 2008).

1.5.2 FAD-associated PS1 mutations.

Linkage studies indicated that FAD also segregated with chromosome 14 (Mullan et al., 1992b, Schellenberg et al., 1992, St George-Hyslop et al., 1992, Van Broeckhoven et al., 1992) and chromosome 1 (Levy-Lahad et al., 1995b). PS1 and PS2 are identified as the causative genes, which are located in chromosome 14 and chromosome 1, respectively (Levy-Lahad et al, 1995; Rogaev et al, 1995; Sherrington et al, 1995). Since then, more than 170 familial AD-related mutations in PS1 and 14 mutations in PS2 genes have been identified (AD Mutations Database).

FAD-associated presenilin mutations result in typical AD, where the clinical and pathological features are indistinguishable from the sporadic AD. The age of onset for FAD caused by PS1 mutations ranges from 24 to 65 years old, with an average age of onset of 41.7 years, about 10 years earlier than that by APP mutations (51.2 years) (Raux et al., 2005). The unique clinical and histopathological features of PS1 mutations include spastic pareparesis, myoclonus, extrapyramidal signs, Levy body and cotton wool plaques (CWP) (Kwok et al., 1997, Lopera et al., 1997, Crook et al., 1998a, Houlden et al., 2001b). CWP is described as large, non-cored, diffuse amyloid plaques, which is composed primarily of Aβ42 with a lack of surrounding neuritic dystrophy and glial activation (Tabira et al., 2002). CWP is often associated with spastic pareparesis (SP) (Karlstrom et al., 2008), both of which are reported in a subset of PS1 mutants like PS1 M233T, PS1 R278T and PS1 ΔE9 (Kwok et al., 1997, Crook et al., 1998b). The mechanism underlying these unique clinical and pathological phenotypes is unknown.

Increased Aβ42/40 ratio is a common feature in PS1 mutations but the underlying mechanism is equivocal. By both *in vitro* (Borchelt et al., 1996, Scheuner et al., 1996) and *in vivo* transgenic mice (Jankowsky et al., 2004a) assays, Aβ42 is found to be the preferred product of PS1 mutants. However, given the wide spread of these known mutations throughout the whole coding sequence of PS1 gene, how these mutations shifts the γ-cleavage site is still elusive.

1.6 Overall goal of this research

APP and PS mutations are helpful tools for researchers to elucidate the AD etiology. In sporadic AD, there are too many uncertainties that make the already intricate AD pathogenesis more complex, for instance, aging, hypoxia, inflammatory disease, hypertension, diabetes, cerebral vascular disease, etc.; whereas FAD, with early-onset, provides informative clues to disentangle the conundrum in specific scenarios, like FAD-associated mutations in APP or PS genes. The overall goal of my dissertation is to dissect two recently discovered mutations – APP_{A673V} and $PS1_{\Delta S169}$, by characterizing these two mutations and exploring how they affect Aβ generation and their contribution to cognitive impairment and memory deficit using transgenic mice models.

1.6.1 Examine the pathogenic effect of APP_{A673V} on AD pathogenesis.

In 2009, Di Fede and colleagues reported the first recessive FAD-associated APP mutation – APP_{A673V} (Di Fede et al., 2009), intensively challenging the hypothesis that all APP point mutations are inherited in an autosomal-dominant pattern. They demonstrated that the A673V mutation promoted Aβ generation

and $A\beta_{A673V}$ in the homozygous state was more amyloidogenic than its heterozygous state with $A\beta_{WT}$, both of which contributed to the recessive inheritance pattern in this APP-mutated FAD family. Considering that the A673V mutation site is only one amino acid away from the APP Swedish mutation (KM670/671NL) but has a distinct inheritance pattern, we are interested in how this A673V mutation would influence APP processing compared with Swedish mutation. ***We hypothesize that APP_{A673V} contributes to its recessive inheritance via affecting APP processing, especially at the β-secretase cleavage site.*** In my dissertation, we examine the effect of APP_{A673V} mutation on APP processing and Aβ production, as well as the underlying mechanisms. We find that APP_{A673V} contributed to AD pathogenesis via comprehensive effect on APP processing, especially the β-secretase and γ-secretase cleavages.

1.6.2 Study of the effects of $PS1_{\Delta S169}$ on APP and Notch processing.

Mutations in the PS1 gene are accounting for the majority of FAD cases. APP and Notch are the most important substrates of γ-secretase with PS as the catalytic subunit. APP is the precursor of Aβ, while Notch signaling is involved in fundamental neurodevelopment and neurodegeneration. Since most FAD-associated PS mutations impair both APP and Notch processing, the contribution of Notch signaling to AD pathogenesis has always been in debate. Most recently, a new PS1 deletion mutation of locus 169 serine was identified in a Chinese FAD family with typical AD symptoms developed in their early 40s (Guo et al., 2010a). In my dissertation, we thoroughly examine the effect of $PS1_{\Delta S169}$ on APP and Notch processing. ***We hypothesize that $PS1_{\Delta S169}$ specifically promotes indicative Aβ42/40 ratio via affecting the γ-cleavage of***

APP and retains its enzymatic activity on ε-cleavage for NICD (Notch Intracellular Domain) production. In chapter 3 of this thesis, the effect of $PS1_{\Delta S169}$ on APP and Notch processing will be explored *in vitro* under different scenarios.

1.6.3 Examining the effect of $PS1_{\Delta S169}$ on plaque formation and memory deficits *in vivo*.

Pathogenic PS1 mutations are not only affect APP processing by increasing Aβ42/40 ratio *in vitro* (Borchelt et al., 1996, Scheuner et al., 1996), but also advance neuritic plaque formation and increase the plaque load in bigenic APP/PS1 mice (Jankowsky et al., 2004a). We investigate the effect of $PS_{\Delta S169}$ on APP processing *in vitro* and determine the effect of $PS_{\Delta S169}$ on amyloid plaque formation and cognitive functions. ***We hypothesize that $PS1_{\Delta S169}$ could promote neuritic plaque formation, and progressively exacerbate cognitive impairment in APP23 mice***. In chapter 4 of this thesis, $APP23/PS1_{\Delta S169}$ transgenic mice will be studied histopathologically and behaviorally.

Chapter 2

APP A673V recessive mutation promotes Aβ generation by promoting β-cleavage at Asp-1 site and enhancing lysosomal degradation of APP

2.1 Introduction.

The mutation in the APP gene was the first genetic factor identified to cause autosomal dominant FAD (Goate et al., 1991). Recently, the first recessive APP mutation (A673V) has been reported in an Italian family, intensively challenging the conception that all APP mutations are inherited in an autosomal-dominant pattern (Di Fede et al., 2009). Genetic studies have revealed that both parental linkages of the homozygous proband were A673V heterozygous carriers. The heterozygous carriers with the A673V mutation did not have any AD symptoms between the ages of 21 and 88; whereas the homozygous proband developed dementia symptoms at the age of 36 and his homozygous sister also showed multi-domain mild cognitive impairment (MCI). The proband died at age 46 and the following neuropathological study detected both neuritic plaques and neurofibrillary tangles and confirmed the diagnosis of AD (Giaccone et al., 2010). The neuropathological study also revealed some distinct features of this APP recessive mutation. The diameter of neuritic plaque was up to 120uM with a high content of Aβ40 and cerebral amyloid angiopathy (CAA) was present and

striking. APP A673V mutation stood out from numerous other APP dominant mutations for its unique recessive inheritance pattern and distinct neuropathology.

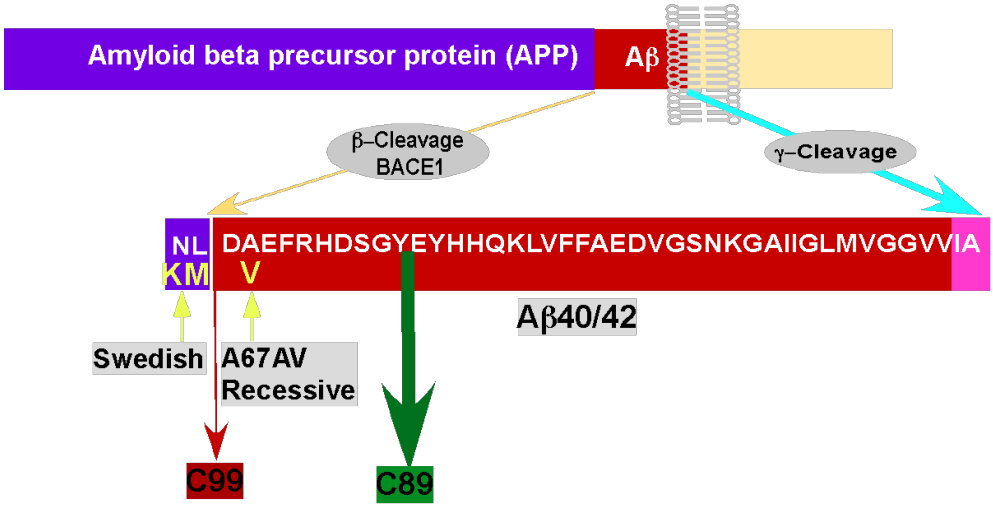


Figure 2.1 Schematic diagram of APP Swedish mutation and A673V mutation.
Red bar represents the Aβ region. Big green arrow represents the major β-cleavage at Asp-1 site (Aβ numbering). Small red arrow represents the minor β-cleavage at Glu-11 site. Swedish mutation (yellow letters) occurs just before the Asp-1 site of β-secretase but outside of the Aβ sequence. APP A673V mutation occurs at one amino acid after the Asp-1 site of β-secretase and within the Aβ sequence. The small peach bar represents the last two amino acids of Aβ42, indicating the heterogeneous γ-cleavages at the C-terminal of APP with Aβ40 or Aβ42 as alternative products.

APP Swedish mutation (KM670/671NL) is a well investigated and a typically dominant APP mutation with full penetrance in the heterozygous state (Mullan et al., 1992a, Felsenstein et al., 1994a). Swedish mutation has also been established as an example to show the effect of APP mutation on β-cleavage. Swedish mutation (KM670/671NL) contains two amino acids alteration juxtaposed to the BACE1 Asp-1 cleavage site, promoting BACE1 to process APP at this site to generate more APP C-terminal fragment 99 (C99), which is the immediate precursor of Aβ (Citron et al., 1992, Felsenstein et al., 1994b, Lo et al., 1994, Haass et al., 1995, Thinakaran et al., 1996b) (Fig 2.1). Given that a higher level of C99 is directly correlated with an increase in the more hydrophobic Aβ42

production (Yin et al., 2007), an up-regulation of C99 production in Swedish mutation provides for an abundance of precursors for Aβ42 generation. Transgenic mice expressing an APP Swedish mutation are widely used in AD research as animal models, e.g. APP23 (Tg.AD147.71H). APP23 mice specifically express human APP751 bearing Swedish mutation in neurons under the control of mouse Thy1.2 promoter and would develop amyloid plaques in the neocortex and hippocampus as early as 6 months old (Sun et al., 2006a).

APP_{A673V} mutation occurs one amino acid after the Asp-1 site of Aβ sequence (Fig 2.1). Although APP_{A673V} mutation and Swedish mutation (KM670/671NL) are very close to each other, being only one amino acid apart, they show distinct inheritance patterns. It is intriguing how this A673V mutation influences APP processing differently from the Swedish mutation. In this chapter, we examined the effect of APP_{A673V} mutation on APP processing and Aβ production and further explore the underlying mechanism. We found that APP_{A673V} contributed to AD pathogenesis by comprehensively influencing on the metabolism of APP.

2.2 Methods.

2.2.1 Materials.

Dulbeccoo's modified eagle medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, Penicillin-Streptomycin, geneticin, zeocin and lipofectamin 2000 were purchased from Life Sciences Technologies. Rabbit anti-C20 recognized the last twenty amino acids on the C-terminal end of APP were made in-house. β-actin was detected using monoclonal antibody AC-15 (Sigma). IRDye™ 680- labeled goat anti-rabbit and IRDye™ 800CM-labeled goat anti-mouse secondary antibodies were obtained from LI-COR Biosciences.

2.2.2 cDNA constructs.

With pcDNA4-APP695$_{WT}$ (wild type) as template, pcDNA4-APP$_{A673V}$ was generated via site-directed mutagenesis. Using customary T7 (Invitrogen) as forward primer and a designed primer containing a A673V mutation (5'-TCGGAATTCTACATCCATCTTCAC) as reverse primer, an APP fragment containing A673V mutation was generated and cloned back between the HindIII and EcoRI sites in pcDNA4-APP$_{WT}$. To generate inducible plasmids pIND-APP$_{WT}$, pIND-APP$_{A673V}$ and pIND-APP$_{SWE}$, full length APP variants were cut from pcDNA4-APP$_{WT}$, pcDNA4-APP$_{A673V}$ and pcDNA4-APP$_{SWE}$ with HindIII and XbaI and cloned into pIND (SP1)/Hygro mammalian expression vector (Invitrogen) between the HindIII and XbaI sites, respectively. Plasmid pVgRXR expresses the heterodimeric ecdysone receptor (VgEcR) and the retinoid X receptor (RXR) (Invitrogen). It is documented that KK sequence in extreme C-terminal tail can act as endoplasmic reticulum (ER) retention signal in transmembrane protein (Nilsson et al., 1989). APP$_{WT-ER}$ and APP$_{A673V-ER}$ were generated with pcDNA4 forward primer T7 and a reverse primer coding KKQN instead of QMQN in the C-terminal tail of APP (5'-GCCTCTAGACTAGTTCTGCTTCTTCTCAAAGAACTTGTAGGTTGG), with pcDNA4-APP$_{WT}$ and pcDNA4-APP$_{A673V}$ as template, respectively. APP$_{WT/F615P}$ and APP$_{A673V/F615P}$ were generated with a pair of primers containing F615P mutation (5'-GTTCATCATCAAAAATTGGTGCCCTTTGCAGAAGATGTGGGTTC and 5'-GAACCCACATCTTCTGCAAAGGGCACCAATTTTTGATGATGAAC), with pcDNA4-APP$_{WT}$ and pcDNA4-APP$_{A673V}$ as template, respectively. The N-terminal 19 peptides (MLPGLALLLLAAWTARALE) of APP serve as signal sequence. With pcDNA4-APP695$_{WT}$ as template, a fragment containing the signal peptide was amplified with forward T7 primer and reverse primer coding the C-terminal sequence of signal peptide and the N-terminal sequence of C99 (5'- GTCGGAATTCTGCATCCTCCAGCGCCCGAGCCGTCC). The amplified

fragment was cloned back between the HindIII and EcoRI sites in pcDNA4-$C99_{WT}$ or pcDNA4-$C99_{A2V}$ to generate $C99_{Sig\text{-}WT}$ and $C99_{Sig\text{-}A2V}$, respectively.

2.2.3 Cell lines, cell culture and transfection.

HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2 mM L-glutamine, 50U/mL penicillin G sodium and 50μg/mL streptomycin sulfate (Invitrogen). NN cells are $PS1^{-/-}/PS2^{-/-}$ mouse embryonic fibroblast (MEF) and N2A cells are mouse neuroblastoma cell line. Stable cell lines were maintained in media containing zeocin (50μg/mL). All cells were maintained at 37°C in an incubator containing 5% CO2. For transfection, cells were grown to 70% confluence and transfected with 2μg plasmid DNA/35 mm plate using 4*u*L of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

2.2.4 APP-inducible expression and Ponasterone A treatment.

To establish APP-inducible cell lines using the ecdysone-inducible mammalian expression system, the cDNA of human APP_{WT}, APP_{A673V} or $APP_{SWE,}$ were cloned into an inducible vector pIND (SP1)/Hygro to generate pIND-$APP_{WT,}$ pIND-APP_{SWE} and pIND-APP_{A673V}, respectively. pIND (SP1)/Hygro contains modified ecdysone response elements and SP1 enhancers, and activation of the APP gene transcription is dependent on the binding of a heterodimer of VgEcR and RXR receptors in the presence of a ligand such as ecdysone analog ponasterone A. HEK293 cells were transfected with pVgRXR and pIND-APP then treated with inducer Ponasterone A or vehicle control. Under different dosages of Ponasterone A treatment, the full length of APP variants could be expressed at different levels under control in HEK293 cells. The basal levels of the APP holo-protein were very low in the absence of Ponasterone A. Ponasterone

A, an ecdysone analog, was obtained from Invitrogen and dissolved in ethanol. HEK293 cells with transient overexpression of APP variants were treated with inducer Ponasterone A to induce APP expression at 0, 0.5μM, 1.0μM, or 3.0μM for 24 h.

2.2.5 Immunoblot analysis.

Cells were lysed in RIPA lysis buffer (1% Triton X100, 1% sodium deoxycholate, 4% SDS, 0.15M NaCl, 0.05M Tris-HCl, pH 7.2) supplemented with 200 mM sodium orthovanadate, 25 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride (PMSF), and a complete mini protease inhibitor cocktail tablet (Roche Diagnostics). The samples were diluted in 4×SDS-sample buffer, boiled, resolved by SDS-PAGE on 8% tris-glycine or 16% tris-tricine gels, then transferred to Immobilon^{TM}–FL phlyvnylidene fluoride (PVDF) membranes (Millipore). For immunoblot analysis, membranes were blocked for 1h in phosphate-buffered saline (PBS) containing 5% non-fat dried milk followed by overnight incubation at 4°C in primary antibodies diluted in the blocking medium. Rabbit antibody C20 (1:2000) was used to detect APP and its C-terminal fragment (CTF) products. Internal control β-actin was detected using monoclonal antibody AC-15 (Sigma). The membranes were rinsed in PBS with 0.1% Tween-20 and incubated with IRDye 800CW-labelled goat anti-mouse or anti-rabbit antibodies in PBS with 0.1% Tween-20 at 22°C for 1 h, and visualized on the Odyssey system (LI-COR Biosciences). All quantification was performed using LI-COR Odyssey system and Image J.

2.2.6 Aβ40/42 enzyme linked-immunosorbent assay (ELISA).

HEK293 cells expressing the human wild type APP, Swedish mutant and A673V mutant were maintained in cell culture media supplemented with 5% FBS. After 24h, conditioned medium was collected and protease inhibitors and AEBSF

(ROCHE Diagnostics) were added to prevent degradation of Aβ peptides. The concentration of Aβ40 and Aβ42 were detected using β-amyloid 1-40 or β-amyloid 1-42 Colorimetric ELISA kit (Invitrogen) according to manufacturer's instructions.

2.2.7 L-685,458, Monensin, cycloheximide (CHX), chloroquine (CHL) and MG132 treatment.

L-685,458 (Sigma) is a potent, structurally novel γ-secretase inhibitor, equipotent inhibitor of both Aβ40 and Aβ42 production. L-685,458 was dissolved in DMSO and applied to cell culture medium at 1μM final concentration for 3h. Monensin (Sigma) is a drug known to block the transport from the medial to the trans cisternae of the Golgi stacks. To block APP in TGN, HEK293 were treated with monensin at 1μM overnight. Cycloheximide (CHX, from Sigma) is an antibiotic produced by *S. griseus*. Its main biological activity is translation inhibition in eukaryotes, resulting in cell growth arrest and cell death. To determine the APP degradation rate, HEK293 were treated with CHX at 100μg/ml for different time course. Chloroquine (CHL, from Sigma) is lysosomal degradation inhibitor and MG132 (from Millipore) is a potent, reversible, and cell-permeable proteasome inhibitor. To determine the effect of APP mutations on APP degradation, HEK293 were treated with CHL at 10μM and MG132 at 1μM overnight, respectively.

2.2.8 Primary neuronal culture, Aβ toxicity treatment and LDH assay.

Hippocampal and neocortical tissues for primary cultures originating from C57BL/6J mice embryos at 14 days of gestation were dissected and gently digested with trypsin (0.025% EDTA; Invitrogen). The cells were suspended in neurobasal medium supplemented with B27 (Invitrogen) and plated at a density of $1–2 \times 10^5$ cells/well onto poly-D-lysine (0.01 mg/ml; Sigma)-coated 24-well plates. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and used for experiments after 10 days. $A\beta42_{WT}$ and $A\beta42_{A2V}$

are synthesized in EZBiolab company. $A\beta42_{WT}$ and $A\beta42_{A2V}$ were prepared in 37°C water bath for 96 hours for aging before treatment. Lactate dehydrogenase (LDH) was used to evaluate the viability of the cells according to the instructions (Sigma). Trypan blue solution was used to stain the dead cells according to the instructions (Sigma).

2.3 Results.

2.3.1 APP_{A673V} recessive mutation moderately increased Aβ generation.

We introduced the A673V mutation into human wild type APP695 isoform by site directed mutagenesis and expressed wild type or mutant APP in transiently transfected HEK293 cells. The expression levels of wild type and mutant full length APP were determined by Western blotting, and HEK293 cells expressing similar amounts of APP were subjected to further analysis. Conditioned media were analyzed using Aβ ELISA assay. Compared to APP_{WT}, APP_{A673V} and APP_{SWE} increased the C99 levels to 155.6%±8.9% and 244.2%±22.8%, respectively ($p<0.05$) (Fig 2.2 A, B). The following Aβ ELISA assay demonstrated that APP_{A673V} and APP_{SWE} increased the Aβ40 levels to 4.48±0.03 folds and 34.8±9.5 folds as compared with APP_{WT}, respectively ($p<0.05$) (Fig 2.2 C). Compared to APP_{SWE}, the lower levels of C99 and Aβ in APP_{A673V} suggested that APP_{A673V} was not as malignant as APP_{SWE}; however, on the other hand, APP_{A673V} did increase C99 and Aβ levels compared to APP_{WT}, indicating the amyloidogenic property of $APP_{A673V.}$ It seems like that Aβ has to be high enough to meet the threshold in order to trigger AD pathogenesis. In the heterozygous state with only one copy of A673V, there is not sufficient Aβ to initiate AD pathogenesis, which accounts for the recessive inheritance pattern of this mutation.

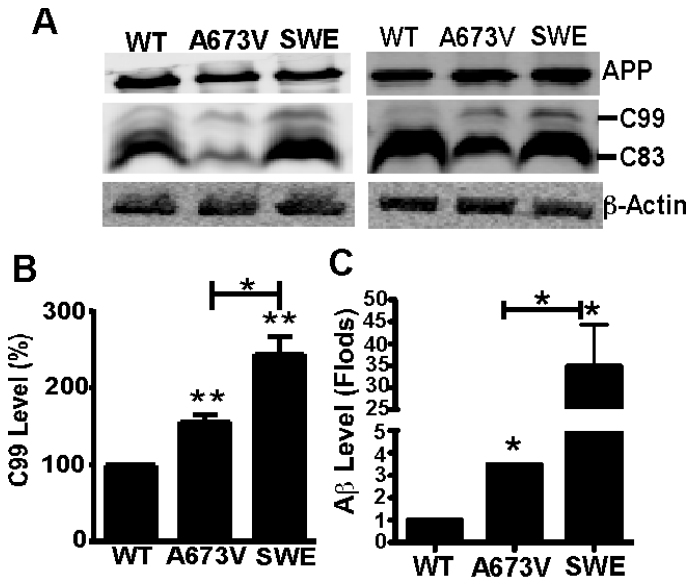


Figure 2.2 Effects of APP $_{A763V}$ mutation on APP processing and Aβ generation.
(A) APP_{WT}, APP_{A673V} or APP_{SWE} were introduced into HEK293; the holo APP protein and its CTFs in cell lysate were detected by rabbit polyclonal antibody C20 that recognized the last 20 residues of APP C-terminals. (B) The levels of C99 were quantified and normalized to that in APP_{WT}. (C) Aβ ELISA (Invitrogen) assay was performed to measure Aβ40 levels in the conditioned cell culture medium. The levels of Aβ40 in APP_{A673V} and APP_{SWE} were normalized to that in APP_{WT}. Values represent mean±SEM, n=6, *p<0.05, **p<0.01 by one-way ANOVA with post-hoc tests.

2.3.2 APP_{A673V} recessive mutation promoted C99 production by shifting the major β-secretase site from Glu-11 to Asp-1 site.

β-secretase cleaved APP at two sites — Asp-1 and Glu-11 — to generate the corresponding products: sAPPβ^{606} (numbered according to APP695 isoform) and C89 at Glu-11 site and sAPPβ^{596} and C99 at Asp-1 site (Vassar et al., 1999, Deng et al., 2013). In HEK293 cells with low BACE1 activity, the majority of APP undergoes α-secretase pathway with C83 being predominant from of APP CTFs; in 293B2 cells that stably overexpress BACE1, the majority of APP undergoes β-secretase pathway with C89 and C99 being predominant forms of APP CTFs, which makes the effect of APP mutants on β-cleavage easier to be detected. Whether C89 or C99 being the predominant form is dependent on different APP

mutants. In wild type APP, the predominant β-secretase product is C89, whereas in the Swedish APP the predominant β-secretase product is C99 (Fig 2.3 A, lane 1 and 3) (Deng et al., 2013). Since APP_{A673V} mutation is close to the β−secretase Asp-1 site (APP D672) like the Swedish mutation (KM670/671NL, APP_{SWE}), we investigated the effect of APP_{A673V} mutation on the β-secretase cleavage of APP and compared it with APP_{WT} and APP_{SWE}. To determine the predominant β-secretase product in different APP variants, we transiently expressed $APP_{WT,}$ APP_{A673V} and APP_{SWE} in 293B2 cells (Fig 2.3 A). In 293B2 cells with overexpression of BACE1, the predominant APP CTFs were β-secretase product — C99 or C89 — in all $APP_{WT,}$ APP_{A673V} and APP_{SWE}, not C83 (Fig 2.3 A, D). Compared to C99 levels in APP_{WT}, APP_{A673V} and APP_{SWE} increased the levels of C99 to 2.37±0.39 and 2.04±0.51 folds, respectively (p<0.05). The levels of C89 in APP_{WT}, APP_{A673V} and APP_{SWE} were 2.23±0.44, 0.71±0.11 and 1.41±0.32 folds, as compared with the C99 levels in APP_{WT}, respectively (p<0.05); and the levels of C83 in APP_{WT}, APP_{A673V} and APP_{SWE} were 1.39±0.17, 0.47±0.11 and 0.94±0.20 folds, as compared with the C99 levels in APP_{WT}, respectively (p<0.05) (Fig 2.3 D). It is noteworthy that APP_{A673V} did not shed more C99 than APP_{SWE} in the presence of BACE1 overexpression, which we will discuss in detail in Discussion section. To determine the shifting effect of APP mutants on β-cleavage, we specifically evaluated the C99/C89 ratio in different APP variants. The C99/C89 in both APP_{A673V} and APP_{SWE} were normalized to that in APP_{WT}. APP_{A673V} significantly increased C99/C89 to 7.51±0.17 folds whereas APP_{SWE} increased C99/C89 to 3.15±0.06 folds (p<0.05), indicating that APP_{A673V} had stronger effect on the preferential cleavage of β-secretase at Asp-1 site (Fig 2.3 E).

293B2 cells are stably overexpressing BACE1 with high β-secretase activity. We next investigated whether the effect of A673V mutation on β-cleavage was dependent of β-secretase activity. We expressed $APP_{WT,}$ APP_{A673V} and APP_{SWE} in

transiently transfected HEK293 cells to investigate the effect of A673V mutation on β-cleavage under conditions with much lower β-secretase activity. In HEK293 cells, the predominant APP CTFs were α-secretase product — C83 — in all $APP_{WT,}$ APP_{A673V} and APP_{SWE} (Fig 2.3 B, F). Compared to C99 levels in APP_{WT}, APP_{A673V} and APP_{SWE} increased the levels of C99 to 1.55±0.39 and 2.37±0.71 folds, respectively (p<0.05) (Fig 2.3 F). The C99/C89 in both APP_{A673V} and APP_{SWE} were normalized to that in APP_{WT}. APP_{A673V} significantly increased C99/C89 to 6.15±0.55 folds whereas APP_{SWE} increased C99/C89 to 2.02±0.09 folds (p<0.05) (Fig 2.3 G). These data indicated that the effect of A673V mutation on CTFβ production is independent of β-secretase activity.

APP CTFs can be further processed by γ-secretase. To eliminate the effect of γ-secretase, we expressed $APP_{WT,}$ APP_{A673V} and APP_{SWE} in transiently transfected NN cells, which are $PS1^{-/-}PS2^{-/-}$ mouse embryonic fibroblast cells (Fig 2.3 C, H). Without the effect of γ-secretase, APP_{A673V} consistently increased C99/C89 to 4.38±0.75 folds whereas APP_{SWE} increased C99/C89 to 2.15±0.58 folds, with normalization to that in APP_{WT} (p<0.05) (Fig 2.3 I). Taken together, the A673V mutation demonstrated stronger effect than Swedish mutation to shift major β-secretase cleavage site from Glu-11 to Asp-1 site independent of the β-secretase activity and γ-secretase cleavage. It is noteworthy that APP_{A673V} generated significantly less α-secretase product C83, compared with wild type APP and Swedish APP in all three 293B2, HEK293 and NN cells (Fig 2.3 D, E, F), which we will discuss in detail in discussion section.

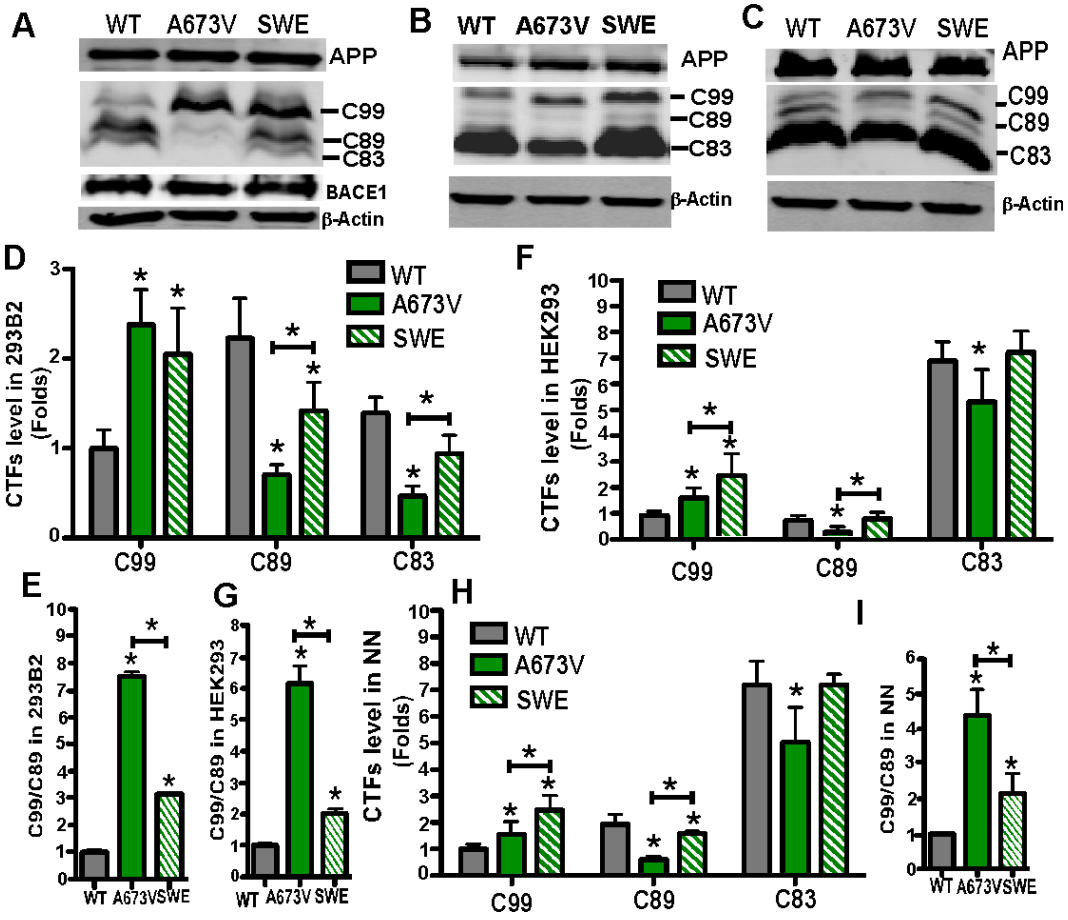


Figure 2.3 APP_{A673V} shifted the major β-secretase product from C89 to C99 in 293B2 cells with BACE1 overexpression.
(A) 293B2 cells were transiently transfected with pcDNA4-$APP_{WT,}$ pcDNA4-APP_{A673V} or pcDNA4-APP_{SWE}. The holo APP and CTFs in cell lysate were detected by C20 antibody. (D, E) Quantification of (A) by Image J and C83, C89 and C99 levels were normalized to C99 levels in APP_{WT}; C99/C89 ratio was normalized with that in APP_{WT}. (B) HEK293 cells were transiently transfected with pcDNA4-APP_{WT}, pcDNA4-APP_{A673V} or pcDNA4-APP_{SWE}. (F, G) Quantification of (B) by Image J and C83, C89 and C99 levels were normalized to C99 levels in APP_{WT}; C99/C89 ratio was normalized with that in APP_{WT}. (C) NN ($PS1^{-/-}PS2^{-/-}$) cells were transiently transfected with pcDNA4-APP_{WT}, pcDNA4-APP_{A673V} or pcDNA4-APP_{SWE}. (H, I) Quantification of (C). C83, C89 and C99 levels were normalized to C99 level in APP_{WT}; C99/C89 ratio was normalized with that in APP_{WT}. Values represent mean±SEM, n=6, *p<0.05, by one-way ANOVA with post-hoc tests.

2.3.3 APP_{A673V} recessive mutation modulated γ-secretase activity.

The recessive A673V mutation is located within the Aβ region (A2V) (Fig 2.1). Therefore, C99 derived from APP_{A673V} is designated as $C99_{A2V}$ (numbered

according to the Aβ sequence). It is well established that APP mutations close to active sites of γ-cleavage in the C-terminal of the Aβ region can modulate γ-cleavage. However, the 'Flemish' mutation (A692G), which is located in the middle of Aβ, has an unexpected effect on γ-cleavage (Tian et al., 2010), suggesting that the interaction between γ-secretase and C99 may also occur at sites far from the active sites of γ-secretase. To investigate the effect of A673V mutation on γ-cleavage, $C99_{A2V}$ and $C99_{WT}$ were each overexpressed in HEK293 cells and the cells were treated with the γ-secretase inhibitor L-685, 458 (Sigma) or a vehicle control (see Methods). C99 variants and their proteolytic product C83 by endogenous α-secretase were detected by Western blotting with C20 antibody. As expected, the pharmaceutical blocking of γ-secretase activity resulted in a significant increase of C99 in both $C99_{WT}$ and $C99_{A2V}$, but the increase in $C99_{A2V}$ (171.8%±12.8%) was significantly less than $C99_{WT}$ (303.6%±31.7%) ($p<0.05$; Fig 2.4 A, B). Meanwhile, Aβ ELISA assay was performed to determine any secreted Aβ production in cell culture medium. Consistent with the increase of C99 levels, the Aβ40 levels in $C99_{A2V}$ was only 36.0%±3.5% of that in $C99_{WT}$ ($p<0.05$; Fig 2.4 C).

APP is a type I transmembrane protein with a signal peptide and both APP and C99 have the transmembrane domain. To ensure the correct insertion of C99 into the membrane of the endoplasmic reticulum and the similar secretary pathway like endogenous C99, the signal peptide of APP was fused to the N-terminus of $C99_{WT}$ and $C99_{A2V}$ ($C99_{Sig\text{-}WT}$ and $C99_{Sig\text{-}A2V}$), which was cleaved during membrane insertion by signal peptidase (Dyrks et al., 1992, Dyrks et al., 1993, Lichtenthaler et al., 1999). Our preliminary data indicated that, with the signal peptide, the expression levels of $C99_{Sig}$ were greatly increased, likely due to the reduced degradation of incorrectly inserted proportion of C99 (data not shown). Thus, $C99_{Sig}$ was transiently transfected into HEK293 cells in the reduced amount

of plasmid DNA (0.1 and 0.25μg), not the routine 2μg plasmid DNA/35 mm plate. Consistent with result in C99 without signal peptide, inhibition of γ-secretase greatly increased the accumulation of both $C99_{Sig\text{-}WT}$ and $C99_{Sig\text{-}A2V}$ in cell lysate, and the increase in $C99_{Sig\text{-}A2V}$ (129.3%±19.3%) was still significantly less than $C99_{Sig\text{-}WT}$ (285.4%±36.9%) ($p<0.05$; Fig 2.4 D, E). These data demonstrated that both $C99_{A2V}$ and $C99_{Sig\text{-}A2V}$ are less efficiently processed by γ-secretase.

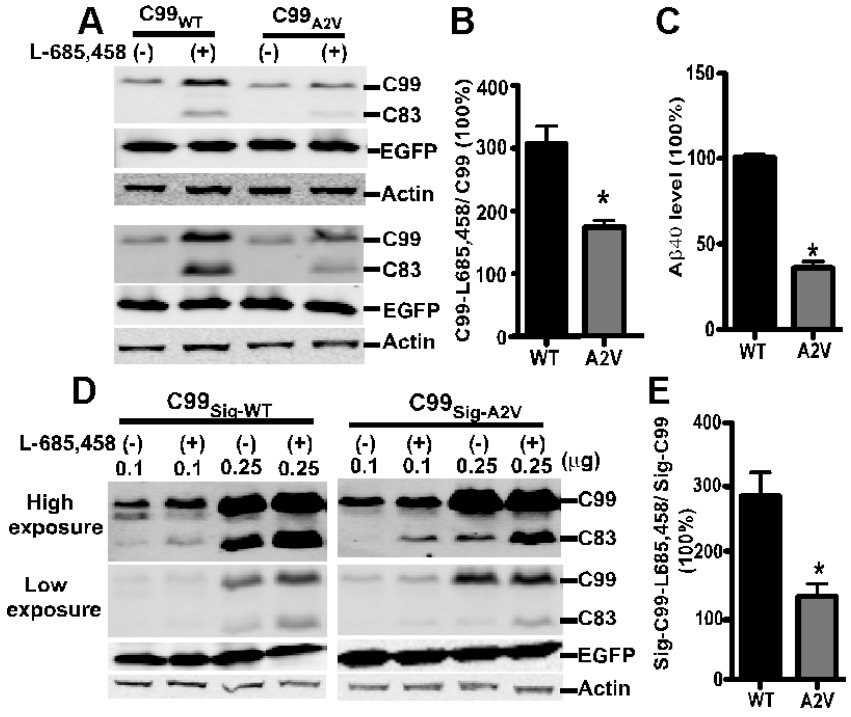


Figure 2.4 Effects of A673V mutant C99 ($C99_{A2V}$) on γ-secretase activity.
(A) $C99_{WT}$ or $C99_{A2V}$ were co-transfected with EGFP into HEK293 with treatment of γ-secretase inhibitor L-685,458 (Sigma) or vehicle control. EGFP was used as internal control to ensure the same transfection efficiency, which was detected via anti-GFP antibody. β-Actin was used as internal control to ensure the same amount of cell lysate applied for analysis. Cell lysate was harvested to determine the accumulation of C99 via Western blotting. Because C99 was substrate of γ-secretase as well as α-secretase (Lichtenthaler et al., 1997, Lichtenthaler et al., 1999), both C99 and C83 can be detected in cell lysate by C20 antibody. (B) The increased folds of C99 levels after L-685,458 treatment in (A) were quantified and compared between $C99_{WT}$ and $C99_{A2V}$. (C) Conditioned cell culture medium was collected to determine the amount of Aβ40 in HEK293 with $C99_{WT}$ and $C99_{A2V}$ overexpression via Aβ ELISA assay (Invitrogen). The amount of Aβ40 in

$C99_{A2V}$ was normalized to that in $C99_{WT.}$ (D) Different amount of $C99_{Sig\text{-}WT}$ and $C99_{Sig\text{-}A2V}$ were co-transfected with equal amount of EGFP into HEK293 with treatment of γ-secretase inhibitor L-685,458 or vehicle control. EGFP and β-Actin were used as internal control to ensure the same transfection efficiency and the same amount of cell lysate in analysis, respectively. (E) The increased folds of $C99_{Sig}$ levels after L-685,458 treatment in (A) were quantified and compared between $C99_{Sig\text{-}WT}$ and $C99_{Sig\text{-}A2V}$. Values represent mean±SEM, n=3, *p<0.05, by student t-test. A

2.3.4 APP_{A673V} demonstrated a higher C99/C89 ratio than Swedish APP independently of its expression level.

APP_{A673V} is a recessive mutation that requires two mutated alleles to initiate AD pathogenesis, whereas in the case of Swedish APP one allele is sufficient to lead to AD when patients are in their early 50s. There is a possibility that the expression level of APP_{A673V} could affect β-secretase processing. We found that APP_{A673V} robustly shifted the major β-cleavage site, resulting in a high C99/C89 ratio. To investigate whether different APP_{A673V} expression levels could affect the β-secretase processing, we used an inducible APP expression system under the control of ponasterone A to achieve the expression of APP expression at different amounts. To assess inducible APP processing, APP_{WT}, APP_{A673V} or APP_{SWE} were introduced to 293B2 cells that strongly express BACE1, and the expression of APP variants was triggered by ponasterone A at 0.5, 1.0, or 3.0 μM for 24 hours. Ponasterone A, an ecdysone analog, would bind to the ecdysone receptor and activate APP gene transcription. While holo APP expression was barely detectable in 293B2cells treated with the vehicle control, ponasterone A treatment resulted in a linear increase of holo APP expression in a similar way among $APP_{WT,}$ APP_{A673V} and APP_{SWE} (p>0.05) (Fig 2.5 A, B, C, D). Ponasterone A treatment had no effect on β-actin protein levels, indicating a specific effect of Ponasterone A on inducible expression of APP. After 24h of ponasterone A treatment, cell lysates were examined by Western blotting to determine the amount of APP CTFs by C20 antibody. Despite of the increasing holo-APP and total CTFs corresponding to the increasing concentration of ponasterone A (Fig

2.5 A, B, C), APP_{A673V} consistently displayed the highest C99/C89 ratio (3.41±0.33) as compared to APP_{WT} (0.45±0.03) and APP_{SWE} (1.42±0.09) (p<0.05) (Fig 2.5 E), indicating that the effect of A673V mutation on β-cleavage shift from Glu-11 to Asp-1 was independent of the expression levels of APP.

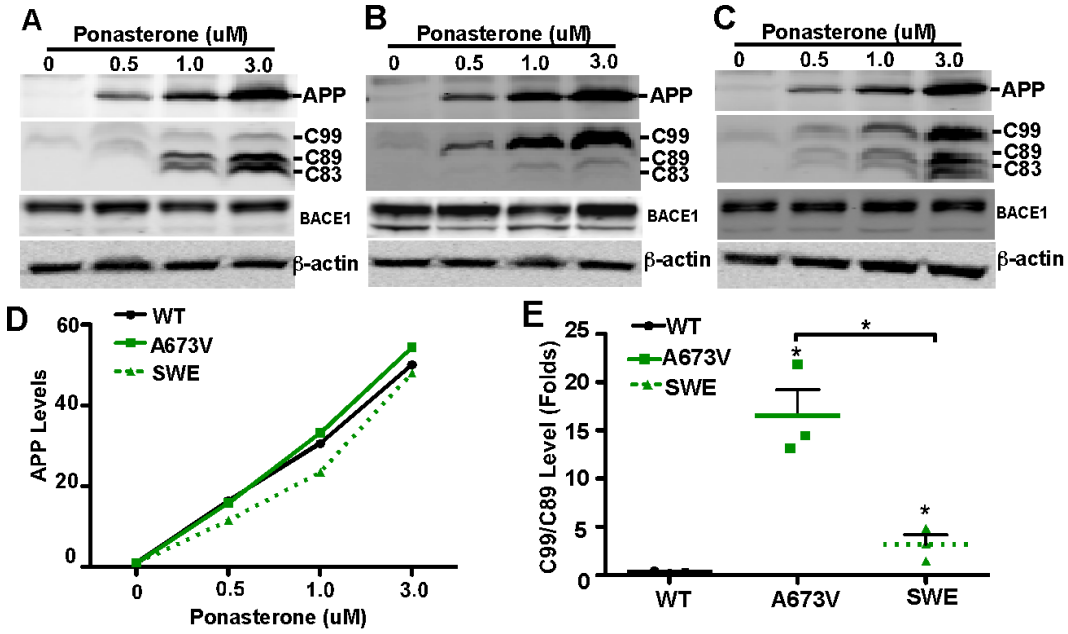


Figure 2.5 Dose-dependent inducible expression of APP variants in 293B2 cells.
293B2 cells were transfected with pIND-APP_{WT} (A), pIND-APP_{A673V} (B) or pIND-APP_{SWE} (C), respectively, then treated with vehicle solution control or ponasterone A at 0, 0.5, 1.0 or 3.0 μM for 24h. Cells were collected at the same endpoint and were lysed in RIPA-DOC buffer. Cell lysates were analyzed by 9% Tris-glycine to detect for full length APP or 16%Tris-tricine to detect for APP CTFs by C20 antibody. BACE1 was detected by 9E10 that would recognize the MycHis tag. Monoclonal anti-β-actin antibody (AC-15) was used to detect β-actin. (D) Full length APP expression levels were quantified and plotted to represent Ponasterone A dosage-dependent expression of APP (Quantification by Image J). (E) C99/C89 ratio under different APP expression levels was quantified and plotted (Quantification by Image J). Values represent mean±SEM, n=3, *p<0.05, by two-way ANOVA and one-way ANOVA with post-hoc tests. All experiments were at least triplicated.

2.3.5 APP_{A673V} structurally facilitated β-secretase cleavage at Asp-1 site in both the endoplasmic reticulum and trans Golgi network.

APP is preliminarily glycosylated in the endoplasmic reticulum (ER) upon synthesis then anterograde transported to the Golgi apparatus for further modification such as sulfation and phosphorylation. A proportion of the fully modified APP (or mature APP, mAPP) is subsequently delivered to the cell surface where the α-cleavages occur (Parvathy et al., 1999); whereas β-secretase mediated cleavages appear to occur in the trans Golgi network or in the endosomal/lysosomal system following the endocytosis of APP from the plasma membrane (Koo and Squazzo, 1994, Tienari et al., 1997). Therefore, it is conceivable that BACE1 mostly, if not exclusively, cleaves mAPP; and nascent APP or immature APP (imAPP) is not a substrate of BACE1. According to this hypothesis, APP with an ER retention signal fused to the COOH-terminus (APP_{ER}) cannot be processed by BACE1; in other words, APP_{ER} does not produce C89 or C99. However, we found that both APP_{A673V} and APP_{SWE} with the ER retention signal can be cleaved into C99, whereas APP_{WT} with the ER retention signal cannot (Fig 2.6 A). Given that all APP variants are synthesized in the ER, imAPP in the ER represents the default forms and structures of APP proteins. Our results indicated that both APP_{A673V} and APP_{SWE} are structurally favored by BACE1 cleavage at the Asp-1 site. We also compared the processing of APP_{WT} and APP_{A673V} in trans Golgi network (TGN) with monensin treatment (see Methods). When the downstream metabolism of APP was blocked and trapped in TGN, APP_{A673V} markedly increased C99 production whereas the C99 in APP_{WT} was barely detected under low exposure conditions (Fig 2.6 B). When APP is trapped in specific organelles such as ER and TGN, the effect of altered trafficking of APP mutations on APP processing is excluded and the abnormal APP processing results from the altered structure imposed by the mutations. The enhanced production of C99 in APP_{A673V} in both ER and TGN implicated that

APP_{A673V} structurally, highly facilitated β-cleavage at Asp-1 site and enhanced the production of C99, resulting in high C99/C89 ratio.

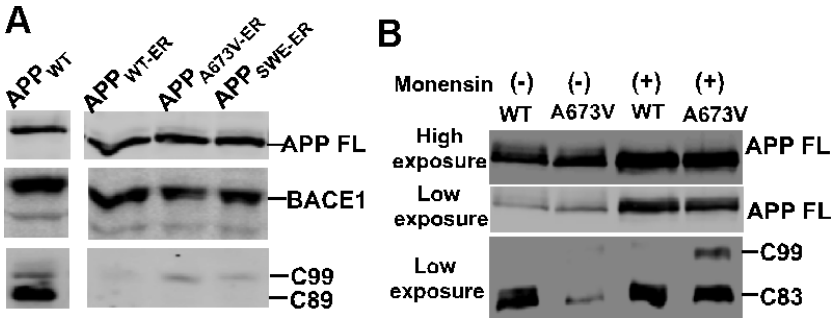


Figure 2.6 APP_{A763V} promoted the production of C99 in both ER and TGN.
(A) APP_{WT}, APP_{A673V} or APP_{SWE} with ER retentional signal (APP_{WT-ER}, $APP_{A673V-ER}$ or APP_{SWE-ER}) were introduced into HEK293 with BACE1 overexpression; the holo APP protein and its CTFs in cell lysate were detected by antibody C20. BACE1 was detected by 9E10 that would recognize the MycHis tag. C99 was produced in both APP_{SWE-ER} and $APP_{A673V-ER}$ by BACE1, whereas C99 was not detected in APP_{WT}. (B) APP_{WT} or APP_{A673V} were introduced into HEK293 cells and treated with 10μM monensin or vehicle control overnight. Cell lysate was harvested to determine the APP CTFs by C20 antibody. After treatment of monensin, C99 production in APP_{A673V} was great increased whereas the C99 in APP_{WT} was barely detected under low exposure conditions. All experiments were at least triplicated.

2.3.6 Modified APP_{A673V} underwent faster lysosome-dependent degradation than APP_{WT}.

It is documented that the half-life of APP is about 60min and the majority of APP is processed by α-secretase or undergoes lysosomal degradation (Caporaso et al., 1994). While we were investigating the processing of APP_{A673V}, we found that APP_{A673V} produced far less α-secretase product (C83) than APP_{WT} (Fig 2.3). This raised the possibility that there is not enough fully modified APP (or mature APP, mAPP) for α-cleavages, as we mentioned that mAPP is the substrate of α-secretase. Consistent with this finding, APP_{A673V} displayed significantly less modified form (53%±5%; $p<0.05$), which is supposed to be the form of APP on the plasma membrane, as compared with APP_{WT}; whereas the modified form of

APP_{SWE} was not significantly affected (p>0.05) (Fig 2.7 A, B). The lower levels of the modified from of APP_{A673V} ($mAPP_{A673V}$) was not likely due to slower ER exiting rate of nascent APP_{A673V}, because the reduction of nascent APP_{A673V} in ER ($imAPP_{A673V}$), resulting from the protein translation inhibition by cycloheximide (CHX), was indistinguishable from that of APP_{WT} (p>0.05) (Fig 2.7 C, D), indicating that nascent APP_{A673V} exited ER normally. Thus the reduced modified form of APP_{A673V} ($mAPP_{A673V}$) was possibly caused by alterations in downstream APP metabolism like faster degradation.

A large proportion of mAPP was processed by α-secretase at plasma membrane. The F615P mutation was documented to increase the levels of the mAPP by inhibiting α-secretase cleavage, increasing mAPP to 356.3%±12.1%, as compared to APP_{WT} (p<0.05) (Fig 2.7 E), which thus greatly increased the sensitivity to detect any alteration in mAPP (Jager et al., 2009). We introduced F615P mutation in both APP_{WT} and APP_{A673V} ($APP_{WT/F615P}$ and $APP_{A673V/F615P}$) and repeated the CHX experiment. As expected, upon CHX treatment, modified APP_{WT} ($mAPP_{WT}$) kept increasing to 315.3%±3.7% until 40 minute then decreased to 231.6%±8.1% at 60 minute; whereas modified APP_{A673V} ($mAPP_{A673V}$) kept increasing to 211.2%±4.7% until 20 minute then quickly decreased to 117.3%±7.3% at 60 minute, as compared with the levels of their each mAPP at 0 minute (p<0.05) (Fig 2.7 F, G), suggesting a stronger non-secretase degradation pathway of modified APP_{A673V}. To determine the affected degradation pathway of $mAPP_{A673V,}$ we treated cells expressing either $APP_{WT/F615P}$ or $APP_{A673V/F615P}$ with proteasome inhibitor (MG132) or lysosome inhibitor (CHL), respectively. While MG132 had little effect on both APP_{WT} and APP_{A673V} (p>0.05), CHL dramatically enhanced mAPP in both APP_{WT} and APP_{A673V} (Fig 2.7 H, I). Upon CHL treatment, $mAPP_{WT}$ increased to 1.4±0.09% folds whereas $mAPP_{A673V}$ increased to 3.57±0.19 folds (p<0.05), as compared with vehicle control, indicating that both

$mAPP_{WT}$ and $mAPP_{A673V}$ undergo lysosome–dependent degradation but the degradation of $mAPP_{A673V}$ is faster (Fig 2.7 H, I). Given that both α- and β-secretase mainly cleave the modified APP (but not exclusively in the case of APP_{A673V} and APP_{SWE}, see Fig 2.6 A), the faster lysosome-dependent degradation of modified APP_{A673V} results in less substrate for α- and β-cleavages and less accumulation of overall CTFs. These data demonstrated that APP_{A673V} altered the metabolism of APP such as lysosome-dependent degradation and the faster degradation of APP_{A673V} resulted in less APP_{A673V} for other metabolism pathway, such as α- and β-secretase pathways.

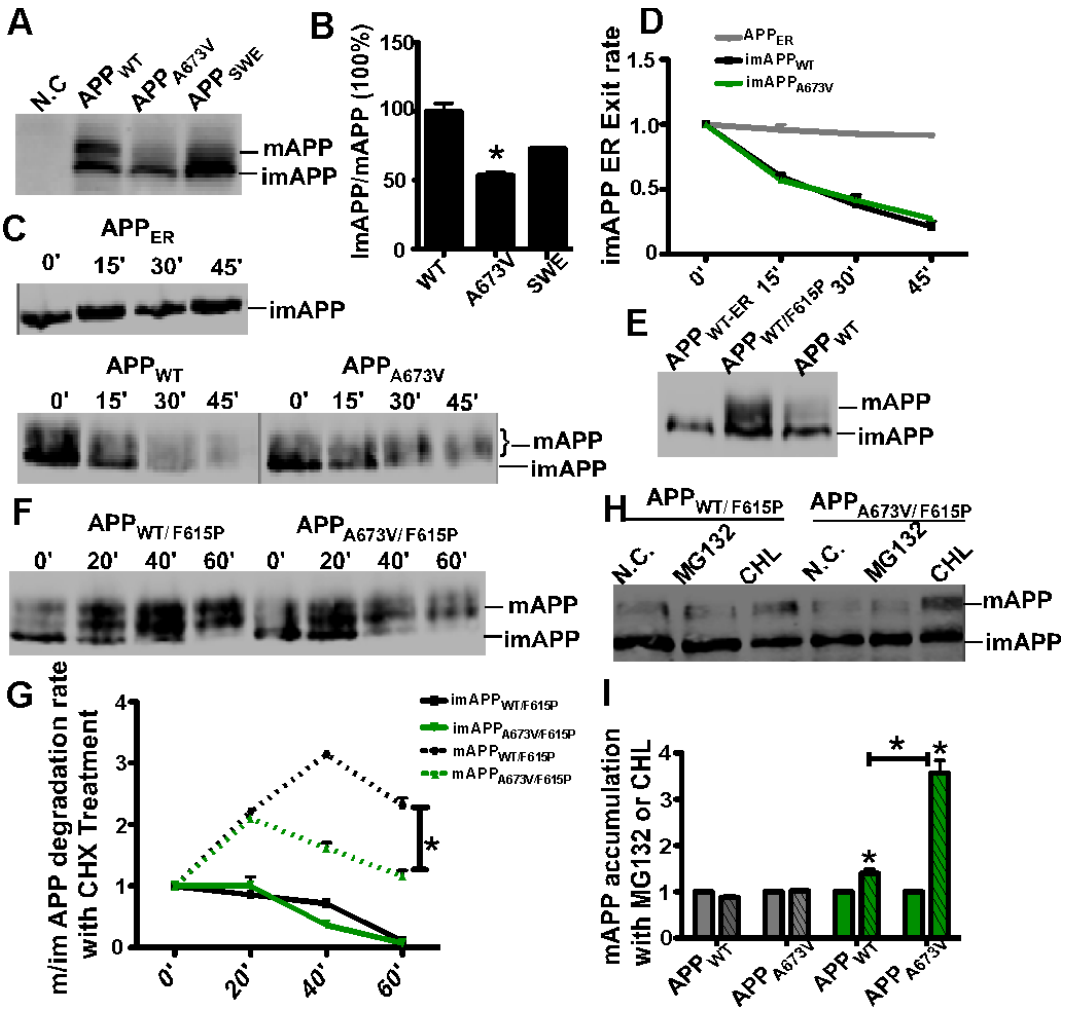


Figure 2.7 APP_{A763V} underwent stronger lysosome-dependent degradation.
(A) APP_{WT} and APP_{A673V} were introduced into HEK293 cells. The nascent APP (imAPP) and modified APP (mAPP) in cell lysate were resolved in 8% glycine SDS-PAGE gel and detected via C20 antibody. (B) The ratio of imAPP/mAPP in (A) was quantified and normalized to that in APP_{WT}. (C, D) APP_{ER}, APP_{WT} or APP_{A673V} were introduced into HEK293 with CHX treatment for 15, 30 or 45 minutes. Both nascent APP (imAPP) and modified APP (mAPP) were resolved in 8% glycine SDS-PAGE gel and detected via C20 antibody. With ER retention signal, APP_{ER} exhibited no modified APP; upon CHX treatment, imAPP in APP_{WT-ER} (gray line) underwent significant slower degradation compared with APP_{WT} (black line) and APP_{A673V} (green line). Upon CHX treatment, both APP_{WT} and APP_{A673V} underwent degradation at similar rate. (E) APP_{WT-ER}, $APP_{WT/F615P}$ or APP_{WT} were introduced into HEK293 to determine the accumulation of modified APP. APP_{ER} was APP with additional ER retentional signal and did not undergo normal modification. $APP_{WT/F615P}$ demonstrated greatly increased accumulation of modified APP compared with APP_{WT}. (F, G) $APP_{WT/F615P}$ and $APP_{A673V/F615P}$ were introduced into HEK293 cells with CHX treatment for 20, 40 or 60 minutes. The nascent APP (imAPP) and modified APP (mAPP) in cell lysate were resolved in 8% glycine SDS-PAGE gel and detected via C20 antibody.

Upon CHX treatment, $imAPP_{WT/F615P}$ and $imAPP_{A673V/F615P}$ demonstrated a similar degradation rate; whereas $mAPP_{A673V/F615P}$ displayed faster degradation compared with $mAPP_{WT/F615P}$; (H, I) $APP_{WT/F615P}$ and $APP_{A673V/F615P}$ were introduced into HEK293 cells with MG132 or CHL treatment overnight. The nascent APP (imAPP) and modified APP (mAPP) in cell lysate were resolved in 8% glycine SDS-PAGE gel and detected via C20 antibody. MG132 treatment did not induced significant accumulation of mAPP in $APP_{WT/F615P}$ and $APP_{A673V/F615P}$; with treatment of CHL, $APP_{A673V/F615P}$ displayed more accumulation of mAPP compared to $APP_{WT/F615P}$. Values represent mean±SEM, n=3, *p<0.05, by two-way ANOVA and one-way ANOVA with post-hoc tests.

2.3.7 The toxicity of $A\beta_{A2V}$ on primary neurons.

Unlike APP_{SWE} that produces $A\beta_{WT}$, APP_{A673V} has an alanine to valine mutation within the Aβ domain (Di Fede et al., 2009), which may affect the toxicity of Aβ. To assess the toxicity of $A\beta42_{A2V}$, we treated E14 primary neurons with either $A\beta42_{WT}$ or $A\beta42_{A2V}$. Vehicle control, soluble $A\beta42_{WT,}$ aged $A\beta42_{WT}$ or aged $A\beta42_{A2V}$ were applied to primary neurons in 96-well plates at 1μM. After 24 hours of treatment, living cells were labeled by trypan blue and counted (Fig 2.8 A). Both aged $A\beta42_{WT}$ or aged $A\beta42_{A2V}$ induced more neuronal death (61%±2% and 74% ± 1%, respectively) than soluble $A\beta42_{WT}$ (41%±2%; p<0.05); however, aged $A\beta42_{A2V}$ appeared to be even more toxic than $A\beta42_{WT}$, because cells challenged with $A\beta42_{A2V}$ displayed the lowest viability (Fig 2.8 B). To further confirm these results, we treated the primary neurons with vehicle control, soluble $A\beta42_{WT}$, aged $A\beta42_{WT}$ or aged $A\beta42_{A2V}$ at much higher concentrations (100uM and 500uM) for 24h, and then performed LDH leakage assay to evaluate cell damage. As compared with 1% triton-100 positive control, $A\beta42_{A2V}$ induced LDH levels to 82%±2% and 68%±3% at both concentrations, respectively; whereas $A\beta42_{WT}$ only increased LDH levels to 52%±1% and 45%±1% respectively, indicating that $A\beta42_{A2V}$ was more toxic to primary neurons (p<0.05) (Fig 2.8 C, D).

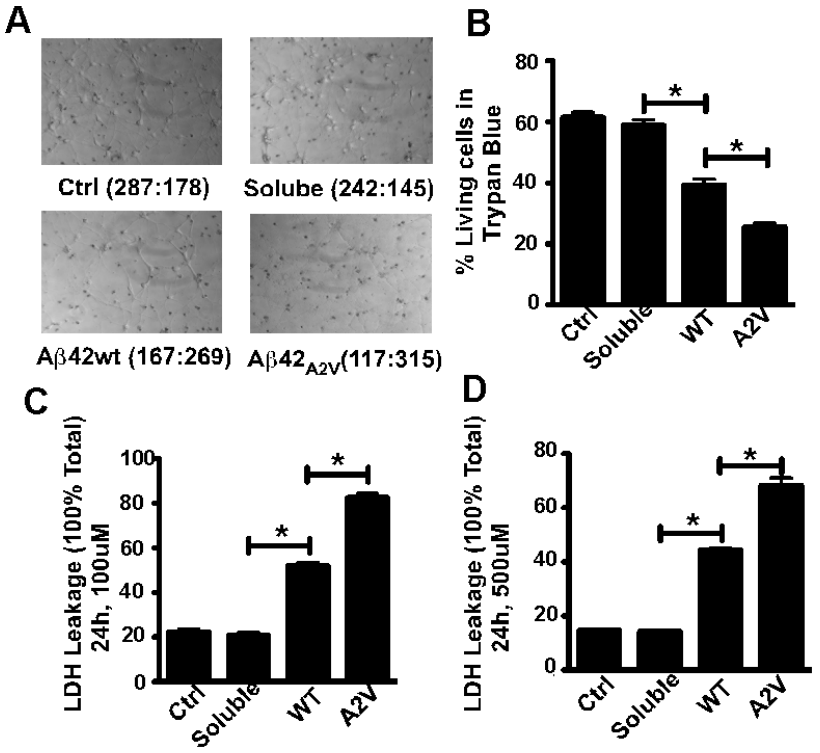


Figure 2.8 $A\beta42_{A2V}$ was more cytotoxic than $A\beta42_{WT}$.
Primary neurons (E14) were cultured for 10 days before Aβ application. (A) Cells were treated with vehicle control, soluble $A\beta42_{WT}$, aged $A\beta42_{WT}$ or aged $A\beta42_{A2V}$ at 1μM for 24 hrs. 10% trypan blue were then added. The number of living cells and dead cells were manually counted. (B) Quantification of (A). (C, D) Primary neurons were treated with vehicle control, soluble $A\beta42_{WT}$, aged $A\beta42_{WT}$ or aged $A\beta42_{A2V}$ at 100μM (C) or 500μM (D) for 24 hrs. LDH kit (Promega) was used to detect cell membrane breakage. Cells treated with 1% triton-100 were positive control. LDH leakage of all four groups was normalized with 1% triton-100 positive control. Values represent mean±SEM, n=6, *p<0.05, by two-way ANOVA and one-way ANOVA with post-hoc tests.

2.4 Discussion.

Due to the complexity of sporadic AD pathogenesis, it is difficult to explore the underlying mechanism. FAD-associated APP mutants have provided great insights into AD pathogenesis and have served as powerful tools for AD researchers. C99 is the precursor of Aβ and a β-secretase product. It has been well established that Swedish APP contributes to AD pathogenesis via promoting C99 generation (Deng et al., 2013). In the cases of the Swedish mutation and APP_{A673V},

C99/C83 ratio does not accurately define the effect of APP mutants to shift APP processing from non-amyloidogenic pathway to an amyloidogenic pathway (Di Fede et al., 2009). C99/C83 defines the competition between α- and β-secretase. The increase of C99/C83 can be interpreted as the result of increased β-secretase activity at the expense of the α-secretase activity. However, the Swedish mutation increases the production of C99 and decreases C89, another β-secretase product; whereas the levels of C83 of Swedish mutation are comparable with that of the wild type APP. The pathogenic effect of APP mutants, like the Swedish on the β-secretase, should be determined and quantified using C99/C89 as an indicator (Fig 2.3).

We found that APP_{A673V} robustly shifted the major β-cleavage site from Glu-11 to Asp-1, with an even higher C99/C89 than Swedish APP. It seems like that APP_{A673V} had a stronger amyloidogenic effect than the Swedish mutation, which was paradoxical concerning the recessive inheritance pattern of APP_{A673V}. However, only when the overall β-cleavage is a constant, C99/C89 is proportional to the severity of amyloidogenesis. In other words, the production of C99 was not only determined by the β-cleavage preference but also by the overall β-cleavage efficiency (or the activity of β-secretase). Our data demonstrated that the C99 production in APP_{A673V} was not more than Swedish mutation even in 293B2 cells with robust overexpression of BACE1, and in HEK293 and in NN cells with endogenous levels of BACE1, the C99 production in APP_{A673V} was less than Swedish mutation (Fig 2.2 and Fig 2.3). BACE1 is highly expressed in pancreases and cortex but is still tightly regulated at both transcriptional and translational levels (Vassar et al., 1999, Li et al., 2006, Zhou and Song, 2006, Sun et al., 2012). We do not know the exact level of β-secretase activity in brain relative to *in vitro* cell lines but we estimate that β-secretase activity in brain falls between that of

HEK293 and 293B2 cells. Thus, we conclude that the production of C99 of APP_{A673V} in brain is less than Swedish but more than wild type APP.

We further explored why β-secretase highly preferentially cleaved APP_{A673V} at Asp-1 site. APP mutations affect its processing via either structural transformation of the active cleavage sites (Sauder et al., 2000) or altering the intracellular trafficking (Felsenstein et al., 1994b, Haass et al., 1995, Thinakaran et al., 1996b). To eliminate the trafficking factors, we trapped APP in particular organelles thus any altered processing of APP should result from the structural effect. We used ER retention signal or monensin treatment to trap APP in ER or TGN and found that both APP_{A673V} and APP_{SWE} can be processed to C99 in ER and TGN, whereas APP_{WT} cannot (Fig 2.6). Our data demonstrated that, at least with respect to the increased C99/C89 in APP_{A673V} and APP_{SWE}, the structural transformation around the Asp-1 β-cleavage site is responsible for shifting the prime β-secretase site of BACE1 from Glu-11 to Asp-1 site, with C99 as predominant β-secretase product in APP_{A673V} and APP_{SWE}. Sauder et al. reported that several hydrophobic residues in BACE1 formed an active pocket with the residues around Asp-1 site, like Leu671 in APP_{SWE}, which affected the preference cleavage of BACE1 in APP (Sauder et al., 2000). Consistent with this study, our data supports that both APP_{A673V} and APP_{SWE} facilitate β-cleavage at Asp-1 site via structural transformation but APP_{A673V} exhibited stronger facilitating effect than APP_{SWE}.

In addition, as reported in section 2.3.2, we also noticed that both the α-secretase (C83) and the overall β-secretase product (C89 and C99) of APP_{A673V} were significantly less than APP_{WT} and APP_{SWE} (Fig 2.3). We explored the underlying mechanism and found that APP_{A673V} underwent faster lysosomal-dependent degradation, resulting that the remaining APP_{A673V} does not suffice for all other metabolism pathways, which nicely explains why there was a general inhibitory

effect on APP processing — the markedly reduced α- and total β-products in APP_{A673V}. Though β-secretase preferentially cleaves APP_{A673V} at Asp-1 site via structural facilitation, the faster lysosome-dependent degradation of APP_{A673V} reduces the substrate for β-cleavages, which results in mild increase in C99 production and contributes the recessive inheritance of APP_{A673V}.

Compared with Swedish mutation, APP_{A673V} is not only located closely to the Glu-11 β-secretase cleavage site but also within the C99 region ($C99_{A2V}$). APP mutations located within the C99 region can affect γ-cleavage via enhancing Aβ42 generation and are usually located around the γ-secretase cleavage site, like APP_{V717I} (London mutation) (Goate et al., 1991, De Jonghe et al., 2001). Though $C99_{A2V}$ occurred far from the γ-cleavage site, $C99_{A2V}$ demonstrated a significant inhibitory effect on γ-cleavage with a reduced Aβ40 generation (Fig 2.4). It should be noted that we also determined the amount of Aβ42 in $C99_{A2V}$. According to our preliminary data, the generation of $Aβ42_{A2V}$ was also decreased as compared with $Aβ42_{WT}$ and the Aβ42/40 in $C99_{A2V}$ ratio was not higher than that in $C99_{WT}$ (data not shown). Nevertheless, in addition to the effect on β-cleavages, the inhibitory effect of APP_{A673V} on γ-cleavage makes another contribution to the recessive inheritance of APP_{A673V}, both of which makes APP_{A673V} require another mutated allele to produce enough Aβ to initiate AD pathogenesis.

APP_{A673V} is also located within the Aβ domain--$Aβ_{A2V}$. Di Fede et al. reported that $Aβ_{A2V}$ in the homozygous state was more amyloidogenic than the equimolar mixture of $Aβ_{WT}$ and $Aβ_{A2V}$, which contributed to the recessive inheritance pattern in this recessive FAD family (Di Fede et al., 2009). The aggregation tendency of

$A\beta40_{A2V}$ was explored by CD Spectrum (Circular Dichroism Spectrum) and atomic force microscopy analysis. Both indicated that Aβ mixture ($A\beta40_{WT}/A\beta40_{A2V}$) prevented aggregate formation but pure $A\beta_{A2V}$ promoted the formation. It is reported that the aggregation of Aβ42 plays a essential role in plaque formation and Aβ40 had a protective role in AD pathogenesis (Yankner et al., 1990, Yang et al., 1995, Zou et al., 2003). Thus the aggregation tendency of Aβ40 may be not appropriately correlated with AD pathogenesis as Aβ42. That is why Aβ42 was applied in primary neurons treatment in our study. We found that $A\beta42_{A2V}$ was more toxic to primary neurons compared to $A\beta42_{WT}$ (Fig 2.8), but the data of Aβ42 mixture ($A\beta42/A\beta42_{A2V}$) was not less toxic than $A\beta_{WT}$ (data not shown). Nonetheless, no matter the aggregation tendency of Aβ or the toxicity of Aβ, both are highly dependent on the physiological microenvironment in the brain. The Aβ experiments from our laboratory and Di Fide's , which were performed *in vitro*, might not reflect the real conditions in the brain.

2.5 Conclusion.

In conclusion, APP_{A673V} facilitates β-cleavage at Asp-1 site while inhibited the general APP processing including all α-/β-/γ-cleavages, due to the intensified lysosome-dependent degradation. The overall effect of APP_{A673V} on the production of Aβ necessitates the homozygous state of APP_{A673V} to produce enough Aβ to initiate AD pathogenesis.

Chapter 3

PS1ΔS169 impairs γ-cleavage of APP but reserves functional ε-cleavage of Notch

3.1 Introduction.

Mutations in Presenilin1 (PS1) are major causes of Familial Alzheimer's Disease (FAD). Unlike FAD-associated APP mutations, which usually cluster around the Aβ domain, PS1 mutations are scattered throughout the entire sequence of this multiple transmembrane protein, including the cytoplasmic domain, luminal domain, and the transmembrane domain. A central and unresolved conundrum in the AD field is how these variously located PS1 mutations lead to the same physiological consequence — initiating AD pathogenesis via altered γ-secretase activity. Given that γ-secretase is the necessary enzyme for Aβ generation, understanding how these PS1 mutations lead to similar consequences could have important implications in AD therapy.

As mentioned above, γ-secretase is the enzyme responsible for the transmembrane cleavage of C-terminal fragments derived from APP, and Aβ is produced from APP by sequential cleavage of β- and γ-secretase. γ-secretase is a protein complex

with presenilin as its catalytic core and Nicastrin, Aph-1 and Pen-2 as assistant subunits. γ-secretase processes its substrates via several sequential cleavages (Fig 3.1); the transmembrane domain of APP is cleaved at two positions: the γ-site, which liberates the N-terminus of Aβ, and the ε-site, which liberates the C-terminus of the APP intracellular domain (AICD) and is located C-terminally to the γ-site (Weidemann et al., 2002). The site of γ-cleavage within the C-terminal fragments of APP is not exact, resulting in a heterogeneous collection of Aβ peptides ranging in size from 39 to 43 residues. Contrarily, cleavage at the ε-site almost exclusively produces a 50-residue AICD. Although the underlying mechanism remains unknown, all clinical PS1 mutations increase the relative amount of Aβ42 versus Aβ40, both *in vitro* and *in vivo* (Borchelt et al., 1996, Duff et al., 1996, Scheuner et al., 1996, Murayama et al., 1998). Thus, the Aβ42/Aβ40 ratio is now widely used as a pathogenic indicator of PS mutations.

A similar γ-cleavage pattern is also observed for Notch, including heterogeneous cleavage within the transmembrane domain at the S3 site (γ-site) to generate Nβ, and homogeneous cleavage further downstream at the S4 site (ε-site) to generate NICD (Okochi et al., 2002). Some PS1 mutations can inhibit cleavage at ε-site of Notch, as seen in certain FAD-associated PS1 mutations, which impair NICD generation in Notch signaling (Song et al., 1999a). While the central role of APP cleavage by γ-secretase has been well established in AD pathogenesis, the impaired Notch signaling seen in FAD-associated PS mutations is still in doubt.

Recently, a novel PS1 deletion, $PS1_{\Delta S169}$, was discovered in a Chinese family; patients with this mutation developed FAD in their early 40s (Guo et al., 2010b). In Chapter 3, we examine the effect of $PS1_{\Delta S169}$ on APP and Notch processing. We found that $PS1_{\Delta S169}$ impairs APP processing but does not affect Notch

proteolysis or signaling, suggesting a mechanism by which $PS1_{\Delta S169}$ contributes to the early on-set of AD in affected members of this family.

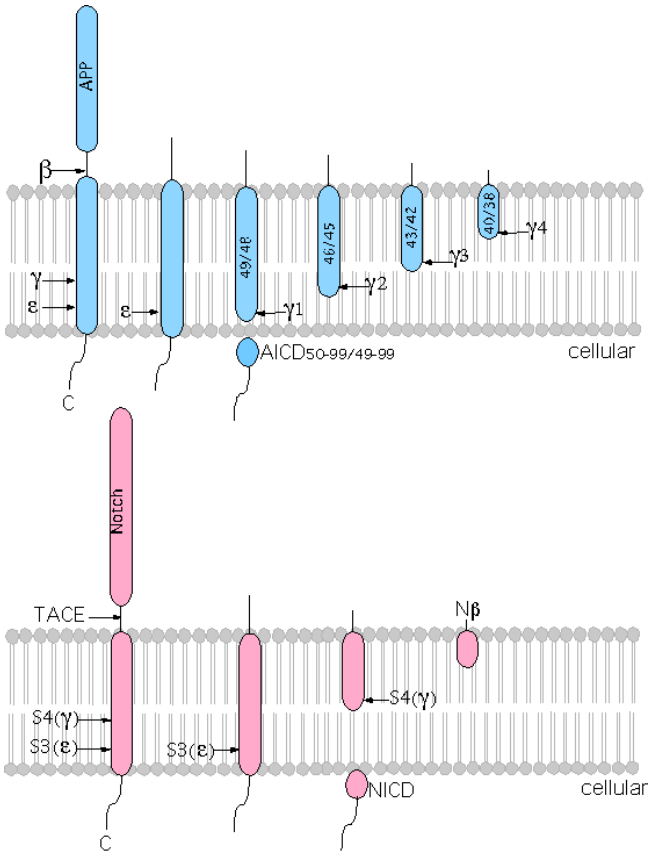


Figure 3.1 Sequential cleavages of γ-secretase.
Top: APP cleavage. γ-secretase first cleaves APP C99 at the ε-site to release AICD, which exists as a 50-residue exclusively. Heterogeneous cleavage at the γ-site generates an Aβ peptide ranging from 39 to 43 residues. Bottom: the same working model occurs in Notch processing at the S3 and S4 sites. Adapted from Wolfe, 2007.

3.2 Methods.

3.2.1 Materials.

Dulbeccoo's modified eagle medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, L-glutamine, Penicillin-Streptomycin, geneticin, zeocin and lipofectamin 2000 were purchased from Life Sciences Technologies. Rabbit anti-

C20 antibody, which recognizes the last twenty amino acids of the C-terminal end of APP and rabbit anti-PS1 N-terminal antibody PS1N, were both made in-house. β-actin was detected using monoclonal antibody AC-15 (Sigma). IRDye^{TM} 680 labeled goat anti-rabbit and IRDye^{TM} 800CM labeled goat anti-mouse secondary antibodies were obtained from LI-COR Biosciences. pcDNA4-Myc-His(A) expression vector was obtained from Invitrogen.

3.2.2 cDNA constructs.

$PS1_{WT,}$ $PS1_{C410Y}$, and $PS1_{Y115H}$ were cut at HindIII and XbaI sites from pRK7-$PS1_{WT}$, $PS1_{C410Y}$, or $PS1_{Y115H}$, respectively; and cloned into pcDNA4-MycHis (A) (Invitrogen) (Song et al., 1999a). $PS1_{\Delta S169}$ was also cloned into pcDNA4-Myc-His (A) expression vector, using a PCR site-directed mutagenesis approach with a pair of complementary primers containing the 3bp deletion coding serine (5'- GCCTGGCTTATTATATCT CTATTG as the forward primer and 5'-GAA CAGCAACAATAGAGATATAAT as the reverse primer). The sequence of $PS1_{\Delta S169}$ was confirmed by Bigdye® Terminator v3.1 Cycle sequencing kit in both directions. Notch cleavage was examined using mNotch1ΔE m/v (NΔE), which expressed a truncated form of mouse Notch1, and undergoes constitutive γ-secretase proteolysis in the absence of ligand (Song et al., 1999a). Expression plasmid ICV, which expresses the truncated form of NΔE from the cleavage site (1744) to the Myc-tag end, was used as a fragment marker corresponding to the Notch-1 intracellular domain (NICD) (Schroeter et al., 1998, Song et al., 1999a).

3.2.3 Cell culture and transfection.

HEK293 cells were cultured in DMEM containing 10% FBS, 1mM sodium pyruvate, 2 mM L-glutamine, 50 U/mL penicillin G sodium, and 50 μg/mL streptomycin sulfate (Invitrogen). NN ($PS1^{-/-}$/$PS2^{-/-}$) cells were cultured in

HEK293 media supplemented with 1mM MEM non-essential amino acids solution, and 1μM β-mecapitolethenol (Invitrogen). Stable cell lines were maintained in media containing Zeocin or G418 (Geneticin). The HAW cell line stably expressed a wild type APP695 in HEK293 cells (Qing et al., 2004b). All cells were maintained at 37°C in an incubator containing 5% CO2. For transfection, cells were grown in 35mm plates to 70% confluence and transfected with 2μg plasmid DNA using 4μL of Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's instructions.

3.2.4 Immunoblot analysis.

Cells were lysed in RIPA lysis buffer (1% Triton X100, 1% sodium deoxycholate, 4% SDS, 0.15M NaCl, 0.05M Tris-HCl, pH 7.2) supplemented with 200 mM sodium orthovanadate, 25 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 30 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride (PMSF), and a complete mini protease inhibitor cocktail tablet (Roche Diagnostics). The samples were diluted in 4× SDS-sample buffers, resolved by SDS-PAGE on 9% tris-glycine or 16% tris-tricine gels, and transferred to Immobilon^{TM} –FL phlyvnylidene fluoride (PVDF) membranes (Millipore). For immunoblot analysis, membranes were blocked for 1 h in phosphate-buffered saline (PBS) containing 5% non-fat dried milk followed by overnight incubation at 4°C in primary antibodies diluted in the blocking medium. Rabbit antibody C20 (1:2000) was used to detect APP and its C-terminal fragment (CTF) products. Rabbit anti-PS1 loop antibody PS1N (1:2000) was used to detect full length PS1 and its N-terminal fragment (NTF). Internal control β-actin was detected using monoclonal antibody AC-15 (Sigma). The membranes were rinsed in PBS with 0.1% Tween-20 and incubated with IRDye 800CW-labelled goat anti-mouse or anti-rabbit antibodies in PBS with 0.1% Tween-20 at 22°C for 1 h, and visualized

on the Odyssey system (LI-COR Biosciences). All quantification was performed suing LI-COR Odyssey system and Image J.

3.2.5 Aβ40/42 Enzyme linked-immunosorbent assay (ELISA).

Stable cells lines were maintained in cell culture media supplemented with 5% FBS. After 24h, conditioned medium was collected and protease inhibitors and AEBSF (ROCHE Diagnostics) were added to prevent degradation of Aβ peptides. The concentration of Aβ40 and Aβ42 were detected using β-amyloid 1-40 or β-amyloid 1-42 Colorimetric ELISA kit (Invitrogen) according to manufacturer's instructions.

3.3 Results.

3.3.1 Generation of $PS1_{\Delta S169}$ and other PS1 mutants.

Although PS1 mutations can be scattered throughout its amino acid sequence, most mutations are in the transmembrane domains and hydrophobic loops. $PS1_{\Delta S169}$ resides in transmembrane domain 3 (TM3) (Fig 3.2). pcDNA4-$PS1_{WT}$, pcDNA4-$PS1_{Y115H}$ and pcDNA4-$PS1_{C410Y}$ have previously been generated in our laboratory. To compare $PS1_{\Delta S169}$ with other PS1 mutations, we generated $PS1_{\Delta S169}$ and 9 other representative PS1 mutations via site-directed mutagenesis ($PS1_{L85P}$, $PS1_{M139V,}$ $PS1_{S169P,}$ $PS1_{S170F,}$ $PS1_{G206S,}$ $PS1_{M233T}$, $PS1_{Y256S,}$ $PS1_{T291P,}$ $PS1_{T354I}$) (Fig 3.2). To generate $PS1_{\Delta S169}$, we used $PS1_{WT}$ as a template and primers containing the 3bp deletion coding serine to generate a full length $PS1_{\Delta S169}$ PCR product, which was then cloned into pcDNA4 between the HindIII and XbaII sites. $PS1_{\Delta S169}$ was generated with

5'- GCCTGGCTTATTATATCTCTATTG and

5'-GAA CAGCAACAATAGAGATATAAT as forward and reverse primers.

Following the same strategy, $PS1_{L85P}$ was generated with

5'-AAGCATGTGATCATGCCCTTTGTC and

5’- GTCACAGGGACAAAGGGCATGA as forward and reverse primers; $PS1_{M139V}$ was generated with 5’- CTGAATGCTGCCATCGTGATCAGT and 5’- AACAATGACACTGATCACGATGGC as forward and reverse primers; $PS1_{S169P}$ was generated with 5’- GCCTGGCTTATTATACCATCTC and 5’- CAGCAACAATAGAGATGGTATAAT as forward and reverse primers; $PS1_{S170F}$ was generated with 5’- TGGCTTATTATATCATTTCTATTG and 5’- GAACAGCAACAATAGAAATGATAT as forward and reverse primers; $PS1_{G206S}$ was generated with 5’- CTGATCTGGAATTTTAGTGTGGTG and 5’- TCATTCCCACCACACTAAAATTCC as forward and reverse primers; $PS1_{M233T}$ was generated with 5’- ATGATTAGTGCCCTCACGGCCCT and 5’- ATAAACACCAGGGCCGTGAGGG as forward and reverse primers; $PS1_{Y256S}$ was generated with 5’-GGCTGTGATTTCAGTATCTGATTTAG and 5’- CAGCCACTAAATCAGATACTG as forward and reverse primers; $PS1_{T291P}$ was generated with 5’- CTCATTTACTCCTCACCAATGGTG and 5’- CCAACCACACCATTGGTGAGG as forward and reverse primers; $PS1_{T354I}$ was generated with 5’- GCCTCATCGCTCTATACCTGAG and 5’- GCTCGTGACTCAGGTATAGAGC as forward and reverse primers. The coding sequence of these PS1 mutants was confirmed via BigDye 3.1 sequencing (Fig 3.3).

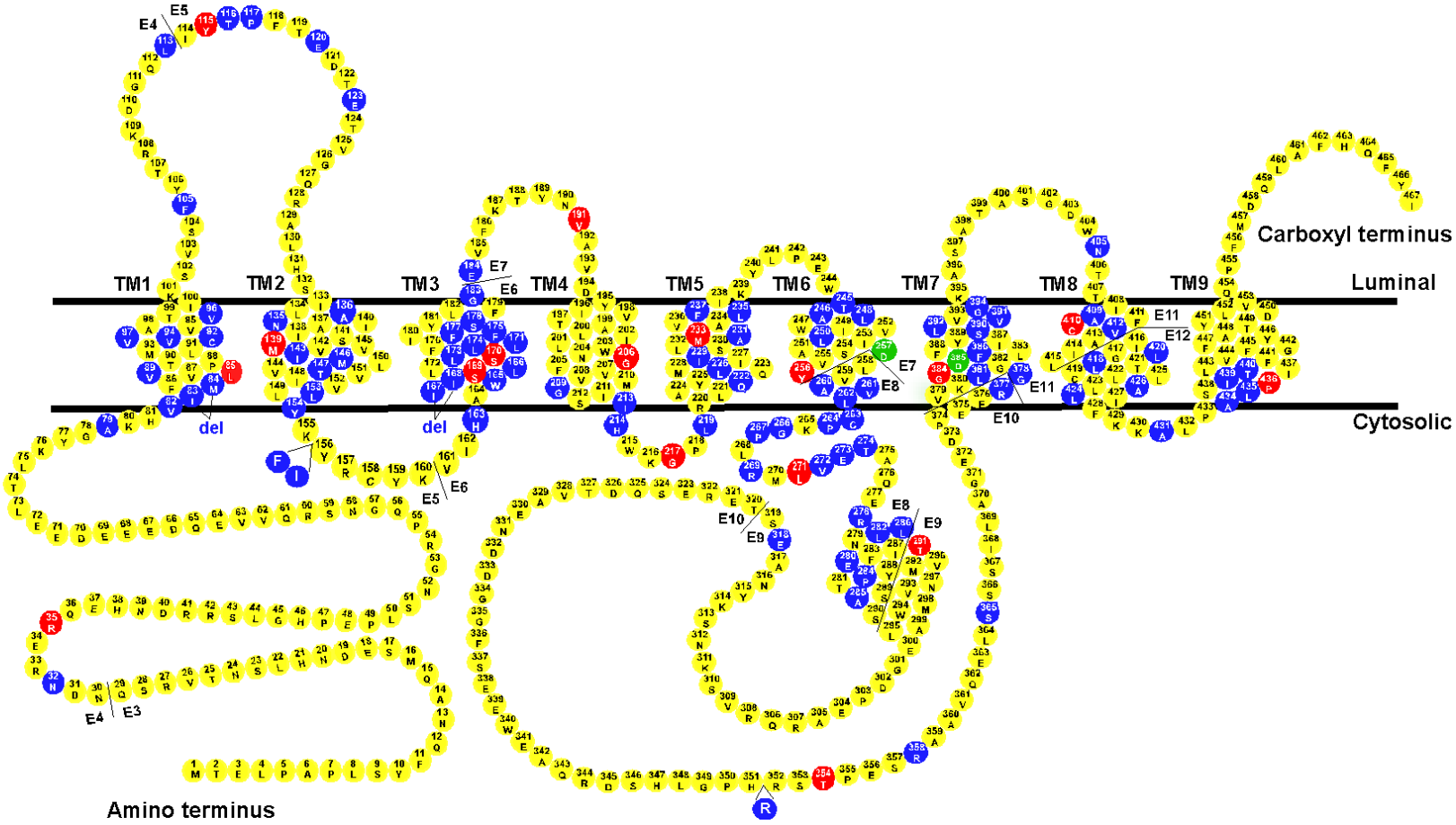


Figure 3.2 Presenilin 1 structure and the locus of PS1$_{\Delta S169}$.
This diagram shows the amino acid sequence of PS1 and the distribution of the FAD-associated mutations (blue and red circles). Green circles indicate the two aspartates residues, which are essential for catalytic activity. Red circles indicate the representative PS1 mutations, which were selected for comparison with PS1$_{\Delta S169}$ in this dissertation.

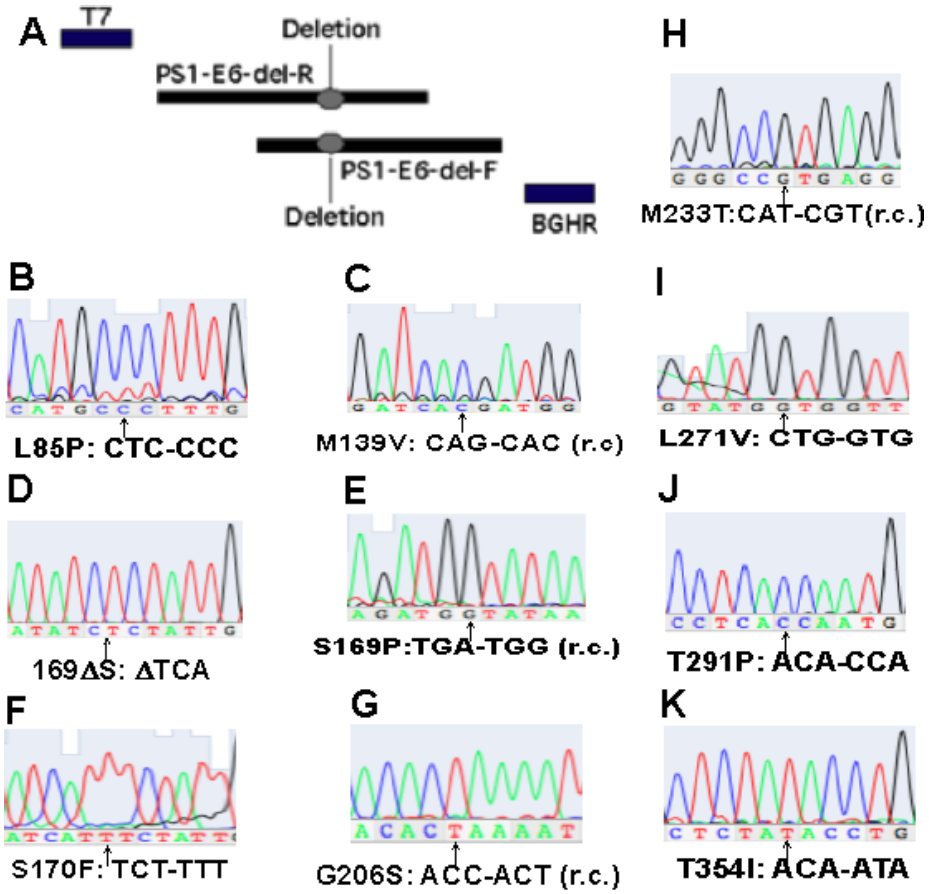


Figure 3.3 Generation of PS1mutants constructs.
(A) Strategy for the generation of PS1 mutants via site-directed mutagenesis: using the cloning of $PS1_{\Delta S169}$ as an example, primers PS1-E6-del-F and PS1-E6-del-R were designed to create the $PS1_{\Delta S169}$ deletion mutation. Primers T7 and primer BGHR were the customized primers used for pcDNA4-mycHis (A). Site-directed mutagenesis PCR was performed as indicated in the schematic diagram with pcDNA4-$PS1_{WT}$ used as the template. (B-K) Sequencing results of the PS1 mutants constructs: $PS1_{\Delta S169}$, $PS1_{L85P}$, $PS1_{M139V,}$ $PS1_{S169P,}$ $PS1_{S170F,}$ $PS1_{G206S,}$ $PS1_{M233T}$, $PS1_{L271V,}$ $PS1_{T291P,}$ $PS1_{T354I}$ (r.m., reverse complementary sequence).

3.3.2 $PS1_{\Delta S169}$ underwent normal endoproteolysis.

Under physiological conditions, presenilin undergoes endoproteolysis to generate N-terminal fragment (NTF) and C-terminal fragment (CTF). Presenilin holo-protein has a short half-life of about 30 minutes and is therefore barely detectable; after endoproteolysis, the NTF and CTF moieties constitute the functional

catalytic core of γ-secretase, with the half-life of 12h (Thinakaran, 2001). During endoproteolysis, the hydrophobic loop between exon8 and exon9 is removed, a necessary step required to activate wild type PS (Thinakaran et al., 1996a). Consequently, $PS1_{\Delta E9}$, a PS1 mutant that lacks the hydrophobic loop, is active even in the holo protein form (Wolfe et al., 1999b). Intriguingly, some FAD-associated PS1 mutations exhibit impaired endoproteolysis, resulting in an accumulation of holo-PS1 protein and a reduction in active NTF/CTF derivatives (Thinakaran et al., 1996a). These observations point to a role of the hydrophobic loop in determining the γ-secretase activity. To investigate the endoproteolysis of $PS1_{\Delta S169}$, expression plasmids, containing $PS1_{WT}$, $PS1_{\Delta S169}$, $PS1_{L85P}$, $PS1_{Y115H}$, $PS1_{M139V}$, $PS1_{S169P}$, $PS1_{S170F}$, $PS1_{G206S}$, $PS1_{M233T}$, $PS1_{Y256S}$, $PS1_{T291P}$, $PS1_{T354I}$, $PS1_{C410Y}$, were transiently transfected into the NN cells, which is $PS1^{-/-}/PS2^{-/-}$ cell line lacking endogenous wild type PS1.

The 50kDa holo PS protein and its 30 kDa NTF were detected via PS1N antibody (Fig 3.4 A). Only $PS1_{T291P}$ and $PS1_{C410Y}$ displayed impaired endoproteolysis, reducing NTF levels to 45.5%±4.1% and 34.5%±5.2% ($p<0.05$) of that seen in $PS1_{WT}$, respectively. In contrast, $PS1_{Y115H}$ and $PS1_{M139V}$ increased the endoproteolysis levels to 133.3%±8.4% and 130.5%±6.2% ($p<0.05$) as compared with wild type PS1, respectively (Fig 3.4 B). $PS1_{\Delta S169}$ and other PS1 mutants underwent normal endoproteolysis without significantly affecting NTF generation. These results demonstrated that the S169 deletion did not affect the endoproteolysis of PS1.

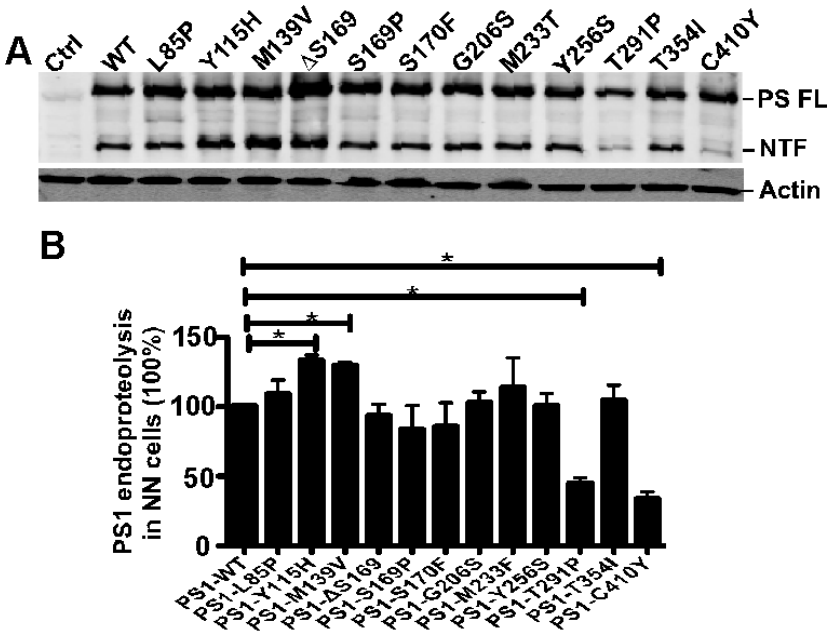


Figure 3.4 $PS1_{\Delta S169}$ undergoes normal endoproteolysis.
NN cells was transiently transfected with pcDNA4-$PS1_{WT}$, $PS1_{\Delta S169}$, $PS1_{L85P}$, $PS1_{Y115H}$, $PS1_{M139V}$, $PS1_{S169P}$, $PS1_{S170F}$, $PS1_{G206S}$, $PS1_{M233T}$, $PS1_{Y256S}$, $PS1_{T291P}$, $PS1_{T354I}$ and $PS1_{C410Y}$. PS1 in cell lysate was detected by rabbit anti-PS1 antibody PS1N. (A) In NN cells transfected with empty vector, both full length PS1 and its NTF could not be detected. With the introduction of PS1 variants, there was an accumulation of full length, holo PS1 at 50kD. PS1 variants underwent varying degrees of endoproteolysis to generate different level of NTF at 30kDa. (B) Quantification of (A) by Image J. The NTFs level in each of the PS1 mutants were normalized to that in $PS1_{WT}$. The values represent mean±SEM. n=3, *p<0.05 by one-way ANOVA with post-hoc tests.

3.3.3 $PS1_{\Delta S169}$ demonstrated impaired APP cleavage activity and an increased Aβ42/40 ratio.

Aβ42, a polypeptide derived from APP by sequential β- and γ- cleavages, is proposed to initiate the amyloid pathogenic cascade. It has been reported that the most investigated FAD-associated PS mutations increase the Aβ42/40 ratio, either by promoting Aβ42 production or reducing Aβ40 generation. To explore the effect of $PS1_{\Delta S169}$ on APP processing, we generated PS-stable cell lines in HAW and NN cells. HAW is a HEK293 cell line, which stably expresses wild type APP, and can be used to investigate APP processing and Aβ generation (Sun et al., 2006a, Deng et al., 2013). NN is the $PS1^{-/-}$/$PS2^{-/-}$mouse embryonic fibroblast cell

line, which has the unique advantage of having no endogenous PS (Song et al., 1999a). $PS1_{WT}$, $PS1_{\Delta S169}$, $PS1_{C410Y}$ or $PS1_{Y115H}$ were transiently introduced into HAW and NN cell line and stable cell lines were selected using Zeocin (800μg/ml) (Invitrogen). HAWPS was HAW cell line stably expressing PS1 variants, while NNPS referred to NN cells expressing stably PS1 variants. As most endogenous PS undergoes endoproteolysis, the holo-protein of PS can barely be detected in HAW cells. After the stable introduction of exogenous PS1, holo-PS1 variants were detected at 50 kDa in HAWPS cells (Fig 3.5 A). In NNPS cells, the introduction of PS1 variants rescued the PS deficiency in NN cells, and the PS1 NTFs were clearly detected at 30kDa by antibody PS1N. In both HAWPS and NNPS cells, $PS1_{\Delta S169}$ underwent normal endoproteolysis similar to wild type PS1 (Fig 3.5 A, E), supporting our previous finding in section 3.3.2.

The conditioned media of HAWPSs cells was collected after 24h of culture and Aβ was measured by ELISA assay. Compared with $PS1_{WT}$, $PS1_{C410Y}$ and $PS1_{Y115H}$ reduced the production of Aβ40 to 32.1%±4.6% and 30.6%±5.2% (p<0.01), respectively; however Aβ42 production of $PS1_{C410Y}$ and $PS1_{Y115H}$ appeared unaffected. In contrast, $PS1_{\Delta S169}$ decreased the production of both Aβ40 and Aβ42 to 24.7%±3.8% and 67.1%±4.2% (p<0.01), respectively (Fig 3.5B, C). Despite this, the overall effect on Aβ42/40 ratio was the same, $PS1_{\Delta S169,}$ $PS1_{C410Y}$ and $PS1_{Y115H}$ all increased Aβ42/40 ratio over that seen in $PS1_{WT}$ to 280.3%±26.2%, 274.1%±14.1% and 286.1%±25.5% (p<0.01), respectively (Fig 3.5 D). We further investigated APP processing in NNPSs cell lines, in which the effect of endogenous wild type presenilins was eliminated. APP_{WT} was transiently introduced into NNPS cells to examine APP processing. Without endogenous PS1, APP CTFs were accumulated in NN cells (Fig 3.5 F, lane 1); when introduced wild type PS1, APP CTFs levels were reduced to 31.6%±2.8% (p<0.01) in $NNPS_{WT}$ cells as compared with NN cells, indicating that $PS1_{WT}$

greatly rescued the γ-secretase processing of APP in NN cells. In $NNPS_{\Delta S169}$ and $NNPS_{C410Y}$ cells, APP CTFs levels were reduced to 74.3%±4.5% and 71.4%±4.2% ($p<0.05$) (Fig 3.5 F, G) as compared with the CTFs levels in NN cells, but the rescuing effect was weaker than $PS1_{WT.}$ The combined analysis of the Aβ and APP CTF assays indicate that $PS1_{\Delta S169}$ impairs γ-cleavage of APP, particularly at the Aβ40 generation site.

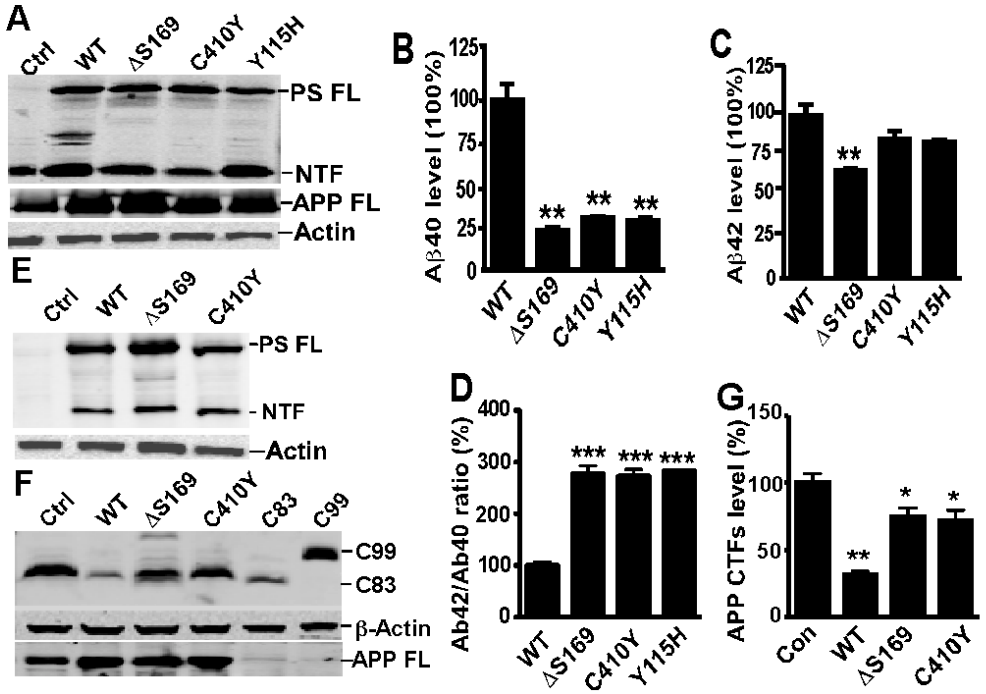


Figure 3.5 $PS1_{\Delta S169}$ promoted accumulation of APP CTFs and increased Aβ42/40 ratio via impaired γ–secretase activity in PS1 stable cell lines.
HAW cells are HEK293 cells which stably overexpress human APP_{WT} (Qing et al., 2004a), and NN cells are PS1/PS2 double-knockout ($PS1^{-/-}/PS2^{-/-}$) mice embryonic fibroblast cells (MEF) (Song et al., 1999b). PS1 stable cell lines were generated by introducing pcDNA4-$PS1_{WT}$, pcDNA4-$PS1_{\Delta S169}$, pcDNA4-$PS1_{C410Y}$ or pcDNA4-PS_{Y115H} into HAW or NN cells and selecting the positive, stably transfected cells with Zeocin (Invitrogen). PS1 was detected by rabbit anti-PS1 antibody PS1N (Song et al., 1999a). APP was detected by rabbit anti-APP antibody C20 (Qing et al., 2004a). Monoclonal anti-β-actin antibody (AC-15) was used to detect β–actin. (A) In $HAWPS_{WT}$, $HAWPS_{\Delta S169}$, $HAWPS_{C410Y}$, $HAWPS_{Y115H}$ cell lines, accumulation of full length PS1 was robustly increased compared with HAW. $PS1_{\Delta S169}$ underwent endoproteolysis similar to $PS1_{WT}$. (B, C, D) Conditioned media from HAWPS cell lines were collected to determine Aβ production. $PS1_{\Delta S169}$, $PS1_{C410Y}$ and $PS1_{Y115H}$ produced significantly less Aβ40 than $PS1_{WT}$. $PS1_{\Delta S169}$ also generated significantly less Aβ42 than $PS1_{WT}$. When the Aβ42/40 ratio was examined, all PS1 mutations lead to a significant increase in the Aβ42/40 ratio. (E) In $NNPS_{WT}$, $NNPS_{\Delta S169}$, $NNPS_{C410Y}$ cells, full length PS1 and PS1 NTFs were robustly detected at 50kDa and

30kDa separately. (F) In $NNPS_{WT}$, $NNPS_{\Delta S169}$, $NNPS_{C410Y}$ cells with wild type APP overexpression, APP CTFs were detected via C20 antibody. (G) The levels of APP CTFs were quantified using Image J. Compared with $NNPS_{WT}$, $NNPS_{\Delta S169}$ and $NNPS_{C410Y}$ exhibited less effect on rescuing the accumulation of APP CTFs in NN. Values represent mean±SEM, n=5, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with post-hoc tests.

3.3.4 $PS1_{\Delta S169}$ cleaved Notch with normal enzymatic efficiency.

APP and Notch were first two identified substrates cleaved by γ-secretase. While Aβ42, generated from APP by γ-secretase, is believed to initiate the amyloid pathogenic cascade; whether altered Notch cleavage also contributes to AD pathogenesis is still not clear. Notch was first implicated in AD in several studies of PS-deficient mice and stem cells. PS-deficient mice were embryonic lethal and displayed CNS and skeletal defects reminiscent of the Notch-knock out phenotype (Shen et al., 1997, Wong et al., 1997). Later studies confirmed that Notch was a substrate of presenilins (De Strooper et al., 1999, Song et al., 1999a) and FAD-associated PS mutations inhibited NICD production(Song et al., 1999a). Notch signaling is one of the most conserved cell signaling pathways in metazoans, and is fundamental in neuroproliferation and differentiation in both embryonic development and adult brain; it still remains elusive how the impairment in Notch processing caused by FAD-PS mutations contributes to AD pathogenesis. Furthermore, as PS is a pharmaceutical target for AD therapy, a major obstacle for γ-secretase inhibitor development is how to avoid inhibition of Notch cleavage.

In 1999, Song et al. discovered that FAD-associated PS1 mutations reduced proteolysis of a truncated form of Notch1, mNotch1ΔE m/v (NΔE), which undergoes proteolysis constitutively in the absence of receptors (Song et al., 1999a). Here, I employed NΔE to assess the effect of $PS1_{\Delta S169}$ on Notch processing by transiently co-overexpressing $PS1_{\Delta S169}$ and NΔE in HEK293 cells. 48h post transfection, cell lysates were resolved by 10% SDS–PAGE and immunoblotted with 9E10 antibody against the C-terminal Myc-tag of NΔE. The cleaved form of NΔE (NICD) was analyzed in different PS1 variants ($PS1_{WT}$,

$PS1_{\Delta S169}$ and $PS1_{C410Y}$). Quantification of the results showed that compared with $PS1_{WT}$, $PS1_{C410Y}$ only produced 36.6% ± 1.3% NICD (p<0.001); whereas $PS1_{\Delta S169}$ and $PS1_{WT}$ were indistinguishable in terms of NICD generation (Fig 3.6 A, B). To confirm these findings, a stable NΔE stable expressing cell line in HEK293, designated V2, was created in HEK293 cells. $PS1_{WT}$, $PS1_{\Delta S169}$, $PS1_{Y115H}$ and $PS1_{C410Y}$ were introduced into V2 cells to determine the NICD generation in different PS1 variants. Consistent with the transient overexpression results, no significant differences were seen in the amount of NICD generated by overexpression of $PS1_{\Delta S169}$ and $PS1_{WT}$ in V2 cells; whereas $PS1_{Y115H}$ and $PS1_{C410Y}$ significantly reduced NICD levels to 67.6% ± 7.4% and 52.7% ± 5.2% (p<0.05) (Fig 3.6 C, D). These data indicate that S169 deletion in PS does not affect γ-cleavage of Notch.

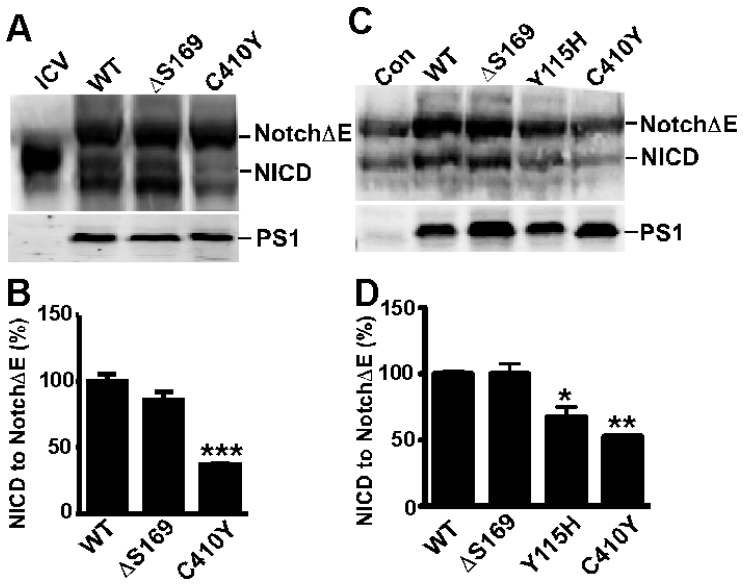


Figure 3.6 $PS1_{\Delta S169}$ retained its enzymatic activity in Notch signaling.
(A) pcDNA4-$PS1_{WT}$, pcDNA4-$PS1_{\Delta S169}$ or pcDNA4-$PS1_{C410Y}$were transiently introduced into HEK293 cells with pcDNA3-mNotch1ΔE m/v (NΔE). pcDNA3-ICV1744 was introduced into HEK293 as gel marker for NICD. Cell lysates were analyzed by Western Blot. NΔE and ICD (NICD) were detected by anti-mouse antibody 9E10. (B) Quantification from (A) by Image J. In comparison with $PS1_{WT}$, $PS1_{C410Y}$ generated significantly less NICD. The levels of NICD in $PS1_{\Delta S169}$ was similar to that in $PS1_{WT}$. (C) NΔE stable cell line (V2) was generated by introducing pcDNA3-NΔE into HEK293 cells and selecting positive colonies with G418 (Sigma). pcDNA4-$PS1_{WT}$, pcDNA4-$PS1_{\Delta S169}$, pcDNA4-$PS1_{C410Y}$ or pcDNA4-$PS1_{Y115H}$were transiently introduced

into V2 cells to analyze the Notch processing efficiency of PS1. Empty vehicle was used as a negative control. (D) Quantification from (C) by Image J. $PS1_{\Delta S169}$ had no effect on NICD (ICV) production, whereas $PS1_{C410Y}$ produced significantly less NICD than $PS1_{WT}$. Values represent mean±SEM, n=5, *p<0.05, **p<0.01, ***p<0.001 by one-way ANOVA with post-hoc tests.

3.4 Discussion.

It has been almost two decades since presenilin was found to linked to early-onset of AD, yet the underlying mechanism of action is still unknown. $PS1_{\Delta S169}$ is a PS1 deletion mutation newly discovered in a Chinese family. $PS1_{\Delta S169}$ is located in TM3, and two missense mutations are also known to occur at this location – $PS1_{S169P}$ and $PS1_{S169L}$ (Taddei et al., 1998, Ezquerra et al., 1999). Both $PS1_{S169P}$ and $PS1_{S169L}$ are characterized with very early-onset AD (mean age of onset is 32 years), rapid progressiveness (mean age of death is 38 years) and the presence of generalized myoclonic jerks and seizures. Except for the early age of onset (as early as 43 in $PS1_{\Delta S169}$ family), other phenotypes of $PS1_{\Delta S169}$ were not distinguishable from typical sporadic AD (Guo et al., 2010a). The serine at the site 169 of PS1 showed high phylogenetic conservation across different species, suggesting that the position is highly conserved and functionally important.

During normal maturation, presenilin undergoes endoproteolysis to generate NTFs and CTFs (Thinakaran et al., 1996a). Although it is difficult to study the crystallization structure of presenilin and the γ-secretase complex, it is proposed that endoproteolysis confers γ-secretase activity to presenilin by removing the cytoplasmic hydrophobic loop (Knappenberger et al., 2004, Fukumori et al., 2010). While mutations like $PS1_{\Delta E9}$, $PS1_{M146L}$ and $PS1_{A246E}$ fail to undergo endoproteolysis, mutations like $PS1_{H163R}$ undergoes endoproteolysis normally (Okochi et al., 1997). Thus, the current consensus is that not all clinical PS mutations impair endoproteolysis, but mutations with significant endoproteolysis deficits usually demonstrate more severe impairments of γ-secretase activity. To investigate the effect of $PS1_{\Delta S169}$ on its endoproteolysis, we compare $PS1_{\Delta S169}$

with 12 other pathogenic PS1 mutations occurring within the transmembrane domain or specific locus near the functional site, such as $PS1_{T291P}$ near the endoproteolysis site. Consistent with previous studies, deficits in endoproteolysis were not an obligatory phenomenon in clinical PS1 mutations, and $PS1_{\Delta S169}$ underwent normal endoproteolysis.

APP is the most important substrate of PS1. The effect of $PS1_{\Delta S169}$ on APP processing was investigated in different scenarios. Aβ42/40 ratio was analyzed in cell lines that stably expressed of both wild type APP and different PS1 variants. As mentioned in 3.3.3, an increased Aβ42/40 ratio is a pathogenic indicator of clinical PS mutations. While we found that an overall increase in Aβ42/40, this resulted from various mechanisms, depending on the PS1 mutations studied. Some mutations, like $PS1_{C410Y}$, specifically impaired the production of Aβ40 without significantly affecting on Aβ42 generation; other mutations produced more Aβ42, with or without an accompanying reduction in Aβ40; while others impaired production of both Aβ40 and Aβ42, but reduced Aβ42 to a lesser degree. We found that $PS1_{\Delta S169}$ had a significantly increased Aβ42/40 ratio compared with wild type PS1, due to deficits in the generation of both Aβ40 and Aβ42. The impaired γ-secretase activity of $PS1_{\Delta S169}$ was also verified using NNPS stable cell lines, the $PS1^{-/-}/PS2^{-/-}$ mouse fibroblast cells lacking endogenous wild type PS but overexpressing the mutant PSs of interest. We transiently introduced wild type APP into NNPSs stable cell lines and investigated the effect of $PS1_{\Delta S169}$ on the accumulation of APP CTFs. Consistent with the Aβ generation, $PS1_{\Delta S169}$ demonstrated impaired γ-secretase activity as indicated by an increased accumulation of APP CTFs compared with wild type PS1 (Fig 3.5).

γ-Secretase processes its substrates at two distinct sites: the ε-site generates ICDs (like NICD), and the γ-site generates Aβ, for example. It has recently been

reported that γ-secretase complex cleavage at the ε- and γ-sites seem to be independently regulated under different pH, temperature, and salt concentration condition (Quintero-Monzon et al., 2011). A kinetic study on ICD-production of FAD-associated PS1 mutants also demonstrated that cleavage efficiency at the ε-site varies among PS1 mutants, and $PS1_{M139V}$ even displays normal ε-cleavage of APP, Notch, and Erb4. This suggests that inefficient processing at the ε-site is not an essential contributor to AD pathogenesis (Chavez-Gutierrez et al., 2012). These studies are consistent with our finding that $PS1_{\Delta S169}$ specifically affected enzymatic activity at the γ-site.

Although several FAD-associated PS1 mutations demonstrate impaired Notch processing, it is hotly debate whether Notch contributes to AD pathogenesis, and if it does, how much. Two recent studies on Notch conditional knock-out mice drew distinct conclusions concerning the contribution of Notch signaling to adult brain function. One suggested that Notch signaling was involved in synaptic plasticity via the Arc pathway (Alberi et al., 2011), while the another suggested that Notch signaling could be absent at least in excitable glutamate neurons (Zheng et al., 2012). Our study demonstrated that pathogenic $PS1_{\Delta S169}$ could process Notch normally, suggesting that aberrations in Notch signaling are not essential in AD pathogenesis. However, we could not exclude the role of Notch signaling in AD, or explain how Notch signaling contributes to the clinical variety of FAD-associated PS1 mutations.

Various PS1 mutations could have differential effects pathologically and clinically. Pathologically, PS1 mutations demonstrate atypical histopathological features, such as cotton wool plaque (CWP) and spastic paralysis (Rogaeva et al., 2003). Clinically, patients with some PS1 mutations suffer from myoclonic jerks, like $PS1_{S169L}$ and $PS1_{S169P}$. The molecular mechanisms underlying these processes are unknown, however, it is possible that variously affected Notch signaling is one

of the reasons. Moreover, PS1 mutations increased Aβ42/40 through different mechanisms (such as $PS1_{\Delta S169}$ promoting Aβ42/40 via disproportionally inhibiting both Aβ; see above for details). The differing amount of Aβ40 may also contribute to the variable consequences of PS1 mutations.

3.5 Conclusion.

$PS1_{\Delta S169}$ was a recently discovered pathogenic PS1 mutation in a Chinese family with an average age of AD on-set of 45 years. We studied the effect of $PS1_{\Delta S169}$ on APP and Notch processing *in vitro*. With respect to APP processing, $PS1_{\Delta S169}$ increased Aβ42/40 via disproportionally impaired γ-cleavage. In the case of Notch processing, $PS1_{\Delta S169}$ retained its enzymatic activity, resulting in normal NICD generation. Our in vitro study suggested that $PS1_{\Delta S169}$ contributed AD pathogenesis via its impaired γ-secretase processing of APP.

Chapter 4

PS1ΔS169 promotes AD pathogenesis *in vivo*

4.1 Introduction.

Animal models are valuable tools to validate the pathogenic mechanism seen in *in vitro* studies, and to evaluate potential therapeutic interventions. The best animal models have pathogenic process and progression similar to the human disease. In the case of AD, transgenic mice should develop amyloid plaques and neurofibrillary tangles, as well as functional cognitive deficits. Moreover, both the neuropathological and behavioral features should progress with aging.

APP protein is highly conserved across species; the human APP sequence is approximately 96% identical to murine APP. Despite this, rats and mice do not develop amyloid pathology with age and the underlying mechanism for this still unclear. It is hypothesized that the three amino acids difference in the rodent Aβ sequence (Selkoe, 1989), the short lifespan of rodents relative to humans (Jankowsky et al., 2004b), and the differences in β-secretase processing of mouse APP (Cai et al., 2001), may be involved. Thus, to create an animal model that mimics the amyloid pathology seen in human AD, transgenic mice were developed which express human APP (Table 4.1). Unfortunately, neurofibrillary tangles are still absent in APP or APP/PS transgenic mice. This obstacle was

overcome by introducing MAPT (microtubule-associated protein tau) mutants into the APP/PS transgenic mouse background (Table 4.1). These triple transgenic mice develop both amyloid plaques and neurofibrillary tangles (Oddo et al., 2003). Although APP/PS/MAPT triple transgenic mice fully recapitulate plaques and tangles, it should be of note that MAPT mutations have never been found in AD patients. There are still no transgenic mice that fully and precisely mimic the comprehensive feature of AD.

In regards to cognitive deficits, most AD transgenic mice display impaired cognitive function with age. The severity and progression of cognitive decline is dependent on multiple factors, including the different transgenes introduced, the expression level of those transgenes, the promoter used, the background strain of the mouse, the transgenic method used, and so on. Our lab maintains APP23 transgenic mice, which are C57BL/6J mice carrying human APP751 cDNA with the Swedish double mutation at positions 670/671 (KM>NL) under control of the murine Thy-1.2 expression cassette (Sturchler-Pierrat et al., 1997, Sun et al., 2006a). To further confirm the pathological effect of $PS1_{\Delta S169}$ on APP processing in an animal model, we generated APP23/ $PS1_{\Delta S169}$ double transgenic mice and examined the pathogenic effect of $PS1_{\Delta S169}$ on APP processing and the cognitive function.

	Gene	Promoter	Background Strain	Pathology				Cognitive Deficits	Reference
				Plaque	NTFs	Hyper-phosphorylated Tau	Neuron Loss		
PDAPP	APP_{V717F}	PDGF	BL6/DBA/SW	YES	NO	YES	YES	YES	Games et al, 1995
Tg2576	$APP_{K670N/M671L}$	PrP	BL6/SJL	YES	NO	YES	YES	YES	Hsiao et al, 1996
APP23	$APP_{K670N/M671L}$	Thy1.2	BL6/DBA/BL6	YES	NO	YES	NO	YES	Sturchler-Pierrat et al., 1997
APPArcSwe	$APP_{E693G,K670N/M671L}$	Thy1	BL6/CBA	YES	NO	YES	NR	YES	Lord et al., 2006
TAPP	$APP_{K670N/M671L}$,hMAPT	PrP	BL6/SJL/DBA2/SW	YES	Spinal Cord	YES	YES	NR	Lewis et al., 2001
APP/PS1	$APP_{K670N/M671L}$,$PS1_{M146L}$	PrP	BL6/SJL/DBA/SW	YES	NO	YES	NO	YES	Holcomb et al., 1998
APP/PS1-21 Resize	$APP_{K670N/M671L}$ $PS1_{L166P}$	Thy1	BL6	YES	NO	YES	YES	Inconclusive	Radde et al., 2006
$APP751^{SL}$/PS1KI	$APP_{K670N/M671L, V717F}$ $PS1_{M233T, L235P}$ KI	Thy1	BL6/CBA/129	YES	NO	NR	YES	NR	Casas et al., 2004
3xTg-AD	$APP_{K670N/M671L}$, $MAPT_{P301L}$ $PS1_{M146V}$KI	Thy1.2	BL6/129SvJ	YES	YES	YES	NO	YES	Oddo et al, 2003
5XFAD	$APP_{K670N/M671L, I716V,V717E}$ $PS1_{M146L, L286V}$	Thy1	BL6/SJL	YES	NO	NO	NO	YES	Oakley et al., 2006

Table 4.1 Summary of AD transgenic mouse models.
APP: amyloid precursor protein; Aβ: amyloid beta; EC: extracellular; IN: intracellular; NR: not reported; PDGF: platelet-derived growth factor; PrP: prion protein; PS: presenilin. Adapted from (Morrissette et al., 2009)

4.2 Methods.

4.2.1 Genomic DNA isolation

Mouse genomic DNA was extracted from ear punch biopsies by overnight digestion in 300μL of proteinase K lysis buffer. The next day, 300μL of 1:1 phenol: chloroform was added, followed by vortex. Samples were centrifuged to separate the aqueous and organic layers, and the aqueous layer was carefully removed to a fresh 1.5 mL eppendorf tube. DNA was precipitated with isopropanol, and the pellet was washed twice with 70% ethanol, dried, and dissolved in 50μL TE buffer (pH 7.4). The quality and quantity of mouse genomic DNA was determined by spectrophotometry at OD260.

4.2.2 Transgenic mouse generation.

All animal experiment protocols were approved by the University of British Columbia Animal Care and Use committee. All transgenic mice used in this study were of the C57BL/6J genetic background, and as stated previously, APP23 transgenic mice carried human Swedish mutant APP 751 (see section 4.1). $PS1_{\Delta S169}$ was cloned into neuron-specific Thy1-promotor-containing vector and the resulting plasmids were confirmed by sequencing. Linearized mini-gene constructs were purified using a Gel Extraction Kit (QIAEX). Prepared DNA was microinjected into fertilized oocytes (CBA X C57Bl/6 F2) and survivors were transferred into pseudo-pregnant recipient female mice (CD-1/ICR). We analyzed 36 pups and identified 3 positive founder mice via PCR. APP23/$PS1_{\Delta S169}$ mice were generated by crossing heterozygous APP23 mice with heterozygous Thy1-$PS1_{\Delta S169}$ mice. The presence of both APP23 and $PS1_{\Delta S169}$ was confirmed by genotyping with primers. Genotyping PCR primers for PS1 were 5'-CACCACAGAATCCAAGTCGG (Thy1-E2 forward) and 5'-

GGTATCTTCTGTGAATGGGG (PS1-D-Tg reverse), with the target band being 420bp. Genotyping PCR primers for APP gene were 5'-CACCACAGAATCCAAGTCGG (Thy1-E2 forward) and 5'-CTTGACGTTCTGCCTCTTCC (App1082 reverse), with the target band being around 1.3kb**.**

4.2.3. Real-time PCR and copy number estimation.

Thy1-PS1$_{\Delta S169}$ plasmid DNA was extracted by Qiagen Hispeed plasmid midi kit (CAT#12643), linearized and quantified by gel electrophoresis. Then a series of 10-fold dilutions of Thy1-PS1$_{\Delta S169}$ plasmid DNA were mixed into 10 ng/μL of genomic DNA from non-transgenic mice to create a standard curve of real-time PCR data from known amounts of DNA template. Custom TaqMan Assays (ABI) were used to generate primers and probes for the mouse β-actin and human PS1 genes. The following primer pairs and probes were used: for the mouse β-actin assay: forward primer: (5'- AGCAAGACAAGATGGTGAATGGT); reverse primer: (5'- CCCTGTGGTTGTCAGAGCAA); probe: (5'-FAM-GAGCTCTCTGGGTGCTGGGATTCCC-NFQ). For human PS1 assay: forward primer: (5'- CACCACAGAATCCAAGTCGG); reverse primer: (5'-CCGTCTGTCGTTGTGCTCC); probe: (5'-FAM-GGATCTCGAGGCCACCATGACAGAG- NFQ). Real-time PCR was performed on ABI PRISM 7000. Ct values were generated from an assay specific to the human PS1 and mouse β-actin genes. Genomic DNA samples from Thy1-PS1$_{\Delta S169}$ transgenic mice or copy number standards were analyzed in a 25μL reaction volume; all reactions were performed in triplicate. Copy number estimates were derived from Ct values of the standard curve samples. The standard curve was created by drawing a scatter plot chart with Ct values

for each standard on the X axis, and the known logarithmic copy number of each standard on the Y axis (see Figure 4.2). Three genomic DNA samples from different Thy1-PS1$_{\Delta S169}$ transgenic mice, which shared a common founder, were used to determine the copy number of the human PS1 gene in these mice.

4.2.4 Body weight measurement and Rotorad test.

Mice were transferred via a specified transferring box from their home cage to a plastic box that was previously zeroed on the weighing scale. Body weight was recorded manually when the value on the scale stabilized. Mice were placed on a standard mouse rotarod with single station (ENV-576M, Med Associates Inc., USA). The shaft diameter was 3.2cm, lane width of 5.7cm, fall height of 16.5 cm, and divider diameter of 24.8cm. The rod was accelerated from 20 to 20,000 rpm over a 300 sec period. Latency to fall was measured manually. Mice were returned to their home cage and the second round test was repeated approximately 30min later. The average of two trials was taken as a measure of balance and motor coordination.

4.2.5 Neuritic Plaque Staining.

Mice were euthanized after the behavioral tests, and half of the brain was fixed and sectioned with a Leica cryostat (Deerfield, IL) to 30 μm thickness. Every sixth slice with the same reference position was mounted onto slides for staining. Slices were immunostained with biotinylated mono- clonal 4G8 antibody (Signet Laboratories, Dedham, MA) at 1:1000 dilutions. Approximately 24 slices were stained for each mouse. Plaques were visualized by the avidin-biotin-peroxidase complex (ABC) and diaminobenzidine (DAB) method, and counted under microscopy with 40×magnification. Plaques were

quantitated by average plaque count per slice for each mouse, and the data were analyzed by student t-test. Thioflavin S staining of plaques was performed with 1% thioflavin S, and the green fluorescence- stained plaques were visualized with fluorescent microscopy (Ly et al., 2011).

4.2.6 Morris Water Maze.

The water maze test was performed in a pool 1.5 m in diameter; the platform was 10 cm in diameter. The procedure consists of 1 day of visible platform tests and 4 days of hidden platform tests, plus a probe trial 24 h after the last hidden platform test. In visible platform tests, mice were tested for five contiguous trials, with an intertrial interval of 30 min; both the position of the platform and the start position varied with each trial. In hidden platform tests, mice were trained for six trials, with an inter-trial interval of 1 h; the platform was placed in the southeastern quadrant of the pool, and the start position varied with each trial. Tracking of animal movement was achieved with an HVS 2020 Plus image analyzer (HVS Image). In the probe trial, the platform was removed and mice were allowed to swim for 60 sec in the pool. The percentage of time spent in the third quadrant was analyzed. Escape latency and path length to reach the platform were analyzed as a measure of spatial learning and memory (Bromley-Brits et al., 2011).

4.2.7 Contextual fear conditioning.

We used a simplified contextual fear conditioning paradigm in our study. On day1, mice were placed in the conditioning chamber for 5min. The walls of the chamber were made of plexiglass and steel. The floor of the chamber was made of stainless steel rods, which were 2mm in diameter, spaced 5mm apart, and

connected to a shock generator. From the beginning of the 3rd min, mice received an unconditioned foot-shock stimulus (1mA, 50Hz) for 3s. 24h later, mice were placed into the same chamber for 4min without the foot-shock. In both trails, freezing behavior was recorded on a second-by-second basis Freeze Frame™ (ActiMetrics Software). Freezing was defined a stereotypical crouching posture combined with the absence of all movements, excluding respiratory-related movements (Blanchard and Blanchard, 1969). Total test time was divided into 60 sec bins. The conditioned response was measured by analyzing the fold-increase in the percentage of time spent freezing on Day2 and dividing by the average of the first 3 bins (0-120 sec, pre-shock) of Day1.

4.3 Results.

4.3.1 Generation and characterization of APP23/PS1$_{\Delta S169}$ double transgenic mice.

To investigate the effect of PS1$_{\Delta S169}$ on AD pathogenesis *in vivo*, we first generated heterozygous PS1$_{\Delta S169}$ mice in a C57BL/6J background strain via pronuclear injection method. Transgenic mice were identified by PCR with primers targeting the human PS1$_{\Delta S169}$, which was expressing under the control of the neuron-specific Thy-1 promoter (see methods). APP23/PS1$_{\Delta S169}$ double transgenic mice were established by crossing APP23 and PS1$_{\Delta S169}$ transgenic mice. PCR was performed to target the cDNA of the human APP Swedish mutant and human PS1$_{\Delta S169}$ genes. In APP23/PS1$_{\Delta S169}$ mice, both human APP fragment (655bp) and PS1 fragment (305bp) were detected in cortical brain tissue (Fig 4.1 A). Furthermore, immunoblot analysis showed that APP23/PS1$_{\Delta S169}$ mice expressed far more PS1 NTF than APP23 mice, which only had endogenous mouse PS expression, confirming the expression of

$PS1_{\Delta S169}$ (Fig 4.1 B). We also determined the difference in body weight and motor ability between APP23 mice and APP23/$PS1_{\Delta S169}$ mice. APP23 mice and APP23/$PS1_{\Delta S169}$ mice displayed similar body weight at 3 months (23.45±0.97 and 25.38±0.98g, respectively, p>0.05) and 6 months (28.93±1.04 and 29.63±1.22g, respectively, p>0.05) (Fig 4.1 C, D). APP23 mice and APP23/$PS1_{\Delta S169}$ mice also displayed similar performance in the rotorad test at 3 months (223.1±14.5 and 204.3±15.6s, respectively, p>0.05) and 6 months (220.8±15.4 and 210.3±15.3s, respectively, p>0.05) of age (Fig 4.1 E, F). These data demonstrated that APP23 mice and APP23/$PS1_{\Delta S169}$ mice are generally comparable and can be trained and tested in later cognitive behavior tests.

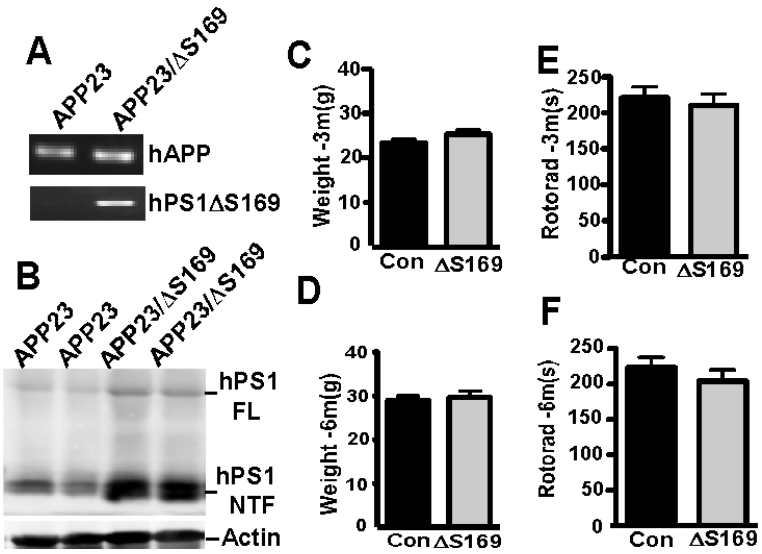


Figure 4.1 Establishment and characterization of APP23/$PS1_{\Delta S169}$ double transgenic mice. (A) Tail tips from APP23/$PS1_{\Delta S169}$ transgenic mice were collected under anesthesia (see Methods) and DNA was extracted via the phenol-chloroform method. Equal amounts of genomic DNA were used as template in a PCR reaction system with human APP or human PS primers (see Methods). (A) The targeted fragment sizes were 655bp for human APP human APP and 305bp for $PSEN1_{\Delta S169}$. (B) $PS1_{\Delta S169}$ expression was detected in the cortex of APP23/ $PS1_{\Delta S169}$ transgenic mice compared to APP23 control, as shown by an accumulation of PS1 NTF at 30kDa. (C, D) APP23/$PS1_{\Delta S169}$ and APP23 mice had similar body weights at 3 months of age. (E, F) APP23/$PS1_{\Delta S169}$ and APP23 mice showed similar performance in the rotorad test at 3months and 6 months of age.

4.3.2 APP23/PS1$_{\Delta S169}$ mice carry two copies of the human PS1$_{\Delta S169}$.

The effect of the transgene in transgenic mice is not only decided by the transgene itself but also by the levels of its expression. In PS1 transgenic mice, it is not optimal to have very high copy number of PS1 gene since the extra copies of PS1 gene might compensate or conceal some pathogenic effect of PS1 mutant. It is also a concern that high expression of PS1 gene would greatly accelerate the originally slow pathogenic progression. Thus, it is important to determine the copy number of PS1$_{\Delta S169}$ in APP23/PS1$_{\Delta S169}$ mice.

Copy number was determined by real-time PCR on samples taken from three PS1$_{\Delta S169}$ transgenic mice, which were from the same founder (see Methods). We plotted the standard curve to determine the copy numbers of β-actin gene and PS1$_{\Delta S169}$ gene with the real-time PCR results from standard curve samples, respectively (Fig 4.2 and Table 4.2). According to the standard curve of PS1$_{\Delta S169}$, we evaluated that APP23/PS1$_{\Delta S169}$ mice carried 2 copies of the human PS1$_{\Delta S169}$ gene (Table 4.2).

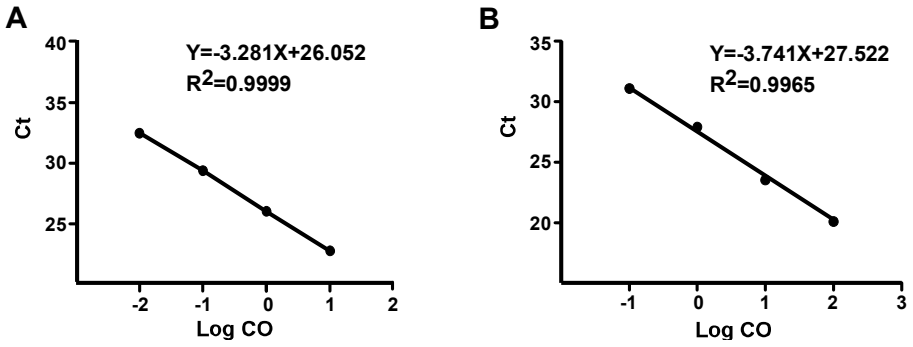


Figure 4.2 Standard curves for absolute quantitation of PS1$_{\Delta S169}$ copy number in APP23/PS1$_{\Delta S169}$ mice.
(A) Scatterplot chart for the mouse β-actin gene, which was used as an internal control. (B) Scatterplot chart for the human PS1 gene. Triplicate experiments provided copy number standards with high reproducibility (R2 = 0.9999, R2 = 0.9965) for estimating the copy number of samples.

β-Actin	Ct (#1)	Ct (#2)	Ct (#3)	Ct avg	DNA (μg)
standard curve sample-1	23.1	22.5	22.6	22.8	10.0
standard curve sample-2	26.0	26.0	26.1	26.1	1.0
standard curve sample-3	29.4	29.2	29.5	29.4	0.1
standard curve sample-4	32.5	32.3	32.7	32.6	0.0
standard curve sample-5	0.0	0.0	0.0	0.0	0.0
sample 1	22.6	22.1	22.3	22.4	10.0
sample 2	22.3	21.9	22.1	22.1	10.0
sample 3	23.1	22.4	22.5	22.7	10.0
PS1	**Ct (#1)**	**Ct (#2)**	**Ct (#3)**	**Ct avg**	**Copy number**
standard curve sample-1	20.9	18.2	21.1	20.1	100.0
standard curve sample-2	23.8	23.5	23.3	23.5	10.0
standard curve sample-3	28.0	27.5	28.3	27.9	1.0
standard curve sample-4	30.8	31.4	31.1	31.1	0.1
standard curve sample-5	0.0	0.0	0.0	0.0	0.0
sample 1	26.2	26.0	26.3	26.1	1.8
sample 2	26.4	25.6	25.9	25.7	1.9
sample 3	26.3	25.2	26.1	26.2	2.1

Table 4.2 Original Ct values for Copy number estimation.

4.3.3 APP processing and Aβ generation in APP23/ PS1$_{\Delta S169}$ double transgenic mice.

We first analyzed the APP processing and Aβ generation in APP23/PS1$_{\Delta S169}$ mice and APP23 mice. The cortical tissue of APP23/PS1$_{\Delta S169}$ mice was homogenized in lysis buffer and APP CTFs were determined by Western blotting. We found that APP23/PS1$_{\Delta S169}$ mice did significantly reduce the accumulation of APP CTFs under equal human APP expression levels, compared with APP23 mice (Fig 4.3 A). This is not conflict with our in vitro data, showing that PS1$_{\Delta S169}$ exhibited impaired γ-secretase activity. Because of the dosage effect, the extra 2 copy of PS1$_{\Delta S169}$ in APP23/PS1$_{\Delta S169}$ mice might compensate its deficits, although PS1$_{\Delta S169}$ is a pathogenic mutation with impaired APP processing. Aβ42 is proposed to initiate the aggregation of Aβ,

all pathogenic PS1 mutations demonstrated increased Aβ42/40 ratio. Brain cortical tissue of APP23 and APP23/PS1$_{\Delta S169}$ mice was prepared according to Aβ ELLISA Kit instructions (Invitrogen). APP23/PS1$_{\Delta S169}$ mice had significantly increased Aβ42/40 ratio to 2.43 ± 0.61 folds ($p < 0.05$), as compared with APP23 mice (Fig 4.3 B). These date demonstrated that APP23/PS1$_{\Delta S169}$ promoted the generation of the more hydrophobic Aβ42 peptide *in vivo*.

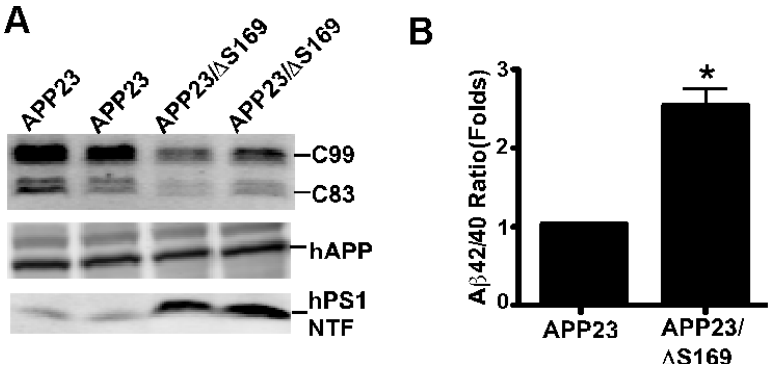


Figure 4.3 APP processing and Aβ generation in APP23/PS1$_{\Delta S169}$ and APP23 mice. (A) Brain cortical tissue was collected from APP23 and APP23/PS1$_{\Delta S169}$ mice and homogenized in RIPA-DOC lysis buffer. APP CTFs and full length APP were detected with C20 antibody; human PS1 NTF was detected with PS1N antibody. (B) Brain cortical tissue of APP23 and APP23/PS1$_{\Delta S169}$ mice was prepared according to Aβ ELISA Kit instructions (Invitrogen), and the Aβ level was determined. The values represent mean±SEM. N=6, *p<0.05, by student t-test.

4.3.4 PS1$_{\Delta S169}$ promoted neuritic plaque formation in the transgenic mice.

Although endogenous APP is expressed in mouse brain, the lack of a critical Aβ region in murine APP prevents amyloid plaque formation in mice. To mimic the most characteristic pathology of human AD, human APP is introduced into mouse brain to initiate plaque deposition. APP23 transgenic mice specifically express human Swedish mutant APP in neurons. Although APP23 mice develop amyloid plaques in the hippocampus and neocortex as early as 6 months of age,

significant plaque deposition is not observed until 12 months of age (Sturchler-Pierrat et al., 1997, Sun et al., 2006a). To test whether $PS1_{\Delta S169}$ could promote plaque deposition, APP23/$PS1_{\Delta S169}$ and age-matched control APP23 mice were sacrificed after behavioral tests at 3 and 6 months.

Amyloid plaques were detected by 4G8 immunostaining and confirmed by staining with thioflavin S (Fig 4.4). From 3 months to 5 months, no plaques were detectable in either APP23/$PS1_{\Delta S169}$ or APP23 mice (data not shown). At 6 months, amyloid plaques were detected in APP23/$PS1_{\Delta S169}$ mice, but not in APP23 mice (Fig 4.4 A, B). APP23/$PS1_{\Delta S169}$ mice had an average plaque number per slice of 9.45 (9.45±1.09 per slice, $p<0.05$; Fig 4.4 C), compared to the 0 plaque per slice seen in APP23 mice, suggesting that $PS1_{\Delta S169}$ significantly accelerated plaque formation in the APP23/$PS1_{\Delta S169}$ mice from 6 months. At 12 months, amyloid plaques could be detected in APP23 mice; however, APP23/$PS1_{\Delta S169}$ mice exhibited significantly more plaque formation in the same hippocampal region (Fig 4.4). APP23/$PS1_{\Delta S169}$ mice had 2.64 fold more plaques than APP23 mice (36.5 ± 4.79 vs. 14.55 ± 2.29 per slice; $p < 0.001$; Fig 4.4 D), indicating that $PS1_{\Delta S169}$ continued to promote the plaque formation at 12 months of age. Thioflavin S staining method also confirmed that $PS1_{\Delta S169}$ accelerated plaque formation and increased plaque number in APP23/$PS1_{\Delta S169}$ mice (Fig 4.4 B).

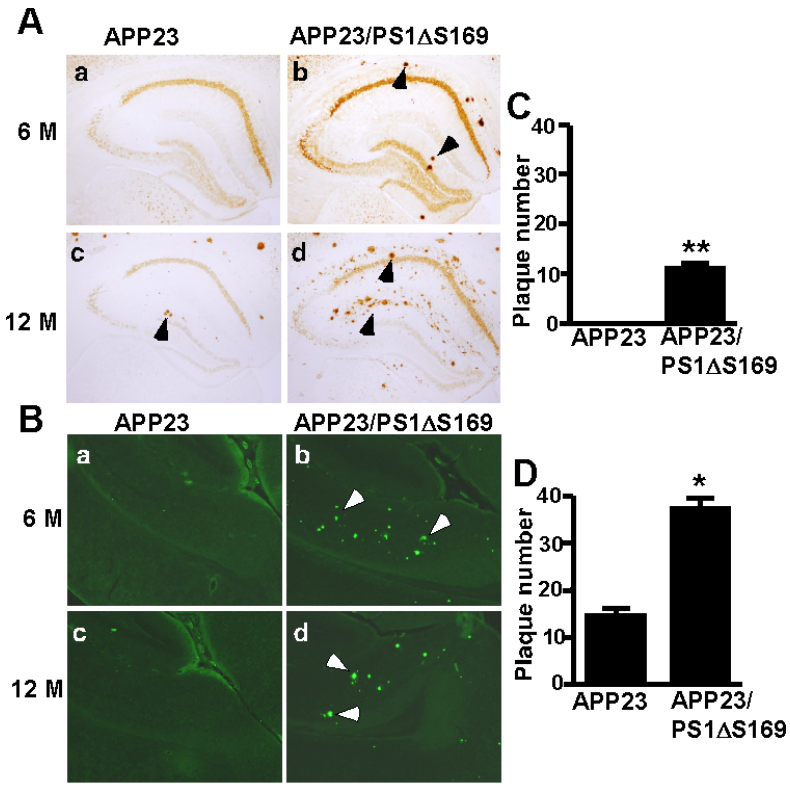


Figure 4.4 $PS1_{\Delta S169}$ significantly promoted amyloid plaque formation in the transgenic mice.
(A) APP23/$PS1_{\Delta S169}$ and APP23 transgenic mice at 6 and 12 months of age were euthanized after behavioral tests, and the brains were dissected, fixed, and sectioned. Amyloid plaques were detected with Aβ-specific monoclonal antibody 4G8 via the DAB method. Plaques were visualized by microscopy with 40×magnification. **a** and **c** showed representative brain sections from APP23 mice at 6 and 12 months of age, respectively; **b** and **d** showed representative brain sections from APP23/$PS1_{\Delta S169}$ mice in 6 and 12 months of age, respectively. Black arrows point to plaques. (B) Amyloid plaques were further confirmed using thioflavin S fluorescent staining and visualized by microscopy at 40×magnifications. There were more amyloid plaques in APP23/$PS1_{\Delta S169}$ mice (**b** and **d**) compared with age-matched APP23 mice (**a** and **c**). White arrows point to green fluorescent neuritic plaques. (C) Quantification of amyloid plaques in APP23 and APP23/ $PS1_{\Delta S169}$ mice at 6 months of age. Numbers represented mean ± SEM; n = 10 each; **, $p < 0.005$ by Student's t-test. (D) Quantification of amyloid plaques in APP23 and APP23/ $PS1_{\Delta S169}$ mice at 12 months of age. Numbers represented mean ± SEM; n = 10 each; *, $p < 0.01$ by Student's t-test.

4.3.5 APP23/PS1$_{\Delta S169}$ double transgenic mice exhibited age-dependent memory impairment at 3 months of age.

To investigate whether PS1$_{\Delta S169}$ affected learning and memory in AD pathogenesis in an age-dependent manner, Morris water maze and fear conditioning tests were performed at 3 months and 6 months of age. At 3 months, APP23 controls and APP23/PS1$_{\Delta S169}$ mice had a similar escape latency (30.46 ± 2.11s, n=21 and 31.51 ± 2.18s, n=15, respectively; $p > 0.05$; Fig 4.5 A) and path length (6.02 ± 0.58s, n=2 and 6.53 ± 0.45, n =15, respectively; $p>$ 0.05; Fig 4.5 B) in the visible platform tests of the Morris water maze, indicating that both groups of mice had similar motility and vision. In the hidden platform and probe trials which followed, no significant differences were seen in escape latency, path length, and number of times the mouse crossed the hidden platform area (Fig 4.5 C, D, E; $p > 0.05$). However, in the contextual fear conditioning test, the APP23/PS1$_{\Delta S169}$ mice froze significantly less on Day 2 (14.8 ± 3.45%) compared with APP23 mice (23.7 ± 3.26%) ($p<0.05$; Fig 4.5 F), suggesting that PS1$_{\Delta S169}$ promoted the memory deficits in APP23 mice at 3 months of age.

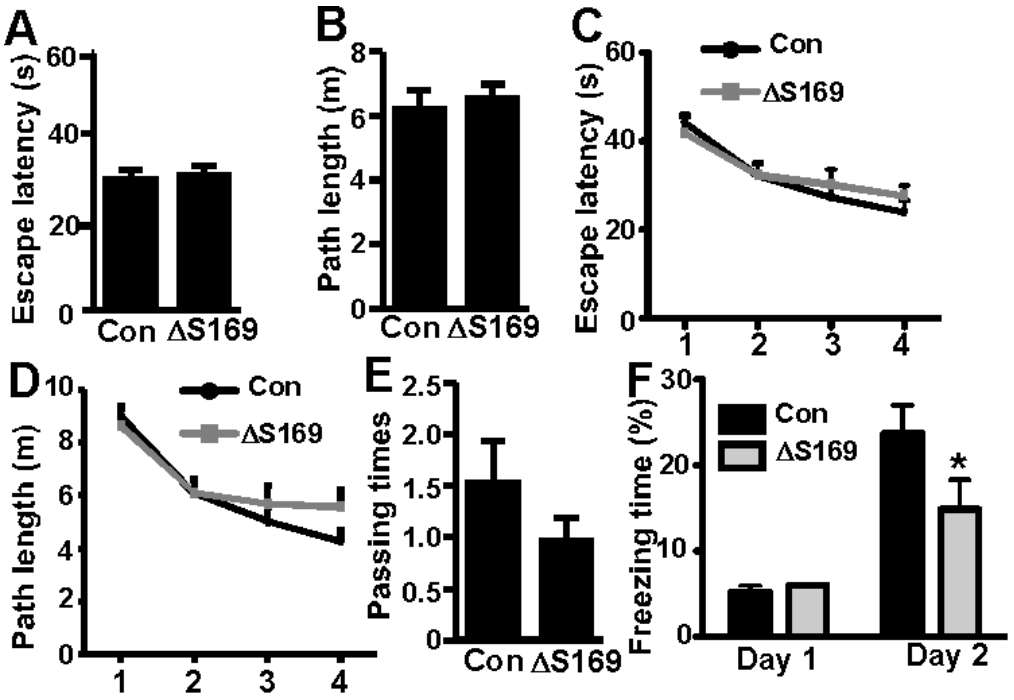


Figure 4.5 PS1$_{\Delta S169}$ negatively affects fear conditioning, but not water maze performance of APP23 mice at 3 months of age.

A Morris water maze test consists of 1 day of visible platform tests and 4 day of hidden platform tests, plus a probe trial 24 h after the last hidden platform test. Animal movement was tracked and recorded by a HVS 2020 Plus image analyzer. APP23/PS1$_{\Delta S169}$ (n=15) and APP23 transgenic mice (n=21) were tested at 3 months of age. (A) During the first day of visible platform tests, APP23/PS1$_{\Delta S169}$ and APP23 mice exhibited a similar latency to escape onto the visible platform. $p > 0.05$ by Student's t test. (B) APP23/PS1$_{\Delta S169}$ and APP23 mice had similar swimming distances before escaping onto the visible platform in the visible platform test. $p > 0.05$ by Student's t test. (C) In hidden platform tests, mice were trained with 5 trials per day for 4 days. APP23/PS1$_{\Delta S169}$ and APP23 mice showed a similar latency to escape onto the hidden platform, $p > 0.05$ by two-way ANOVA; (D) APP23/PS1$_{\Delta S169}$ and APP23 mice had a similar swimming length to escape onto the hidden platform. $p > 0.05$ by two-way ANOVA. (E) In the probe trial on the 6th day, APP23/PS1$_{\Delta S169}$ and APP23 mice traveled similar times into the third quadrant, where the hidden platform was previously placed. $p > 0.05$ by Student's t-test. (F) In the contextual fear conditioning test (see Methods), on day1, APP23/PS1$_{\Delta S169}$ and APP23 mice demonstrated indistinguishable freezing level immediately after foot shock; $p > 0.05$ by Student's t-test. On day 2 (24 h later), APP23/PS1$_{\Delta S169}$ mice demonstrated significantly reduced freezing levels compared with APP23 mice in the same contextual cage without shock; $p < 0.05$ by Student's t-test.

APP23 and APP23/PS1$_{\Delta S169}$ mice were also subjected to the Morris water maze and contextual fear conditioning tests at 6 months of age. On day1 of the visible platform test, APP23 and APP23/PS1$_{\Delta S169}$ mice had a similar escape latency (27.81 ± 1.86 s, n =14 and 32.47 ± 2.90s, n =12, respectively; $p > 0.05$) (Fig 4.6 A) and path length (5.94± 0.39s, n =14 and 6.38 ± 0.57s, n =12, respectively; $p > 0.05$) (Fig 4.6 B), suggesting normal motility and vision. In the hidden platform tests on day 2-5, APP23/PS1$_{\Delta S169}$ mice showed significant deficits compared with APP23 controls. The escape latency on the third and fourth days of APP23/PS1$_{\Delta S169}$ mice (38.01 ± 2.75 s and 36.07 ± 2.96s, n =12, respectively; $p < 0.05$) was longer than APP23 mice (29.69 ± 2.44 s and 22.85 ± 2.13s, n =14, respectively; $p < 0.05$) (Fig 4.6 C). APP23/PS1$_{\Delta S169}$ mice also swam significantly longer distances (6.44 ± 0.65s and 5.49± 0.37s, n =12, respectively; $p < 0.05$) to reach the platform as compared with APP23 mice (5.43 ± 0.4s and 4.16 ± 0.36s, n =14, respectively; $p < 0.05$) (Fig 4.6 D). In the probe trial on the last day of testing, the platform was removed and the number of times the mice passed the platform's previous position was measured. APP23/PS1$_{\Delta S169}$ mice made significantly fewer passes compared with controls (1.45 ± 0.15 and 3.63 ± 0.37, respectively; $p < 0.005$; Fig 4.6 E). These data indicated that APP23/PS1$_{\Delta S169}$ mice have significantly worse memory deficits compared with APP23 mice. In the contextual fear conditioning test, the APP23/PS1$_{\Delta S169}$ mice froze significantly less on Day 2 (13.4 ± 2.91%) compared with APP23 mice (27.9 ± 3.8%) (Fig 4.6 F). Together with the behavioral performance of 3 months old mice, the impaired performance of 6 months old APP23/PS1$_{\Delta S169}$ mice in the Water maze and fear conditioning tasks indicated that PS1$_{\Delta S169}$ continues to exacerbate memory deficits of 6 months old APP23 mice in an age-dependent manner.

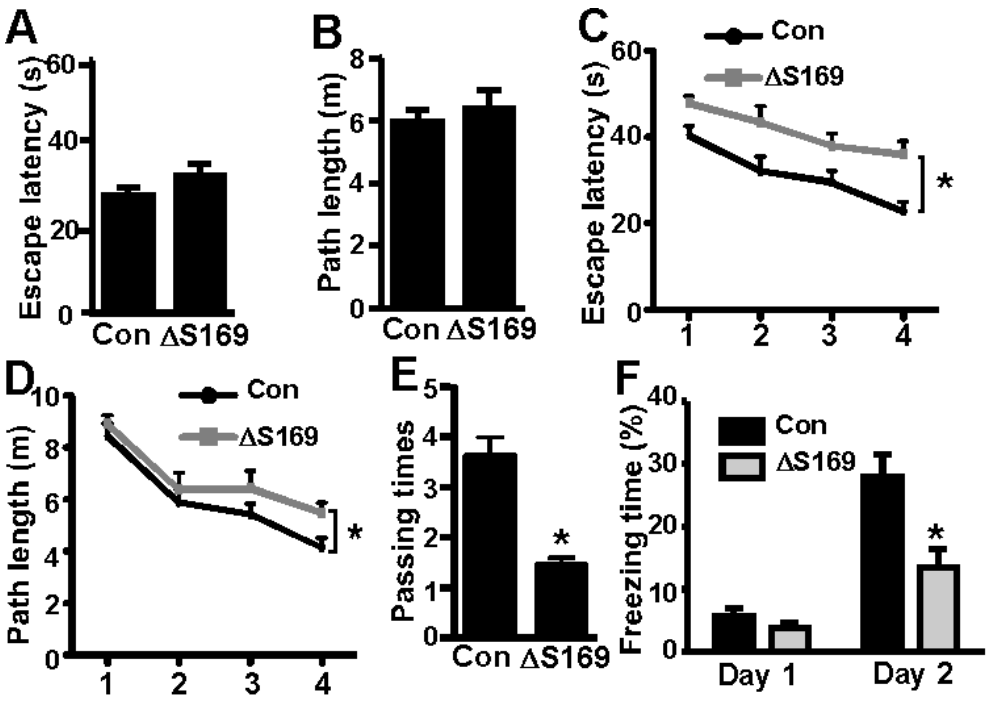


Figure 4.6 PS1$_{\Delta S169}$ exacerbates memory deficits in APP23 mice at 6 months of age. APP23 transgenic mice (n=14) and APP23/PS1$_{\Delta S169}$ (n=12) were tested at 6 months of age. (A, B) On day 1, APP23/ PS1$_{\Delta S169}$ and APP23 mice exhibited a similar escape latency and swimming path length to escape onto the visible platform. $p > 0.05$ by Student's *t*-test. (C, D) In hidden platform tests on day 2 to 5, APP23/PS1$_{\Delta S169}$ mice showed a longer escape latency and swimming path length to escape onto the hidden platform. $p < 0.01$ by ANOVA. (E) In the probe trial on the sixth day, APP23/PS1$_{\Delta S169}$ mice traveled into the third quadrant, where the hidden platform was previously placed, significantly less times than controls. *, $p < 0.01$ by Student's *t*-test. (F) On day1 of the contextual fear conditioning test, APP23/PS1$_{\Delta S169}$ and APP23 mice demonstrated indistinguishable freezing levels immediately after foot shock. $p > 0.05$ by Student's *t*-test. 24h later, APP23/ PS1$_{\Delta S169}$ mice demonstrated significantly reduced freezing level compare with APP23 mice in the same contextual cage without shock at Day2. $p < 0.05$ by Student's *t*-test.

4.4 Discussion.

To investigate the effect of PS1$_{\Delta S169}$ on AD pathogenesis *in vivo*, we generated PS1$_{\Delta S169}$ transgenic mice and crossed them with APP23 transgenic mice. The APP23 mice overexpress human APP-751 cDNA harboring the human Swedish

mutation (KM670/671NL) under control of a neuron-specific mouse Thy-1 promoter. In APP23 mice, Swedish APP is expressed 7-fold higher than endogenous mouse APP, with higher levels obtained in the deep cortical layers and the hippocampus, and lower in other brain regions such as the thalamus (Sturchler-Pierrat et al., 1997). From 6 months of age, small amounts of amyloid plaques can be detected in the cerebral cortex of APP23 mice, and the size and number of plaques progresses with age (Sturchler-Pierrat et al., 1997).

Pathogenic PS1 mutants have enhanced Aβ deposition and plaque formation in APP23 mice due to increased Aβ42 production, like $PS1_{M146L}$. This effect was not the result of elevated PS levels, because introduction of wild type PS would not alter the Aβ42/40 ratio or promote plaque formation (Duff et al., 1996). It has been reported that, compared with APP transgenic mice, $APP/PS1_{mutant}$ mice have an increased Aβ 42/40 ratio, earlier plaque formation, and more rapid progression of memory deficits (Borchelt et al., 1997, Citron et al., 1997, Holcomb et al., 1998, Lamb et al., 1999). In chapter 4, we found that as compared with APP23 littermates, $APP23/PS1_{\Delta S169}$ mice developed plaques sooner (6 months) and displayed a more severe plaque load by 12 months of age. Using Morris water maze and contextual fear conditioning tests, we found that $PS1_{\Delta S169}$ promoted the memory impairment of APP23 mice starting from 3 months of age. The time interval between plaque detection and abnormal performance in behavioral tests could be interpreted as the toxicity of soluble or oligomerized Aβ prior to plaque formation (Haass and Selkoe, 2007).

A good animal model should be able to mimic the onset and progression of the disease. Taking C57BL/6J as an example, 6 months old mice are equivalent to 30 years old humans, and 12 months equivalent to 42.5 years old humans (Fox,

2006). APP23/PS45 double transgenic mice overexpress both Swedish APP and $PS1_{G384A}$. The APP23/PS45 transgenic mice maintained in our laboratories develop plaque pathology in the neocortex and hippocampus as early as 1 month of age, equivalent to 12.5 years in a human. A human-equivalent age of onset of 12.5 years is too young even for FAD, and this discrepancy in age of onset may not accurately characterize the real picture of AD pathogenesis, which often occurs in an aging body. APP23/PS45 mice develop plaques at 1 month of age because the $PS1_{G384A}$ transgene is artificially overexpressed at a very high level, which could give rise to false results due to the robust overexpression. APP23/$PS1_{\Delta S169}$ mice showed plaques development from 6 months of age (no plaque detection in 4 and 5 months of age, data not shown), equal to approximately 30 of human years; this is much closer to findings in human PS1 mutations. Moreover, animal models such as APP23/PS45 are not suitable for testing therapeutics testing due to the rapid development of plaques. Because APP23/$PS1_{\Delta S169}$ mice develop plaques at a much slower rate, these mice would be better animal model to observe the slow effect of drug treatments.

One potential pitfall in our study was that we used transgenic mice instead of knock-in (KI) mice. To make our mice comparable to APP23/PS45 mice and to shorten the research time, we utilized pronuclear injection method to overexpress human $PS1_{\Delta S169}$ gene in brain. It was previously shown that overexpressed human PS1 could replace endogenous murine PS1 and act as the predominant γ-secretase, resulting from competition for limiting cellular factors (Thinakaran et al., 1997). Although APP23/$PS1_{\Delta S169}$ mice carried only 2 copies of the human $PS1_{\Delta S169}$ gene, the effect of the endogenous wild type mouse PS1 could not be excluded. The virtue of KI mice is the elimination of endogenous

target genes and the similar transgene expression level, as compared with physiological levels. Thus, KI mice could reflect the effect of $PS1_{\Delta S169}$ on APP processing and plaque pathology more accurately.

The limitations in relating the animal studies to the human disease need specific attention. According to the European statistical report on the (2010), mouse is accounting for 59% of the total number of animals used for experimental and other scientific purpose. However, mouse is not an appropriate model for neurodegenerative model, strictly. First and foremost, the short lifespan of mice is around 2 years, which is quite short relative to humans (Jankowsky et al., 2004b); whereas aging is the well-established risk factor for most neurodegenerative disease. Second, it is very hard to mimic the pathogenesis of complex disease (e.g. AD) in mice model with such short lifespan in the simplified experimental conditions. Third, the pure genetic ground of transgenic mice simplifies experimental conditions but poses the obstacle to apply the results to the following clinical trials in human patients. Taken AD as an example, it is currently accepted that FAD and sporadic AD are the same disease in different forms. While FAD is considered as Mendelian, the late-onset sporadic AD is non-mendelian Alzheimer's disease. Sporadic AD is classified as a complex disease, resulting from the comprehensive effect of multiple factors, such as complex genetic background and environment. However, the majority of AD transgenic mice are established based on the FAD studies, like our APP23/$PS1_{\Delta S169}$ mice. A therapeutics, which is significant efficient in APP23/$PS1_{\Delta S169}$ mice, might has insignificant effect in AD patients, such as semagascestat (Lanz et al., 2006, Bateman et al., 2009).

4.5 Conclusion.

In chapter 4, we confirmed the impaired processing of APP by $PS1_{\Delta S169}$ in APP23/$PS1_{\Delta S169}$ transgenic mice. Compared with APP23 mice, APP23/$PS1_{\Delta S169}$ mice have an advanced plaque formation time and increased plaque load. Behaviorally, $PS1_{\Delta S169}$ mice displayed enhanced memory deficits in fear conditioning at 3 months of age, and impaired performance in Morris water maze at 6 months of age, indicating that $PS1_{\Delta S169}$ also promotes the cognitive impairment seen in aging APP23 mice.

Chapter 5

Discussions and future directions

5.1 Revisiting the "Amyloid hypothesis".

The "amyloid hypothesis" is the central dogma in current AD field (Hardy and Selkoe, 2002). Despite the debate on whether its steady-state form (plaque) or its metastable form (oligomers) is the real toxic species, Aβ itself is established as the culprit of AD pathogenesis (Haass and Selkoe, 2007). However, since it was proposed, "amyloid hypothesis" has been strongly challenged by several lines of evidence. First, if Aβ is the cause of AD, why is amyloid plaques/amyloid load poorly correlated with cognitive function decline in AD patients? Second, how should we explain the substantial amyloid deposition in approximately 20%-30% of cognitively normal elderly (Aizenstein et al., 2008)? Third, what mechanism underlies the reduced Aβ42 in the cerebrospinal fluid (CSF), which has been established as a biomarker for AD diagnosis (Motter et al., 1995, Galasko et al., 1998)? Last but not the least, how should we interpret the failure in all of the amyloid-β-centric therapeutic approaches that reached Phase III clinical trials? For instance, Eli Lilly and Company had recently announced to discontinue the phase III trial of semagascestat (LY450139), the first blood-brain barrier permeable γ-secretase inhibitor. In phase III, semagascestat displayed worse cognitive performance as well as daily activities

compared with the placebo controls; moreover, semagascestat also brought intolerable side effects like skin cancer (Cummings, 2010).

5.1.1 Possible explanations to the paradox in the "Amyloid hypothesis".

The aggregation of Aβ is a dynamic procedure, progressively going from monomers to dimers/trimers, and then through oligomers and protofibrils to the final formation of plaques. Given the mounting evidence in support of the toxicity of soluble amyloid oligomers, it is proposed that the amyloid plaques act as the inert reservoir, whereas the oligomers are the real culprit that initiates AD pathogenesis. Moreover, some studies demonstrated that the intracellular Aβ accumulation precedes its extracellular deposition (Oddo et al., 2003, LaFerla et al., 2007), indicating the contribution of "the toxicity of intracellular Aβ" in AD pathogenesis. If "amyloid oligomers" and "intracellular Aβ" are the actual toxic amyloids, it explains why the number of amyloid plaques is poorly correlated with the cognitive impairment in AD patients.

Besides, recent therapeutic studies shed some light on the exact role of Aβ in the complex pathogenesis of AD. It was reported that the cognitive decline continues in the Aβ vaccination despite the effective clearance of plaques (Holmes et al., 2008). Similarly, amyloid imaging via Pittsburgh compound B (PIB) binding PET scan confirmed that the amyloid burden changed little once significant cognitive impairment occurred (Wang et al., 2002, Sojkova et al., 2011, Villemagne et al., 2011). The reduction of Aβ42 in cerebrospinal fluid (CSF) is widely used as a biomarker in AD diagnosis, which is inversed related with the increased PIB bind (Fagan et al., 2006). Further studies find similar amyloid load between MCI (mild cognitive impairment) and AD, and thus

postulated that the majority amyloid deposition occurred before significant cognitive decline (Shaw et al., 2009, De Meyer et al., 2010, Jack et al., 2010). These clinical findings indicated that Aβ is more likely to act as a trigger than a driver, which is not consistently required throughout the whole AD pathogenesis. Again, the "amyloid-trigger" scenario also explains the poor correlation of amyloid load and cognitive decline in AD patients, as mentioned above.

5.1.2 "Amyloid hypothesis" and novel risk factors in late-onset sporadic AD.

While FAD is considered as Mendelian, the late-onset sporadic AD is non-mendelian Alzheimer's disease. Sporadic AD is classified as a complex disease, resulting from the comprehensive effect of multiple factors, such as complex genetic background and environment. The advances in GWAS in sporadic AD postulate an alternate hypothesis to the amyloid-centric dogma. The SORL1, also known as a neuronal apolipoprotein E receptor, was reported to be associated with both familial and sporadic AD (Rogaeva et al., 2007). Earlier studies implicated that SORL1 was involved in the regulation of the endocytosis of APP. Meanwhile, PICALM, coding a protein involved in clathrin-mediated endocytosis, was found as a risk factor for sporadic AD (Harold et al., 2009). As mentioned in chapter 1, the classical theory on APP trafficking proposed that the majority of Aβ was produced in the endocytosis pathway (Andersen et al., 2005). Taken together, it is possible that the abnormal endocytosis of APP plays a role in AD pathogenesis. Besides, the new findings on TREM2 emphasized the role of inflammation in AD pathogenesis (Guerreiro et al., 2013, Jonsson et al., 2013). All these novel discoveries in sporadic AD

support AD as a complex disease and also provide a new therapeutic target for AD treatment.

5.1.3 Linking early-onset FAD with late-onset sporadic AD.

One of the conundrums in AD field is connecting the findings in FAD with sporadic AD cases. FAD-associated mutations are the most powerful evidence in support of the "amyloid hypothesis". APP is the precursor of Aβ, whereas PS is the enzyme responsible for the generation of the more hydrophobic Aβ42. In early-onset FAD, mutations on APP and Presenilins, both of which synergistically promotes the production of the more aggregation-prone Aβ42, building a full "amyloid story" that embraces both the substrate (APP) and the enzyme (γ-secretase) in amyloid pathway. Although patients with FAD-associated mutations develop AD symptoms around 10 years earlier than sporadic AD, FAD and sporadic AD are undistinguishable both clinically pathologically. It is currently accepted that FAD and sporadic AD are the same disease in different forms.

But strikingly, as is mentioned above, there is no successful case in the clinical trials based on the discoveries from the studies of FAD. Taking semagascestat as an example, it was reported a significant reduced amyloid deposition in PDAPP transgenic mice and lower level of newly synthesized Aβ in human volunteers (Lanz et al., 2006, Bateman et al., 2009). However, the phase III clinical trial of semagascestat demonstrated worse cognitive performance than controls. Using stable isotope labeled kinetic (SILK) techniques, the clearance of Aβ was reduced in sporadic AD, which is a distinguishable mechanism compared with the abnormal production of Aβ in FAD. Thus, it is proposed that

abnormal Aβ generation is the culprit in FAD, whereas in sporadic AD it is the disturbed clearance of Aβ.

According to the "amyloid-trigger" scenario mentioned in 5.1.1, once the pathogenesis is initiated by Aβ, it is hard to reverse the whole progression. Thus the most important thing in AD therapeutics is timing, timing and timing. The best timing is probably before the amyloid deposition or the development of MCI clinically. Maybe that is why there has been no successful amyloid-centric therapeutics and the ideal subjects should be the populations with higher risk but no signs of MCI. With the appropriate subjects, the therapeutics based on FAD studies, like robust γ-secretase inhibitor, should take effect in treating the sporadic cases.

5.2 The significance of the research.

The overall goal of the studies on AD is to find the approaches to prevent the onset of this disease or ameliorate the symptoms. Why millions of funding investments on the studies of the only 5% FAD cases? The first reason is that there are too many contributors in sporadic AD, although it is accounting for 95% of AD cases. From the pharmaceutical perspective, it is very hard to develop an effective drug only targeting a small piece of the disease pathogenesis. The second reason is that the limited established genetic factors in FAD greatly narrow down the drug targets to several potential ones – APP (substrate) and Presenilins (enzyme). To meet the demands, this dissertation aims to elucidate how mutations in the APP and PS genes affect APP processing and the resulting amyloidogenesis *in vitro* and *in vivo* as well as the potential to develop the effective therapeutics for AD.

5.2.1 The comprehensive effect of APP_{A673V} on the metabolism of APP.

APP is synthesized in ER and delivered through the classical secretory pathway. Along its trafficking pathway, APP undergoes serial modifications including N- and O-glycosylation in the ER and Golgi compartments respectively (Weidemann et al., 1989, Suzuki et al., 2006). This process is also called APP maturation; the N-glycosylated APP in ER is defined as immature APP (imAPP), whereas the O-glycosylated APP in the Golgi is mature APP (mAPP). It has been documented that only mature APP is a substrate for later secretase cleavages (Tomita et al., 1998).

It has been established that the Swedish APP mutation contributes to AD pathogenesis by promoting β-cleavage at the Asp-1 site, predominantly producing C99 production, as opposed to the C89 production seen in wild type APP (Deng et al., 2013). However, the underlying mechanism of this shift is not clarified. There are two plausible working mechanisms. First, the trafficking pathway of APP might be altered by the Swedish mutation. For decades, it has been proposed that the trafficking of APP_{SWE} and APP_{WT} are distinguishable. In wild type APP, α-cleavages occur on the plasma membrane and β-cleavages and Aβ generation occur in the endocytic organelles such as endosomes; whereas in the Swedish mutation, APP can be processed by β-secretase and generates Aβ in the medial Golgi and compartments close to the plasma membrane in the secretory pathway of delivering APP to the plasma membrane (Lo et al., 1994, Haass et al., 1995, Thinakaran et al., 1996b). Second, the structure of APP might be altered by the Swedish mutation. Given that the locus the Swedish mutation (KM670/671NL) is near the Asp-1 site of β-cleavage, another possibility is that the two mutated residues (NL) confer structural transformation to APP, which facilitate the β-secretase cleavage at Asp-1 site.

Sauder et al. reported that several hydrophobic residues in BACE1 form an active pocket with the residues around Asp-1 site, like Leu671 in APP_{SWE}, which contributes to the preference cleavage of BACE1 in the Swedish APP (Sauder et al., 2000). Antibodies against the region around Glu-11 site have been shown to block β-cleavage at this site without significantly affecting its trafficking (Arbel et al., 2005, Paganetti et al., 2005, Thomas et al., 2006, Boddapati et al., 2011, Thomas et al., 2011). These studies imply that the structure of APP plays an important role in determining the processing.

Although we are still in need of the stronger genetic evidence, APP_{A673V} is the first recessive APP mutation to be proposed, based on current available genetic and molecular evidence. Our study confirms the possibility that APP_{A673V} is a recessive mutation but in a novel working mechanism: firstly, the immature APP_{A673V} in ER as well as the mature (modified) APP_{A673V} in TGN can be processed to C99, whereas APP_{WT} cannot, indicating that the structure of APP_{A673V} facilitates the β-secretase cleavage regardless of the maturation. Secondly, APP_{A673V} undergoes faster lysosome-dependent degradation, possibly because the structural transformation makes APP_{A673V} a better substrate for lysosome-dependent degradation. The two findings not only support that APP_{A673V} is a recessive mutation, but also provide potential the potential pharmaceutical strategy. More specifically, an antibody against alanine at APP 673 could be developed to regulate amyloidogenesis.

5.2.2 The effect of $PS1_{\Delta S169}$ on APP processing and Notch signaling.

$PS1_{\Delta S169}$ was a recently discovered pathogenic PS1 mutation in a Chinese FAD family, and this thesis thoroughly studied and confirmed the amyloidogenic effect of $PS1_{\Delta S169}$ both *in vitro* and *in vivo* in chapter 3 and chapter4. Our study

confirmed the pathogenic effect of $PS1_{\Delta S169}$ on APP processing, and mimicked its amyloidogenic effect in transgenic mice, establishing a potential AD animal model for later studies on AD pathogenesis. Furthermore, we demonstrated that $PS1_{\Delta S169}$ preserved the processing of Notch, indicating that the structural alteration imposed by $PS1_{\Delta S169}$ specifically affected Aβ generation. This not only excluded the arguable contribution of impaired Notch signaling to AD pathogenesis, but also offered the potential for researchers to develop specific γ-secretase inhibitors that spare Notch signaling in future.

In the development of γ-secretase inhibitors, the biggest obstacle is to circumvent their side effects on Notch-signaling. As we discussed in chapter 1, it was a technological challenge to decipher its crystal structure using traditional methods, because PS1 is a multiple transmembrane protein that requires assistant subunits to assemble the γ-secretase complex. The current understanding of the structure of presenilins comes from indirect methods such as cysteine mutagenesis with cross-linking of chemical probes, which proposed that the PS1 transmembrane domain was important in constituting the catalytic pore of γ-secretase in the hydrophobic environment (Sato et al., 2006a, Tolia et al., 2006a, Sato et al., 2008a, Tolia et al., 2008). $PS1_{\Delta S169}$ is located in the transmembrane domain and demonstrated reserved processing of Notch, and thus the clarification of the structure of $PS1_{\Delta S169}$ shed light on developing specific γ-secretase inhibitors that spare Notch signaling.

5.3 Limitations of the research.

5.3.1 Lack of strong genetic evidence for APP_{A673V} being as the recessive mutation.

Di Fede et al. discovered the homozygous APP_{A673V} mutation in an Italian family by the analyzing the entire coding sequence of PSEN1, PSEN2, and the genes encoding the microtubule-associated protein tau, progranulin, prion protein and huntingtin (Di Fede et al., 2009). However, it lacks the linkage disequilibrium study in this family and the SNP analysis in normal controls. Back in 1993, Peacock et al. reported that heterozygous APP_{A673V} was a novel polymorphism in APP gene in a patient who had ischemic cerebrovascular disease but on no evidence of AD (Peacock et al., 1993). Recently, a novel mutation on the same locus of APP_{A673V} was reported – APP_{A673T} (Jonsson et al., 2012). This group analyzed the whole genome sequence of 1775 Icelanders and found that APP_{A673T} protected against AD pathogenesis. Given such multifaceted behavior of the polymorphism in this locus, the genetic evidence is quite weak based on a single consanguineous sib-pair that shares much of the genome "identical-by-decent', not only the APP gene. Further analysis such as linkage disequilibrium and exclusion of SNPs should be included as we discussed in 5.3.1.

5.3.3 Lack of evidence of APP trafficking in neuronal model.

Neuron is a highly polarized and specialized cell type, characterized with multiple specialized structures (dendrites and axons) and microdomains (presynaptic and postsynaptic region) with distinguishable functions. It is no exaggeration to say that neurons are the most complicated and also most powerful cells in nature. For our concerns, simple delivery of protein from

somite to the presynaptic region of the long axon is unbelievably complicated. APP was reported to be involved in anterograde axon transport as a kinesin-1 receptor(Lazarov et al., 2005). All the molecular studies we have done are based on non-neuronal cell types like HEK293. The caveats are that the "behaviors" of APP is different between HEK293 cells and neurons. Earlier studies indicated that Madi-Darby canine kidney (MDCK) cells, a prototypical epithelial cell line, shared similar mechanism to sort the surface protein, in a polarized fashion (Dotti and Simons, 1990, Lo et al., 1994). Thus, the trafficking of APP could be analyzed in MDCK cells. Indeed, the best way should be generating knock-in transgenic mice of APP A673V, and the primary neurons could be extracted to see the difference.

5.3.4 Limitations in the investigation of the effect of $PS1_{\Delta S169}$ on ε-cleavage.

In chapter 2, we investigated the effect of $PS1_{\Delta S169}$ on ε-cleavage on Notch. In addition to APP and Notch, PS has numerous other substrates, such as N-cadherin and Erb4. Previous work had found that FAD- associated PS1 mutations have various effects on the ε-cleavage of different substrates, despite displaying consistently impaired γ-cleavage (Chavez-Gutierrez et al., 2012). For example, $PS1_{\Delta E9}$ demonstrated significantly impaired APP, Notch and E-cadherin ICDs generation in *in vitro* γ-secretase activity assays, but displayed increased Erb4 ICDs generation, indicating that the effect of FAD-associated PS1 mutations on ε-cleavage are quite heterogeneous and substrate-dependent. To compile an integrated story of the effect of $PS1_{\Delta S169}$ on γ-secretase, it would be beneficial to investigate the effect of $PS1_{\Delta S169}$ on other substrates in addition to APP and Notch.

5.3.5 Development of $PS1_{\Delta S169}$ knock-in transgenic mice.

In Chapter 4, we generated transgenic mice that overexpress $PS1_{\Delta S169}$ with copy number of approximately 2. One limitation of $PS1_{\Delta S169}$ overexpressing transgenic mice is that the effect of wild type mouse PS1 and the second copy of $PS1_{\Delta S169}$ could not be excluded, especially when exploring the effect of $PS1_{\Delta S169}$ on Notch signaling. The best solution to this problem is to generate $PS1_{\Delta S169}$ knock-in transgenic mice. The gene knock-in method is defined as insertion of cDNA encoding for a specific protein back to its original locus on the genome chromosome. This method is superior to other methods, because the protein expression level is comparable with the endogenous protein, and substitution of the endogenous protein excludes the confounding effect of the endogenous protein. Those superior qualities of the knock-in method greatly reduce the false-positive errors seen in the traditional transgenic method.

5.4 Potential application and future research.

The ultimate goal in studies of AD is to prevent the onset of AD or to treat the disease. In the study of APP_{A673V}, apart from further genetically characterizing of APP_{A673V}, therapeutic strategy could be developed based on the unique features of APP_{A673V}. In the study of $PS1_{\Delta S169}$, the effect of $PS1_{\Delta S169}$ on Notch should be tested in animal model, to further confirm the normal Notch signaling and following behavior *in vivo*. Moreover, the therapeutic strategy could be developed based on the effect of $PS1_{\Delta S169}$ on structure of presenilin.

5.4.1 Linkage disequilibrium analysis of the family carrying APP A673V mutation and PCR-restriction endonuclease length polymorphism analysis (PCR-RLFP) of normal controls.

To exclude A673V as a polymorphism, PCR-restriction endonuclease length polymorphism analysis (PCR-RLFP) using a mismatch primer pair will be used. In brief, DNA samples are amplified using a pair of the forward primer: 5′ -GAAGTGAAGATGGATGTAGAATTC -3′ (underline: mismatch position) and reverse primer: 5′ -GTCATGTCGGAATTCTACATCCAT -3′), followed by PCR-RLFP using HpyCH4V. The allele of the A673V variant gains an artificial HpyCH4V site. In the PCR-RLFP using Bgl II, if the A673V variant is not be found in 200 normal Italian control subjects (400 normal alleles), we could exclude the possibility of A673V being as a SNP.

5.4.2 Alanine at APP 673 as the potential therapeutic target.

Recently, a novel mutation on the same locus of APP_{A673V} have been reported – APP_{A673T} (Jonsson et al., 2012). This group analyzed the whole genome sequence of 1775 Icelanders and found that APP_{A673T} protected against AD pathogenesis. It is a very intriguing phenomenon that mutations at the same locus can exhibit completely opposite effects on disease pathogenesis, and furthermore that one of them on this locus was the only recessive FAD-associated mutation.

The alanine (A) at APP 673 is replaced with valine (V) in APP_{A673V}, whereas with threonine (T) in APP_{A673T}. Both alanine and valine are hydrophobic amino acids with side chains, but the side chain of valine is bigger; threonine is a hydrophilic amino acid with a polar uncharged side chain. Jonsson et al. (2012)

reported that sAPPβ and Aβ generation were reduced in APP_{A673T}. It may be that the more hydrophilic residue at this locus makes APP undergo the less amyloidogenic pathway. We hypothesize that alanine in locus APP 673 was important for the regulation of APP processing, possibly by altering the structure of APP at functional sites. Next, the effect of APP_{A673T} on APP metabolism needs to be confirmed and if possible, the structural differences between APP_{A673T} and APP_{A673V} should be analyzed. These findings could be used to develop a small peptide, which could specifically bind to the alanine at APP 673 to prevent Aβ generation by affecting its processing and metabolism.

5.4.3 Analyzing the ε-cleavage of $PS1_{\Delta S169}$ on APP and other γ-secretase substrate in cell-free system.

Our present study on $PS1_{\Delta S169}$ examined the two most interesting γ-secretase substrates: APP and Notch. We found that Aβ generation and NICD production are differentially regulated by $PS1_{\Delta S169}$. In chapter 1.4, we noted that Ihara and colleagues (2008) have provided compelling evidence for the sequential cleavages in the transmembrane domain of γ-secretase substrates, namely, ε-cleavage and subsequent γ-cleavage (Sato et al., 2003, Qi-Takahara et al., 2005b, Kakuda et al., 2006, Yagishita et al., 2008). The transmembrane cleavage by γ-secretase is actually an enzyme catalysis event, which can be achieved in an *in vitro* assay with both enzyme and substrate in solution ($PS1_{\Delta S169}$ and APP or Notch in our case). It was reported that the intrinsic pathogenic properties of PS1 mutations are retained in the cell-free γ-secretase activity assay (Ahn et al., 2010b). Characterizing the enzyme kinetics of $PS1_{\Delta S169}$ could yield robust mechanistic insights into how this mutation affects the basic function of PS1.

In future research, we would like to analyze the catalytic efficiency of $PS1_{\Delta S169}$ for ε-cleavages on Notch and γ-cleavage of APP. Due to the quantification needs of this assay and the relatively large size of APP and Notch, APP-C99 and truncated Notch (which is similar in size to APP-C99) will be used in this assay. The $PS1_{\Delta S169}$ -comprising γ-secretase extract and the substrates (APP-C99 and truncated Notch) will be incubated in optimized conditions, and the resulting products will be assessed by SDS-PAGE as well as Coomassie staining. The characterization of the catalytic efficiency of $PS1_{\Delta S169}$ on ε-cleavage will give further compelling evidence to support the reserved ε-cleavage in $PS1_{\Delta S169}$.

5.4.4 To determine the effect of $PS1_{\Delta S169}$ on Notch processing in *C. elegans*.

Presenilins are crucial for Notch signaling. However, knock-out of PS2 was not sufficient to induce the typical Notch deficient phenotype in PS2 knock-out transgenic mice (Herreman et al., 1999), indicating that Notch signaling is highly redundant to meet its fundamental developmental requirements. Most FAD-associated PS mutations still only partially impair Notch processing activity. Thus it has been difficult to define and evaluate impaired Notch signaling in AD pathogenesis in human and transgenic mice. Alternatively, since both Notch and PS are conserved in metazoans, researchers turned to simpler organisms such as *Drosophila* and *Caenorhabditis elegans* (*C.elegans*) to investigate the effect of FAD-associated PS1 mutations on Notch signaling *in vivo*.

In collaboration with Dr. Catherine Rankins's lab, we chose *C.elegans* as an animal model to investigate the effect of $PS1_{\Delta S169}$ on Notch signaling. *C.elegans*

is an excellent model system due to its short generation time, clear genetic map and well-established genetic manipulation approaches. LIN-12 is the *C.elegans* Notch homolog, while SEL-12 (suppressor/enhancer of LIN-12) is the PS homolog. A SEL-12 defective strain reduced LIN-12 activity with an egg-laying defective (Egl) phenotype (Levitan and Greenwald, 1995), which could be rescued by expression of wild-type human presenilins; in contrast, six different FAD-linked mutations reduced this rescuing ability to various degrees. Since our *in vitro* results indicated normal Notch proteolysis in $PS1_{\Delta S169}$, we predict that expression of $PS1_{\Delta S169}$ in a SEL-12 defective strain could rescue the Egl phenotype in a similar manner as wild type human PS1.

In *C.elegans*, pathogenic PS1 mutants exhibit an impaired ability to rescue the Egl phenotype when compared with wild type PS1. Notably, some PS1 mutants showed a dose-dependent rescuing ability, such as $PS1_{\Delta E9}$, which exhibited almost full rescuing (similar to wild type PS1) with high dosages and significant impairments with low dosages (Levitan and Greenwald, 1995). Thus, we will choose different microinjection dosage to distinguish the dosage effect (20μg/ml & 2μg/ml). Based on our *in vitro* study that $PS1_{\Delta S169}$ retains its Notch cleavage activity in mammalian cells, we anticipate that $PS1_{\Delta S169}$ could rescue the Egl phenotype even at low dosages (2μg/ml). This finding would fully support the hypothesis that $PS1_{\Delta S169}$ functions normally in Notch processing *in vitro* and *in vivo*. Since our *in vitro* study used a constitutively active form of Notch (NΔE) to investigate the effect of $PS1_{\Delta S169}$ rather than Notch itself, it is possible that we may not see normal Notch processing in $PS1_{\Delta S169}$ in *C.elegans*. Nevertheless, if $PS1_{\Delta S169}$ does function normally in Notch signaling in *C.elegans*, we still need to take into consideration that *C.elegans* is less complex than mammals, and all results should be critically examined.

5.4.5 TM3-targeted γ-secretase inhibitor.

Most γ-secretase inhibitors target presenilins. Inhibition of Notch signaling by nonselective γ-secretase inhibitors has been a potential limiting issue for the clinical development of γ-secretase inhibitors as an AD treatment. Hence, a major issue for γ-secretase inhibitors is the development of selective inhibitors , which could reduce Aβ peptide production without significantly interfering with the processing of other substrates of γ-secretase, especially Notch.

Our present study demonstrated that $PS1_{\Delta S169}$ displays separate catalytic efficiencies at the ε- and γ-sites, with normal Notch signaling. The serine 169 residue is located in the third transmembrane domain (TM3) of PS1, and our findings are in agreement with a report that TM3 is involved in the determination of a selective γ-secretase inhibitor (Zhao et al., 2008). Utilizing an alanine scanning mutagenesis method, Zhao and colleagues found that $PS1_{S169A}$ specifically affected some class of γ-secretase inhibitors, such as ELN318463. They found that $PS1_{S169A}$ significantly increased the potency of this inhibitor, indicating that the serine 169 residue was important in the inhibitor/PS interaction. Thus, it would be informative to elucidate the structural differences between $PS1_{\Delta S169}$ and wild type PS1, to clarify the working mechanism of PS1 mutations, and to aid development of Notch-sparing γ-secretase inhibitor. The ideal approach would be to analyze the crystal structure of $PS1_{\Delta S169}$, however, given its multi-transmembrane structure and the multi-subunits of γ-secretase, it has been difficult to analyze the crystal structure of presenilin. Recently, the Shi laboratories reported the crystal structure of a presenilin/SPP homologue (PSH) from the archaeon Methanoculleus marisnigri JR1, and made predictions of presenilin's structure based on the conserved

sequence between the two homologues (Li et al., 2012). Since serine 169 was one of the conserved residues between human PS1 and PSH (corresponding to alanine 80 on PSH), we could design a $PSH_{\Delta A80}$ mutation corresponding to the $PS1_{\Delta S169}$ mutation and analyze the crystal structure of $PSH_{\Delta A80}$ instead. The structural difference between $PSH_{\Delta A80}$ and wild type PSH would give direct clues to help develop specific γ-secretase inhibitors.

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