STUDYING PHYSIOLOGICAL FUNCTIONS OF APP USING MICE MODELS

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DEDICATION

To my parents, my friends, and all of my teachers!

STUDYING PHYSIOLOGICAL FUNCTIONS OF APP USING MICE MODELS

by

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DISSERTATION

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STUDYING PHYSIOLOGICAL FUNCTIONS OF APP USING MICE MODELS

Hongmei Li, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2008

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Beta-amyloid precursor protein (APP) is sequentially cleaved by α/β-secrease and γ-secrease into three pieces: a soluble ectodomain (sAPPα or sAPPβ), a p3 or Aβ peptide, and an APP intracellular domain (AICD). Mounting evidence indicates the neuroprotective and neurotrophic effects of sAPP domain. In order to find out whether sAPP domain carries out the major physiological function of APP, we have generated knock-in mice that express truncated ectodomain of APP at beta-cleavage site (sAPPβ) with FLAG tag at C-terminus. The knock-in mice were viable and fertile, with no obvious phenotype. However, when sAPPβ-FLAG knock-in mice were bred to APLP2 knockout background ("knock-in/knockout" mice), the expression of sAPPβ-FLAG failed to fully rescue the postnatal lethal phenotype of APP/APLP2 double knockout pups, suggesting sAPPβ alone cannot substitute for the function of full length APP. We quantified

the expression levels of a series of synaptic proteins and AICD-interacting proteins in the brains of new born APP/APLP2 double knockout (DKO) pubs, as well as in the "knock-in/knockout" (KI/KO) pubs, and found that Fe65 protein expression level is upregulated in brains from DKO pubs but not the KI/KO pubs. Collaborating with Dr. Yi Sun's lab investigating the DNA sequences in genome that potentially bind to AICD binding partners has shown that various promoters of a broad set of genes can bind to Fe65 and their expressions might be influenced by Fe65/AICD.

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AD – Alzheimer's Disease

Aβ - amyloid-β peptide

AICD – APP intracellular domain

APL-1 – amyloid protein-like protein 1 (C. elegans)

APLP 1 and 2 – amyloid precursor-like protein 1 and 2

APP – amyloid-β precursor protein

APPL – amyloid precursor protein like protein (Drosophila)

apoE - apolipoprotein E

BACE - β-site APP cleaving enzyme

CA - cornu ammonis (in hippocampus)

CAA - congophilic amyloid angiopathy

Cdk - cyclin dependent kinase

DKO – double knockout

FAD - familial Alzheimer's disease

FTD - frontotemporal dementia

GDI - GDP-dissociating inhibitor

GSK-3β – glucogen synthase kinase 3β

KIKO – knock-in and knockout

KPI - Kunitz protease inhibitor domain

NFT – neurofibrillary tangles

PHF - paired helical filaments

PP-2A - protein phosphatase-2A

PS 1 and 2 – presenilin-1 and 2

SP - senile plaques

sAPPα or β – soluble (secreted) APP α or β form

CHAPTER ONE
GENERAL INTRODUCTIONS

1.1 ALZHEIMER'S DISEASE BASICS

1.1.1 History and Symptoms

Alzheimer's disease (AD) is named after German physician Dr. Alois Alzheimer (1864-1915) who first reported the observation of connection between dementia with neurofibrillary tangles (NFTs) and senile plaques, from postmortem examining the brain of his patient, Auguste D. who developed cognitive deficits in her late 40s (Alzheimer et al., 1995) [an English translation of Alzheimer et al. 1907 article]. Now, one hundred years after the initial discovery, we know that AD is a progressive neurodegenerative disease with hippocampus and entorhinal cortex appear being the first site of pathological changes. There are two types of AD, less than 10% AD patients are the early onset (before age 65) familial type caused by gene mutations in the family; the vast majority of AD patients are late-onset or "sporadic" which seem to be closely related with aging process (Bertram and Tanzi, 2005). AD is a neurodegenerative disease that causes loss of memory, language ability, and other intellectual abilities severe enough to affect daily life. Symptoms of AD begin with progressively increased difficulty in short-term memories, disorientation to time, space, and location; followed by afflicted long-term memories, poor and impaired judgments, problems with language, difficulties in performing familiar tasks.

Eventually, AD patients lose their ability to communicate, cannot recognize their family faces, and have problems in performing any essential activities in daily livings. AD is an ultimately fatal disease with no cure available for the time being.

1.1.2 Prevalence and Costs

AD is a terminal disease with severe prevalence. According to the 2008 report from Alzheimer's association (http://www.alz.org/alzheimers_disease_facts_figures.asp), AD is the most common form of dementia and the 7th leading cause of death in United States. It is estimated that there are more than 5 millions of Americans are suffering with AD in 2007, and 10 million baby boomers will be afflicted by AD in their life time. One in every eight persons 65 age old has AD. There are more women than men having AD because women have longer life span in average, not that female gender is a risk factor (Barnes et al., 2003; Hebert et al., 2001; Miech et al., 2002). It seems that people with more years of education will have less risk of developing AD, which is not entirely because other factors such as higher education is usually associated with higher income, higher occupational attainment, or physical conditions in adulthood (Evans et al., 1997; Hall et al., 2000; Ngandu et al., 2007; Stern et al., 1994).

Without the proper cure, the number of AD patient will continual to grow with the steady growth of average life span of the society. The economic and social burden of AD to families and society are enormous. It is calculated that

there are 9.8 million Americans provided unpaid care for a person with AD or another dementia. These unpaid caregivers are mostly family members, friends and neighbors, and they give 8.4 billion hours of unpaid care. It cost Medicare $91 billion in 2005 for care of beneficiaries with AD and other dementia, and this figure is expected to rise to $160 billion in 2010 (http://www.alz.org/national/documents/report_alzfactsfigures2008.pdf).

1.1.3 Diagnosis and Treatments

Diagnosis of AD mostly relies on the combination of symptom reports, neuropsychological assessments and laboratory tests to give a "probable" conclusion when the full dementia had already set in. The only way to be absolute certain with the diagnosis is through the unhelpful postmortem histological verification of neurofibrillary tangles and amyloid plaques at autopsy. In recent years, remarkable advances have been achieved using neuroimaging techniques to provide more accurate and early detection of AD [recently reviewed by (O'Brien, 2007; Ries et al., 2008; Scahill and Fox, 2007)]. These progresses not only make it possible for early detection and intervention at predementia stage called mild cognitive impairment (MCI), but will also be helpful in screening for more effective therapies and monitoring the progress or the treatment of the disease.

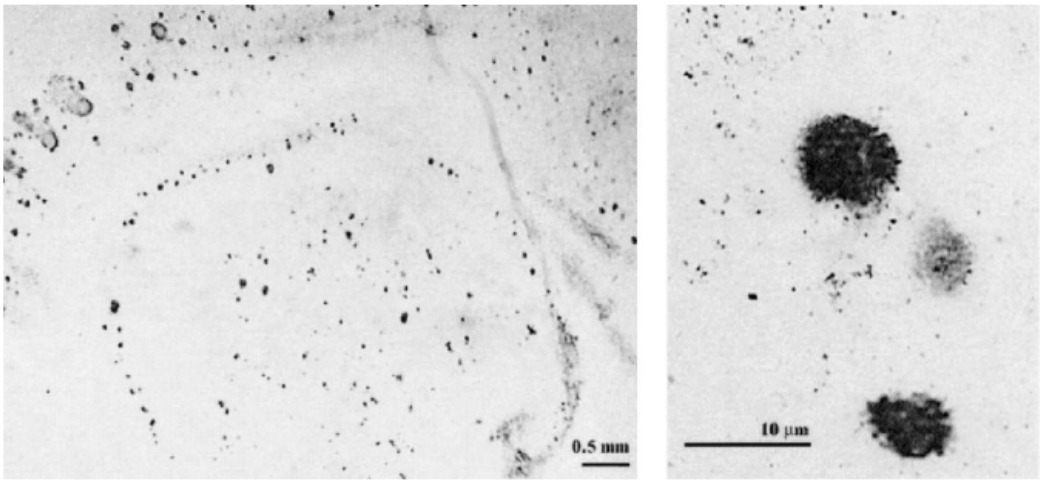
So far, even though there is no treatment succeeded in clinical trials to cure or stop the neurodegeneration in AD, there are five approved drugs that

temporarily slow down cognitive symptoms [all with their adversary side effects, recently reviewed (van Marum, 2008)]. Four of these drugs, Aricept (donezepil HCL), Exelon (rivastigmine), Razadyne (galantamine), and Cognex (tacrine) are cholinesterase inhibitors that can increase the levels of acetylcholine in the brain, which is very important for memory and learning; the other one, Namenda (memantine), is a low-affinity non-competitive NMDA receptor blocker that would hopefully regulate the excessive neuronal excitotoxicity in the AD brains [recently reviewed (Lipton, 2006)]. More therapeutic approaches based on the amyloid hypothesis (details in later section *1.2.3*) and other targets are at various stages of clinical testing. In addition to the drug treatment, there are various non-drug treatments in helping to cope with behavior symptoms (http://www.webmd.com/alzheimers/guide/alzheimers-disease-treatment-care). Growing number of evidences suggested that healthy diet (low-fat and rich in fruits and vegetables), healthy life style (less stress and moderate exercises), life-time intellectual curiosity, and cognitive stimulations support brain health, and thus, be helpful in fighting AD [see recent reviews (Burgener et al., 2008; Gatz et al., 2006; Hooijmans and Kiliaan, 2008; Kivipelto and Solomon, 2008; McDonald, 2008; Spector et al., 2008; Szekely et al., 2007; Zakharov and Yakhno, 2008)].

1.2 THREE TYPES OF LESIONS ASSOCIATED WITH AD

The cause and the progression of AD are still not fully understood yet. At autopsy of brains from AD patients, people usually would observe cerebral atrophy and classical histological lesions in various brain areas, such as hippocampus, entorninal cortex, amygdale, cerebral association cortices, and certain subcortical nuclei that project to these areas. The two diagnostic neuropathological lesions of AD are intracellular deposit of neurofibrillary tangles (NFTs) and extracellular deposit of senile plaques in the autopsy of afflicted brains. Lewy bodies are also observed in some familial AD cases. There are still many questions regarding to relationships among these lesions and the observed cell-loss; and whether they are "causes" or "consequences" of the pathogenesis of the disease.

1.2.1 Senile plaques (SP) - APP



Klucken, et. al., 2003

Figure 1. 1 Aβ staining of the hippocampus AD brain.

The left image is imaged in the dentate gyrus region, while the right images is the higher magnification showing the amyloid plaques. [Figure 3 from Klucken, J. et. al Neurochem. Res. 2003 (Klucken et al., 2003)]

Senile plaques are extracellular deposit of insoluble protein aggregation (amyloid), either in a dense-core spherical form containing dystrophic dendrites and axons, or a diffuse form free of dystrophic neurites (Figure 1.1)[reviewed (Selkoe, 1994)]. Almost all AD patients also have scattered microvascular amyloid deposits in their brains, and a small minority of them, can turn into severe cerebral amyloid angiopathy (also called congophilic amyloid angiopathy, CAA). Protein component of the plaques was isolated from both CAA and parenchymal sources, and sequenced to be almost identical ~4 kd peptide, Aβ, indicating the common origin of the peptide (Glenner and Wong, 1984; Masters et al., 1985). These findings led to the cloning of the gene encoding the parent protein of Aβ peptide, amyloid-β precursor protein (APP), in 1987 (Goldgaber et al., 1987; Kang et al., 1987; Robakis et al., 1987; Tanzi et al., 1987). Location of human APP gene was mapped to Down's syndrome region of chromosome 21 (Tanzi et al., 1987), which provided genetic explanation why trisomy 21 (Down's syndrome) patients invariably developed early-onset of AD (Wisniewski et al., 1978).

1.2.1.1 Genetics of Alzheimer's disease

The early-onset type of AD (typically in the late 40s to mid 50s), which makes up less than 10% of all AD cases, appears to be clustered in families and inherited in an autosomal dominant manner. Genetic causes of FAD have been mapped to three genes: APP (on Chromosome 21), Presenilin 1 (Chromosome 14), and Presenilin 2 (Chromosome 1). Study on these familial Alzheimer's disease (FAD) provided strongest evidences for amyloid cascade hypothesis, which considers AD is initiated by progressive abnormal accumulation of Aβ in limbic and association cortices that not only constitutes senile plaques but also leads to hyper-phosphorylation of tau, formation of NFT, and eventually neurodegeneration (Hardy, 1997; Hardy and Selkoe, 2002).

Besides above three genes that are strongly related with early-onset of AD, different genetic variants are implicated in the vast majority of late-onset of AD. The most well-established risk genetic factor for late-onset of AD is the ε4 allele of APOE on chromosome 19 (Strittmatter et al., 1993). More recently, another genetic risk factor was identified on the polymorphism of CALHM1 gene resided at chromosome 10, which encodes a pore component of a new cerebral ion channel family, and thus influences Ca^{2+} permeability and cytosolic Ca^{2+} level.

1.2.1.2 APP processing

Amyloid precursor protein (APP) is a type I membrane- protein ubiquitously expressed but most prominently expressed in central nerve system (CNS). In mammalian genome, APP gene contains 18 exons in total and the full

length isoform has 770 residues. Alternative splicing of exons 7, 8, and 15 will generate eight different isoforms (Konig et al., 1992; Monning et al., 1992). Exon 7 encodes 56-residue Kunitz protease inhibitor (KPI) domain. Exon 7-contain isoforms are expressed mostly in peripheral organs (Kitaguchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988). Isoform APP695, where both exon 7 and 8 are missing, is the most abundant form expressed in the brain (Golde et al., 1990; Kang and Muller-Hill, 1990; Neve et al., 1988).

APP has a large extracellular domain, a single transmembrane region (TM), and a short intracellular domain with less than 60 residues. It is sequentially cleaved by first α, or β-secretases outside the TM region on the extracellular side and then by γ-secretase inside TM (Figure 1.2) [for recent reviews, see (Nathalie and Jean-Noel, 2008; Zhang and Xu, 2007)]. The sequential proteolytic cleavage by β/γ-secretases in CNS will generate β- amyloid (Aβ) peptides, whereas α/γ-secretases pathway will produce a non-amylogenic peptide p3 that is 17 residues shorter than Aβ. The Aβ has been considered to play a central role in the pathogenesis of Alzheimer's disease by strong genetic,

biochemical, and neuropathological data (Selkoe, 2003).

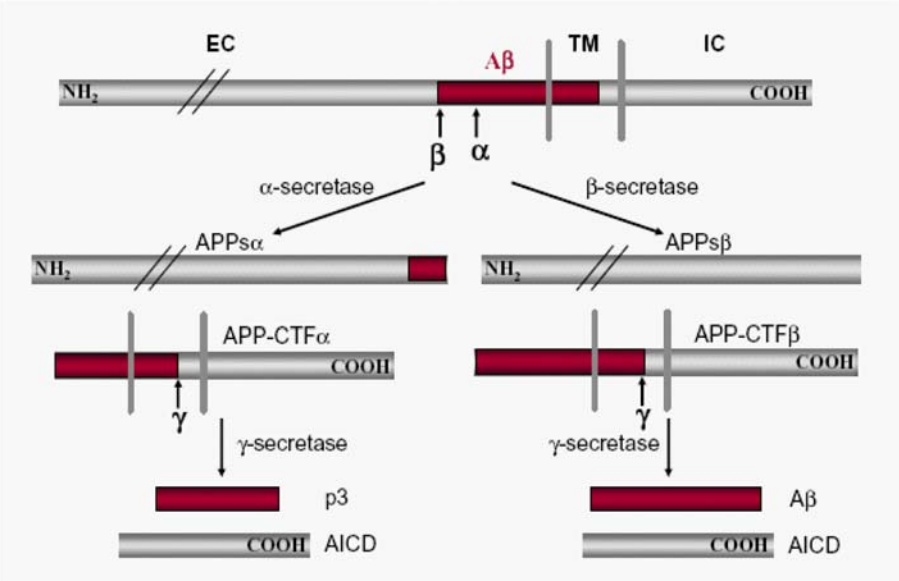
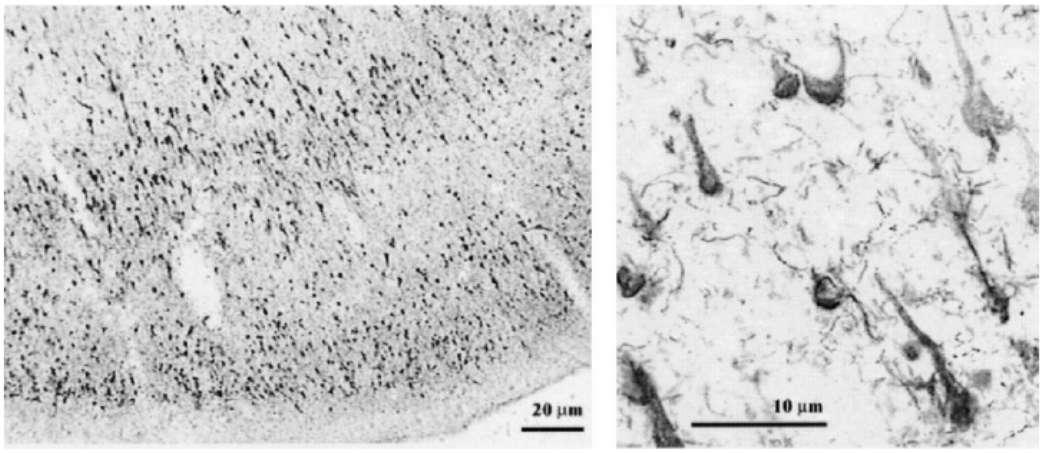


Figure 1. 2 Schematic drawing of APP processing.
[From the review of Zheng and Koo, 2006 (Zheng and Koo, 2006)].

APP missense mutations detected from various FAD cases were shown to enhance the amyloidogenic properties of Aβ; and FAD mutations on PS1 and 2 genes were found to increase production and oligomerization of Aβ42 peptide [reviewed by (Selkoe, 2002)].

1.2.2 neurofibrillary tangles (NFTs) - Tau



Klucken, et. al., 2003

Figure 1. 3 Phospho-tau staining of the entorhinal cortex of AD brain.

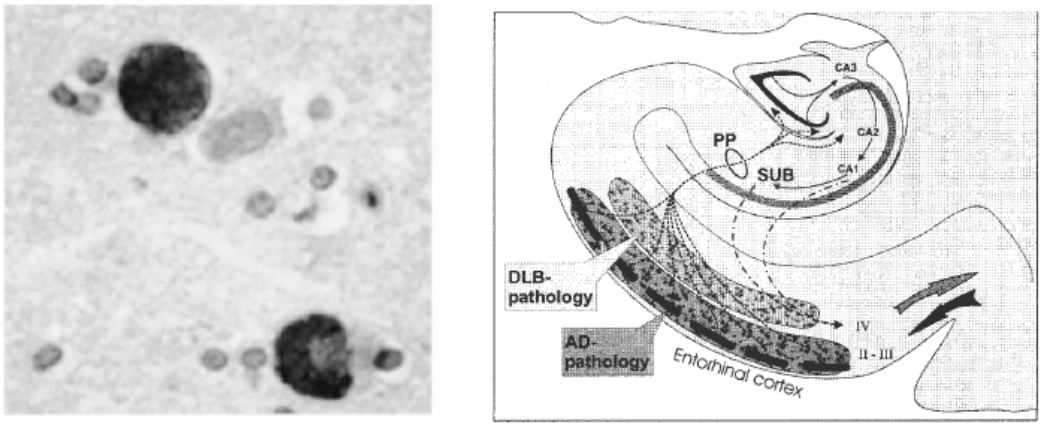
The left image in lower magnification showed the NFTs mainly in layers II and IV, whereas the right one is a higher magnification image. [Figure 2 from Klucken, J. et. al Neurochem. Res. 2003 (Klucken et al., 2003)]

The second hallmark of AD is NFTs. Briefly, NFTs consist of aggregates of hyper-phosphorylated tau protein bundling into paired helical filaments (PHF) in selected cell bodies (Grundke-Iqbal et al., 1986a; Grundke-Iqbal et al., 1986b). Tau normally binds to microtubules and thereby promotes the assembly and stabilizes their structure. Hyper-phosphorylated tau has decreased abilities in binding to microtubules, however, increased abilities in promoting the self-assembly into PHF and thus NFTs. A number of kinases have been found to phosphorylated tau, among which Glycogen synthase kinase-3 (GSK-3) and cyclin dependent protein kinase 5 (cdk5) are most implicated in the abnormal hyper-phosphorylation of tau. The major phosphotase for tau is protein

phosphatase-2A (PP-2A), whose activity was down-regulated in AD brain [for recent review, see (Ballatore et al., 2007; Iqbal and Grundke-Iqbal, 2008)].

There isn't any concrete explanation yet to explain the simultaneous appearance of SP and NFTs in the AD brains. So far, the amyloid hypothesis is the dominant hypothesis in the cause of AD because mutations in APP that increase Aβ production can cause autosomal dominant AD; in contrast, mutations in tau that enhance tau aggregation can cause autosomal dominant frontotemporal dementia (FTD) but not AD. Studies using transgenic mice with APP-swedish mutations (KM 670, 671 → NL, APP770 numbering) and tau (P301L) mutations suggested that NFT desposition is more likely to occur after the altered APP process (Gotz et al., 2001; Lewis et al., 2001; Lewis et al., 2000). The exact mechanisms of how APP and tau influence each other under physiological conditions have not been understood yet, however, cdk5 and GSK-3 have been proposed as links between the two histopathological hallmarks of Alzheimer's disease.

1.2.3 Lewy bodies – α-Synuclein



Klucken, et. al., 2003

Figure 1. 4 Lewy bodies.

Left image is α-synuclein-positive LB in the entorhinal cortex of dementia with Lewy bodies (DLB) brain. And the right one shows the location difference of AD and DLB within the entorhinal cortex. [Figure 4 and 5 from Klucken, J. et. al Neurochem. Res. 2003 (Klucken et al., 2003)]

Besides the two histopathological hallmarks of AD mentioned above (NFT and senile plaques), Lewy bodies are also observed in many cases of familial AD (Lantos et al., 1994; Lippa et al., 1998). There are ~60% of familial and sporadic AD patients whose brains are also burdened by Lewy bodies (Kazee and Han, 1995; Lippa et al., 1998; Weiner et al., 1996).

Lewy bodies are abnormal intracellular protein aggregates with α-synuclein as their primary structural component (Mann et al., 1998). Lewy bodies observed in substania nigra are the pathologic hallmark of the second most common neurodegenerative disease, Parkinson disease; and they can also occur in

cells scattered in limbic and paralimbic areas throughout the cortex as well as in spinal cord (Bloch et al., 2006). There are various degenerative disorders associated with occurrence of Lewy bodies, such as dementia with Lewy bodies, multiple system atrophy and diffuse Lewy body disease, etc [reviewed in (Dev et al., 2003)].

Occurrences of Lewy bodies in FAD and Down syndrome patients are not accidental but rather the result of abnormally excessive APP processing (Lantos et al., 1994; Lippa et al., 1998). The molecular mechanisms involving synuclein and APP processing remain to be elucidated. Studies on Tg2576 strain of APP transgenic mice breeding to α-synuclein knockout background showed that α-synuclein-deficiency has no effect on Aβ level or the onset of the plaque pathology, but substantially increased the formation of plaque formation at a later stage, suggesting α-synuclein's role in progression of plaque deposition rather than the initiation of the process (Kallhoff et al., 2007). On the other hand, transgenic mice that over-expressing human α-synuclein, or human α-synuclein with A53T or A30P mutants were found to have increased ApoE level and increased insoluble mouse Aβ levels (Gallardo et al., 2008). Further study is needed for us to fully understand the relationship between synuclein and APP processing.

1.3 PHYSIOLOGICAL FUNCTIONS OF APP

Many different possible functional roles have been ascribed to APP as reviewed in recent articles (Senechal et al., 2006; Wolfe and Guenette, 2007; Zheng and Koo, 2006), such as involving in axonal transportation, associating with various cellular and developmental processes, being putative receptors, serve as adhesion molecules, affecting gene expressions, calcium homeostasis, and membrane trafficking, etc. Nonetheless, our knowledge of the normal functions of APP is still limited. It is also unclear whether these varieties of functions are caused by a common cellular and molecular mechanism, and how the physiological functions of APP relate to the pathogenesis of Alzheimer's disease.

As APP holo-protein been cleaved into three pieces by β/γ-secretases, the extracellular domain, Aβ peptide, and AICD, each fragment has been shown to be involved in a variety of physiological mechanisms. Increasing evidences have shown that the soluble and secreted domain of APP (sAPP) can exert neuroprotective effect. Aβ peptide was mostly implicated as a neurotoxic factor, however, the toxicity might be resulted from the over-production and accumulation of Aβ. Measurement of human Aβ synthesis and clearance rate in cerebrospinal fluid has shown that Aβ is rapidly produced and cleared away from central nerve system in humans (Bateman et al., 2006). Thus, small perturbations to the production and clearance rates might have huge accumulative effects over time. So, we are still uncertain of the physiological functions of Aβ under normal healthy conditions. There are published results implicating physiological

functions of Aβ include : (1) production is critical requirement for the viability of central neurons (Plant et al., 2003). (2) regulating NMDA receptor trafficking (Snyder et al., 2005) ↔NMDA receptor activation inhibits α-secretase and promotes neuronal Aβ production (Lesne et al., 2005). (3) Regulation of cholesterol and sphingomyelin metabolism (Grimm et al., 2005).

For the third piece from APP processing, AICD, which is hightly conserved among APP, APLP1 and APLP2, multiple important roles have been associated with this domain, including cell migration, cell signaling, apoptosis, and axonal transport, etc. [reviewed (Zheng and Koo, 2006)]. Various proteins have been identified to interact with AICD through NPTY motif, such as Mints (X11s), Fe65 family of proteins, Jun N-terminal-kinase-interacting protein 1 (JIP-1), kinesin 1, Numb, mDab1, and Pat1a, etc. Fe65 is especially interesting, in that knockout of Fe65 (feh-1) in worm or Fe65-Fe65L1 in mice produce the remarkably similar phenotypes seen in knockouts of APP family of genes (Guenette et al., 2006; Zheng and Koo, 2006). Tripartite complex of Fe65, AICD and the histone acetyltransferase Tip60 has been shown to activate transcription (Baek et al., 2002; Cao and Sudhof, 2001; Cao and Sudhof, 2004). A few AICD-targeted genes have been proposed, such as tetraspanin CD82, APP, glycogen synthase kinase-3beta (GSK3β), and neprilysin, however, there is also a report showing Fe65 can transactivates a wide variety of different promoters independent of AICD coexpression (Hebert et al., 2006).

In conclusion, great advances have been achieved in understanding the pathogenesis of AD and the physiological functions of APP in the past two decades, which provided great hope for humankind to eventually conquer the disease. Still, we might need to better understand how the spectrum of APP physiological functions is orchestrated and how they are related to the pathogenesis of AD in order to achieve the final success over the disease.

CHAPTER TWO

ANALYSIS ON APP/APLP2 DOUBLE KNOCKOUT MICE

INTRODUCTION – KNOCKOUT MODELS IN DIFFERENT ANIMAL SYSTEMS

There is a single gene encodes the APP-related protein, APL-1, in the nematode *Caenorhabditis elegans*. Knockout one copy of APL-1 leads to pharyngeal pumping defects, and loss of two copies causes molting defects and larval lethality (Hornsten et al., 2007; Zambrano et al., 2002). In *Drosophila melanogaster*, there is one gene for APP-like protein, APPL. APPL-knockout flies are viable, fertile, with only minor behavioral deficits which can be rescued by transgenes expressing either wild type APP and partially by human APP (Luo et al., 1992). Later it was found that knockout APPL in flies can disrupts axonal transportation and neuronal viability (Gunawardena and Goldstein, 2001) and decrease synaptic bouton numbers at neuromuscular junction (Torroja et al., 1999). Over expression of APPL in flies can induce neuronal apoptosis (Gunawardena and Goldstein, 2001), partial stalling of axonal transport vesicles (Rusu et al., 2007), increasing post-developmental axonal arborization (Leyssen et al., 2005), as well as affecting development of the peripheral nervous system interaction with Notch signaling pathways (Merdes et al., 2004). In zebrafish

(*Danio rerio*), two homologues of human APP were identified, APPa and APPb (Musa et al., 2001). Observations that knock-down of APPb by injection of morpholino antisense oligonucleotides can cause embryonic death in zebrafish were presented at the satellite meeting of 2007 Annual Meeting of Society for Neuroscience (San Diego) by Dr. Pimplikar (Lerner Res. Inst., Cleveland Clinic, Cleveland, OH, USA), however, the results were not officially published yet till today.

There are three members in the APP family in mammalian genome, APP, APP-like-protein 1 (APLP1), and APP-like-protein 2 (APLP2). In mice, single knockout of anyone of them doesn't cause any severe deficits. APP-knockout mice are viable, but exhibit minor phenotypes such as reduced brain and body weight, behavior abnormalities such as reduced grip strength, and impairment in spatial learning and long-term potentiation (LTP) (Dawson et al., 1999; Muller et al., 1994; Zheng et al., 1995). These phenotypes can be rescued by a knock-in allele of sAPPα (Ring et al., 2007). Double knockout of APP-APLP2 or APLP1-APLP2 die early postnatally (Heber et al., 2000; von Koch et al., 1997), while triple APP-APLP1-APLP2 knockout are also postnatal lethal, with cranial dysplasias resembling human type II lissencephaly (Herms et al., 2004). Study on APP-APLP2 double knockout mice (DKO) have revealed that these mice can develop defects in neuromuscular junction (Wang et al., 2005), exhibit less synaptic vesicle density and active zone size, and number of docked vesicles per

active zone in submandibular ganglion synapses as observed by EM (Yang et al., 2005), suggesting an important role of APP family of proteins in development of synapse. In addition, DKO mice also exhibit reduced proliferation rate and impaired cell adhesion and thus cell migration rate in keratinocytes (Siemes et al., 2006), together with the results from triple knockout mice (Herms et al., 2004), and the data from utero electroporation of shRNA to knowdown APP in mice cortex (Young-Pearse et al., 2007), indicating APP family of proteins have critical roles in cell adhesion and cell migration. Besides, APP-KO and APP/APLP DKO mice have lowered ApoE level and increase cholesterol level in their brains, pointing to the importance of APP family in cholesterol metabolism (Liu et al., 2007). Furthermore, a recent publication illustrated that DKO pups have abnormally low glucose level and excessively high insulin level in their plasma, and significantly reduced plasma magnesium, calcium and phosphate, linking APP and APLP2 genes with glucose homeostasis and growth (Needham et al., 2008).

Among all these reported physiological roles of APP, our primary focus is on its function at synapse. Instead of using a single synaptic marker to examine the synapse density as most studies did, in the study below, we looked at a spectrum of synaptic proteins for their global expression levels in brains of DKO mice, together with comparison of micro array analysis for the mRNA composition between DKO pups and their less mutant littermates. We found no

statistically significant changes in any of the synaptic protein assayed, nor in the few AICD interacting proteins that we had the antibodies against. The only significant changes at protein level in DKO brains is the upregulation of an AICD interacting protein, Fe65, providing further evidence of the tight physiological link between Fe65 and APP family of proteins.

MATERIALS AND METHODS

Generation and maintenance of APP APLP2 double deficient mice

$APP^{+/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). $APLP2^{-/-}$ mice were kindly provided by Dr. D. Borchelt (John Hopkins University, currently at University of Florida). Offspring with double mutations ($APP^{+/-}$ $APLP2^{+/-}$) were selected from cross-breeding of these two lines. Survival analysis of adult offspring was done by mating $APP^{+/-}$ $APLP2^{+/-}$ mice. Survival analysis of new born pups was done by collecting P1 pubs from breeding pairs of $APP^{+/-}$ $APLP2^{-/-}$ mice. Genotyping of APP was down by PCR using three-primer single tube reaction: Primer A = agtctgccagtatcatggctt (common primer); Primer B = ctgccttgggaaaagcgcctc (KO specific, neo cassette sequence); and Primer C = ctggaaggataggtaattcatcc (wild type specific sequence). The wild type allele produces PCR product of 539 bp, while the knockout allele will generate a product of ~ 400 bp. (Note, this reaction system works very well at the beginning,

but failed to give good results in a single tube reaction in the later years, which might due to the quality of primers. So we later use two-primer two tube reactions of Primer A & B for mutant allele, and A and C for the wild type allele. Genotyping of APLP2 was determined by PCR of tail DNA using primers previously described (von Koch et al., 1997).

Quantitative Western blot analysis

Three litters of pups were sacrificed at birth, and brain tissues were collected and tails were used for genotyping. Brains from selected genotyping pups were homogenized in PBS, 5 mM EDTA, 1 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ml Leupeptin, and 1 µg/ml Pepstatin A. The whole brain lysates (25 ug per lane) were analyzed by SDS/PAGE, transferred to nitrocellulose membrane for immunoblotting. Quantitative immunoblotting was carried out by using ^{125}I-labeled secondary antibodies and Storm 860/ImageQuant (GE Healthcare, Piscataway, NJ) for detection with GDP-dissociation inhibitor (GDI) or vasolin-containing protein (VCP) as internal standard. Most antibodies that were used were described previously (Rosahl et al., 1995; Schoch et al., 2002). Results are presented as mean ± SEM. All statistical analyses are based on a two-tailed t-test.

RNA preparation, Microarray analysis, Northern Blot.

APP and APLP2 double knockout pubs were dissected at E18. Brain total RNA was prepared using Trizol method (Invitrogen). The total RNA was analyzed by

agarose gel with formaldehyde for Northern Blot. For Genechip experiement, the total RNA was further analyzed by Agilent Bioanalyzer before proceeded to Genechip experiments. For Northern Blot, ten micrograms of total brain RNA was loaded in each lane of the 1.2% agarose gel (1× MEA buffer: 20 mM morpholineethanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate, 5% formaldehyde, 0.1% diethyl pyrocarbonate (DEPC)). The gel was run at 2.5 V/cm for approximately 2 h and then washed with DEPC-treated water for 30 min. The RNA was then transferred to a nylon transfer membrane (MAGNA, 0.45 μm; MSI) using the Posi-blotter from Stratagene. Probe was labeled with Random Primer Kit from Invitrogene. Mouse genome 430 2.0 Array from Affymetrix GeneChip was used for Genechip experiments. 1μg of total RNA was used for cDNA synthesis, cDNA was then purified and analyzed. Successful cDNA was used for amplified RNA (aRNA) synthesis. The aRNA was purified and hybridized to Genechip. After hybridization, the Genechip was washed, stained and scanned to acquire the expression profile. The protocol from Affymetrix was followed for the whole procedure. Expression data from wild type and knockout brains were analyzed by Genespring software. A set of housekeeping genes were used for normalization (- Qiming Li).

RESULTS

2.1 Survival Analysis

APP and APLP2 double knockout pups would exhibit postnatal lethal phenotype, which was published previously (Heber et al., 2000; von Koch et al., 1997). One report observed that 26% of the expected APP/APLP2 null mice escaped lethality till ~ 3 weeks old (von Koch et al., 1997), where the other group reported much lower survival rate for double knockout pups. Only 1 out of 355 offspring from $APP^{+/-}$ $APLP2^{-/-}$ breeding that survived to adulthood, where 89 DKO mice were expected (Heber et al., 2000). This difference might be due to the difference in the genetic background. In order to see how our husbandry of mice would behave, we bred $APP^{+/-}$ single mutant mice with $APLP2^{-/-}$ to generate double mutant offspring. Then, the resulting $APP^{+/-}$ $APLP2^{+/-}$ mice were breed with each other and their offspring were used for survival analysis at weaning age (Figure 2.1, left panel). Out of 270 offspring we analyzed, we didn't find any APP/APLP2 null mice survived to adulthood from these double heterozygous breeding pairs. We found a single APP/APLP2 double null mouse survived lethality till adulthood among more than 300 offspring from $APP^{+/-}$ $APLP2^{-/-}$ breeding pairs. No morphological change in brain was found in this survived double null mouse comparing with the littermate control (Data not shown). In consistence with previous published results, the lethality of APP/APLP2 double null pups was unlikely due to the developmental defects because these pups were born alive, and the number of the double null mice matched very well with expected Mendelian segregation at birth (Figure 2.1, right panel).

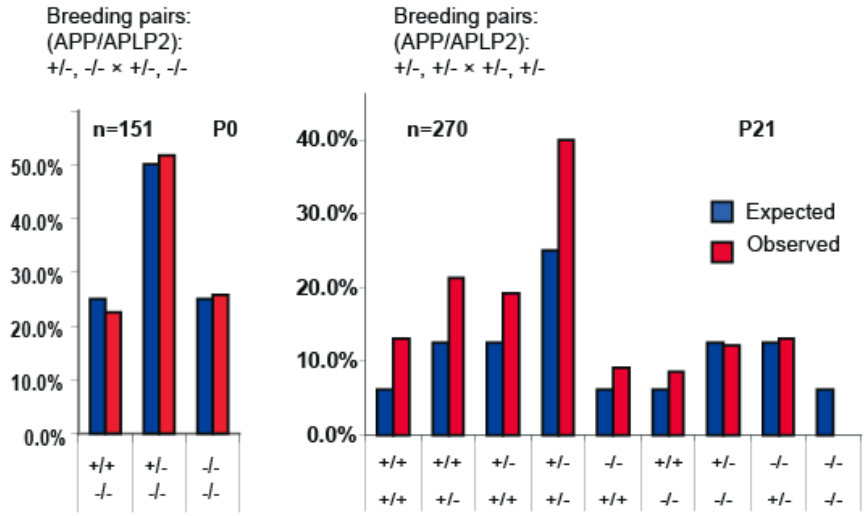


Figure 2. 1. Survival analysis of APP APLP2 DKO mice.

Left panel, genotypes of 151 offspring from mating of $APP^{+/-}$ $APLP2^{-/-}$ mice were analyzed at birth. Right panel, mice that were $APP^{+/-}$ $APLP2^{+/-}$ were mated, and genotypes of 270 surviving adult offspring were analyzed. Grey bars represent frequency of each genotype plotted as percentage of the total. Open (white) bars represent expected frequency according to Mendelian inheritance.

2.2 Protein Quantification in the DKO brain:

In order to evaluate the role APP family proteins might play in neuronal and synaptic functions, brains were removed from DKO pups and their less mutant littermates at birth. Total brain lysates were used for quantitative immonoblot analysis using ^{125}I-labeled 2nd antibodies. Among29 proteins examined, the levels of the Fe65 family proteins (Fe65, Fe65 L1, Fe65 L2) were compensatory increased by ~ 20 - 40% ($p<0.05$) in the APP/APLP2 DKO pups

(**Table 1, Figure 2.2 a & b**). None of the other AICD interacting proteins examined shown any significant changes in APP/APLP2 null mice (**Figure 2.2b**). No significant changes were observed in the expression levels of SNARE proteins or other synaptic proteins. Fe65 was known to be an adaptor protein interacting with APP C-terminus domain (AICD) to play a possible role in transcriptional activation (Cao and Sudhof, 2001; Cao and Sudhof, 2004) (Sumioka et al., 2005), as well as mediating APP processing through unclear mechanisms, although there were some discrepancies in whether overexpression of Fe65 would increase or decrease APP processing in previously published results(King et al., 2004) (Pietrzik et al., 2004) (Santiard-Baron et al., 2005). Our findings here are consistent with results published on APP/human-Fe65 (hFe65) transgenic mice, where APP overproduction could lower Fe65 expression level and expression of hFe65 reduced β-amyloid load (Santiard-Baron et al., 2005). These observed selective increase of expression of Fe65 family proteins in APP/APLP2 double DKO mice strongly suggested that Fe65 and APP were functionally connected.

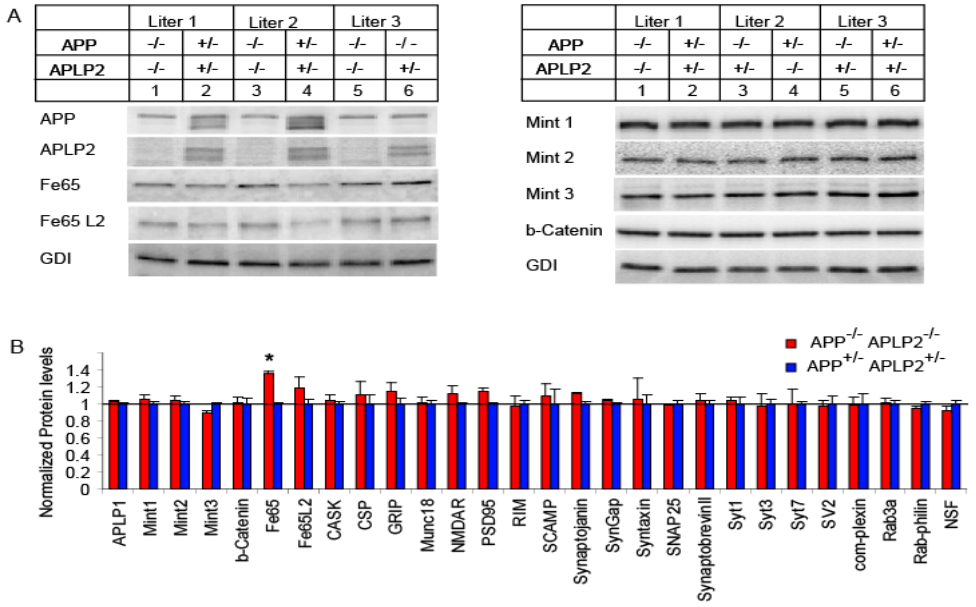


Figure 2. 2. Effect of the APP/APLP2 deletion on Fe65-family protein levels.

(A) Whole brain lysates from three sets of liter mates were used in quantitative western blot using ^{125}I-labled 2nd antibodies to validate APP/APLP2 genotypes and to measure the expression level of Fe65 family of protein. (B) Quantification of AICD interacting proteins and other synaptic proteins using PhosphorImager. * $p < 0.05$. Data represent means ± SEMs. GDI, GDP-dissociating inhibitor, was used as internal loading control.

Table 2. 1 Quantitative levels of brain proteins in APP/APLP2 compound heterozygous and double homozygous knockout mice.

Protein	$APP^{+/-}$ $APLP2^{+/-}$	$APP^{-/-}$ $APLP2^{-/-}$
APLP1	100 ± 1	102 ± 1
Mint1	100 ± 2	104 ± 5
Mint2	100 ± 2	104 ± 5
Mint3	100 ± 0	89 ± 2
Fe65	100 ± 1	136 ± 2
Fe65 L1	100 ± 2	128 ± 8
Fe65L2	100 ± 5	118 ± 13
β-Catenin	100 ± 6	101 ± 5
CASK	100 ± 2	104 ± 6
CSP	100 ± 10	110 ± 15
GRIP	100 ± 6	115 ± 10
Munc18-1	100 ± 3	101 ± 5
NMDAR	100 ± 1	112 ± 8
PSD95	100 ± 1	114 ± 4
RIM	100 ± 3	97 ± 12
SCAMP	100 ± 17	108 ± 15
Synaptojanin	100 ± 2	111 ± 1
SynGap	100 ± 0	104 ± 0
Syntaxin	100 ± 11	105 ± 25
SNAP25	100 ± 3	98 ± 1
Synaptobrevin 2	100 ± 4	103 ± 8
Synaptotagmin 1	100 ± 7	103 ± 5
Synaptotagmin 3	100 ± 4	97 ± 14
Synaptotagmin 7	100 ± 2	99 ± 17
SV2	100 ± 8	97 ± 7
complexin	100 ± 11	99 ± 8
Rab3a	100 ± 3	101 ± 5
Rabphilin	100 ± 2	95 ± 2
NSF	100 ± 3	92 ± 5

2.3 Microarray analysis reveals no dramatic transcriptional effect of APP.

Previous data has implicated that APP and FE65 can activate transcription of certain unknown genes and initiate a signal transduction pathway (Cao and Sudhof, 2001). The hypothesis was further supported by identifying KAI1 as a possible target of APP/Fe65 regulation (Baek et al., 2002). Other genes such as

BACE1, TIP60, GSK3β and even APP itself were purposed to be novel targets (von Rotz et al., 2004). However all the experiments were done in cell lines with over-expression of APP or AICD. To elucidate the complete set of genes downstream of this pathway, we carried out a large scale expression array analysis in the brain of APP/APLP2 double knock-out compared with wild type controls. Two sets of experiments were carried out to eliminate the possible noises in the microarray analysis. Genes with expression affected in both experiments were considered further.

The expression of the majority of genes were unchanged (**Figure 2.3**). There are no genes with expression levels altered over 4-fold in either experiment, indicating that there are no dramatic changes in gene expression level. We then lowered the threshold to 2-fold, we were unable to detect any genes with expression level increased over 2-fold in the double knockout, however we have a list of 8 genes which were down-regulated over 2-fold in the double knockout (Table 2.2 A). Because the changes of these genes are minor, we reasoned that they may not be a result of APP as a transcriptional regulator. These genes may be involved in the functions of APP and APLP2 genes and their expression level was changed as a result of APP/APLP2 deletion.

The protein level of FE65 was increased substantially in the knockout brain. We therefore wonder if this up-regulation happens at the transcriptional level. We analyzed the mRNA levels of most genes which interact with APP C-

terminus both in the wild type animal and the double knock-out. We have seen no increases of FE65 on the mRNA level. In addition, FE65L1, Fe65L2, Mint1, Mint2, Mint3, Numb, Dab, Jip1 and Jip2 remain unchanged (Table 2.2 B). Since the mRNA level of Fe65 was not altered between wild-type and double knockout, the protein level increase of Fe65 must occur at post-transcriptional level.

–Qiming Li

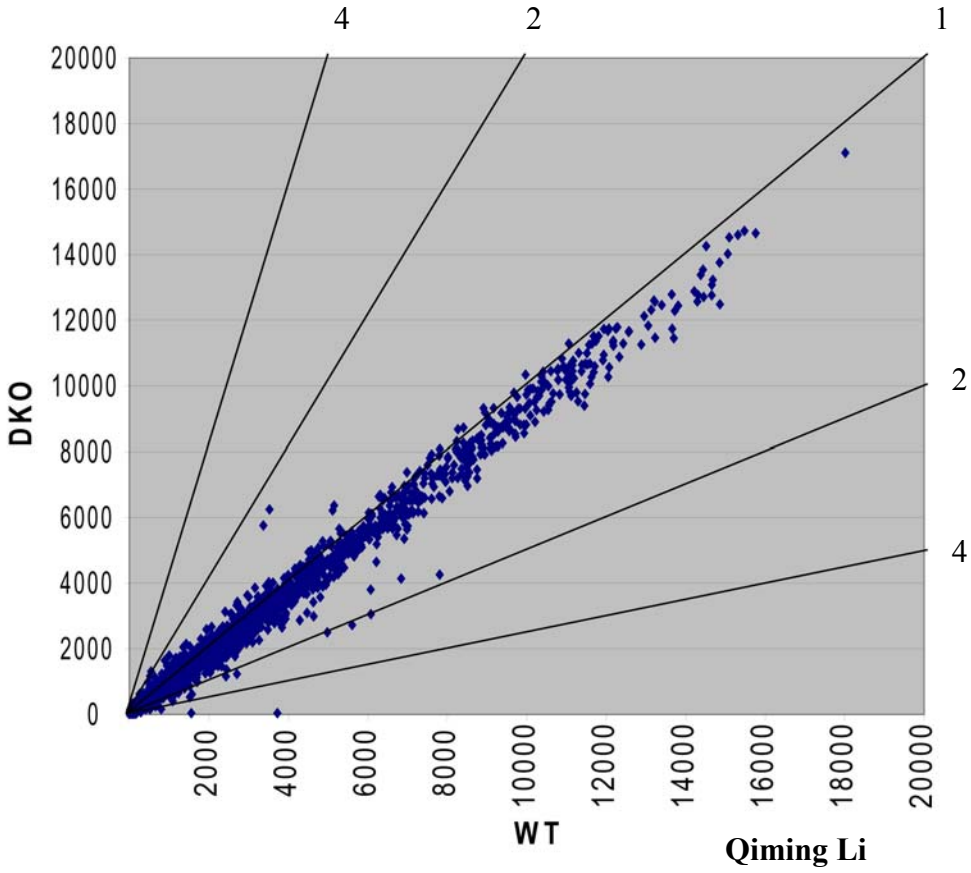


Figure 2. 3. Microarray analysis of wild type and APP/APLP2 double knock-out brains.

The expression profile of the wild-type is plotted on the horizontal axis while the expression profile of the double knockout is plotted on vertical axis. The two spots on the horizontal-axis were APP and APLP2 respectively as their expressions were reduced more than 50-fold to the background. The 4-fold and 2-fold change lines were also drawn.

Table 2. 2 Summary of micro array data

(A) The list of genes which are down-regulated at least two-fold in the APP/APLP2 double knock-out. Two independent Microarray experiments were performed. Genes which were reduced in both experiments with an average of 2-fold were further studied. WE found none of the genes were up-regulated by at least 2 fold. E1R1 and E2R2 represents the ratios of mRNA level of a candidate gene in the knockout mice over that in the wild-type in Experiment 1 and Experiment 2 respectively. P-values were also listed. **(B)** Expression level of APP C-tail interacting. DKO stands for APP/APLP2 double knockout. The stand errors were generated by various probe sets of these genes from two independent Microarray experiments.

A. Differentially Expressed Genes between WT and DKO mice

Probe Set	Gene	Accession	E1R1	Pval	E2R2	Pval	CR
1420534_at	Guanylate cyclase 1 alpha3 (Gucy1a3)	AK004815.1	0.28	0.003	0.46	0.0007	NA
1441226_at	F-Spondin	AI503604	0.48	0.038	0.32	0.150	0.45
1435415_x_at	MARCKS-like protein (Mlp1)	BB491008	0.32	0.0002	0.67	0.0024	NA
1449661_at	Mnt	AW536442	0.35	0.002	0.39	0.0002	0.42
1436301_at	leukotriene A4 hydrolase	BB435342	0.45	0.014	0.48	0.024	0.52
1437433_at	B3galt2	BB254922	0.50	0.014	0.46	0.003	0.55
1453998_at	Putative GTPase Activating protein	AK014888.1	0.48	0.06	0.49	0.06	0.73
1419972_at	SLC35a5	C86506	0.39	0.01	0.48	0.014	0.39

B. Expression levels of APP interacting proteins

Genes	WT	p-value	DKO	p-value	DKO/WT Ratio
Fe65	755±59	0.0002	766±89	0.0002	1.03±0.18

Fe65L1	222±9	0.0002	212±13	0.001	0.95±0.12
Fe65L2	169±20	0.03	165±4	0.03	0.98±0.14
Mint1	198±7	0.002	186±33	0.002	0.94±0.13
Mint2	457±1	0.002	424±23	0.002	0.93±0.05
Mint3	129±15	0.002	110±27	0.001	0.88±0.31
Numb	222±9	0.002	212±13	0.001	0.95±0.12
Dab1	946±24	0.0002	875±105	0.002	0.93±0.13
Dab2	218±2	0.0002	203±36	0.002	0.93±0.16
Jip1	278±18	0.002	233±8	0.001	0.84±0.03
Jip2	178±25	0.006	197±13	0.01	1.12±0.23

2.4 Northern Blot confirmed that Gucy1a3 and Mlp1 were down regulated in APP/APLP2 double knock-out.

We then performed Northern Blot analysis for two genes, Gucyla3 and Mlp1, which are expressed at highest level among the genes that were modestly down-regulated. Gucyla3, Guanylate cyclase 1, soluble, alpha 3, in complex with beta subunit, form the heterodimeric protein, soluble guanylate cyclase (sGC) (Giuili et al., 1992). SGC catalyzes the conversion of GTP to the second messenger cGMP, which regulates the activity of protein kinases, phosphodiesterases, and ion channels. It functions as the main receptor for nitric oxide and nitrovasodilator drugs (Zabel et al., 1998), and has implication of its role in generating long term potentiation (LTP) in the brain (Monfort et al., 2002). How sGC is related with APP and AD is not clear. Mlp1, MARCKS (myristoylated alanine-rich C-kinase substrate)-like protein 1, is a widely distributed substrate for protein kinase C (PKC) (Blackshear, 1993). Homozygous knockout of Mlp develops neural tube

defects, which causes high rate in prenatal lethality (Wu et al., 1996). Micro array analysis on prefrontal cortex of mice has shown that mRNA level of Mlp 1 is progressive reduced after birth (Semeralul et al., 2006), so this protein might related with physiological function of APP in some way, but unlikely to be involved in the pathogenesis of AD. There is a report suggesting Mlp1's function in vesicular trafficking in neurons (Yang et al., 2002).

Gucyla3 mRNA migrated at ~5.5k and its level was reduced to about 70% in knockout brains (Figure 2.4.a, Compare Lane 2, 4 and 6 with 1, 3 and 5). Mlp1 mRNA migrated at ~1.5 kbp and its level was reduced to 75% in the knockout brains (Figure 2.4.b, Compare Lane 1, 6 and 7 with Lane 2, 3, 4 and 5).

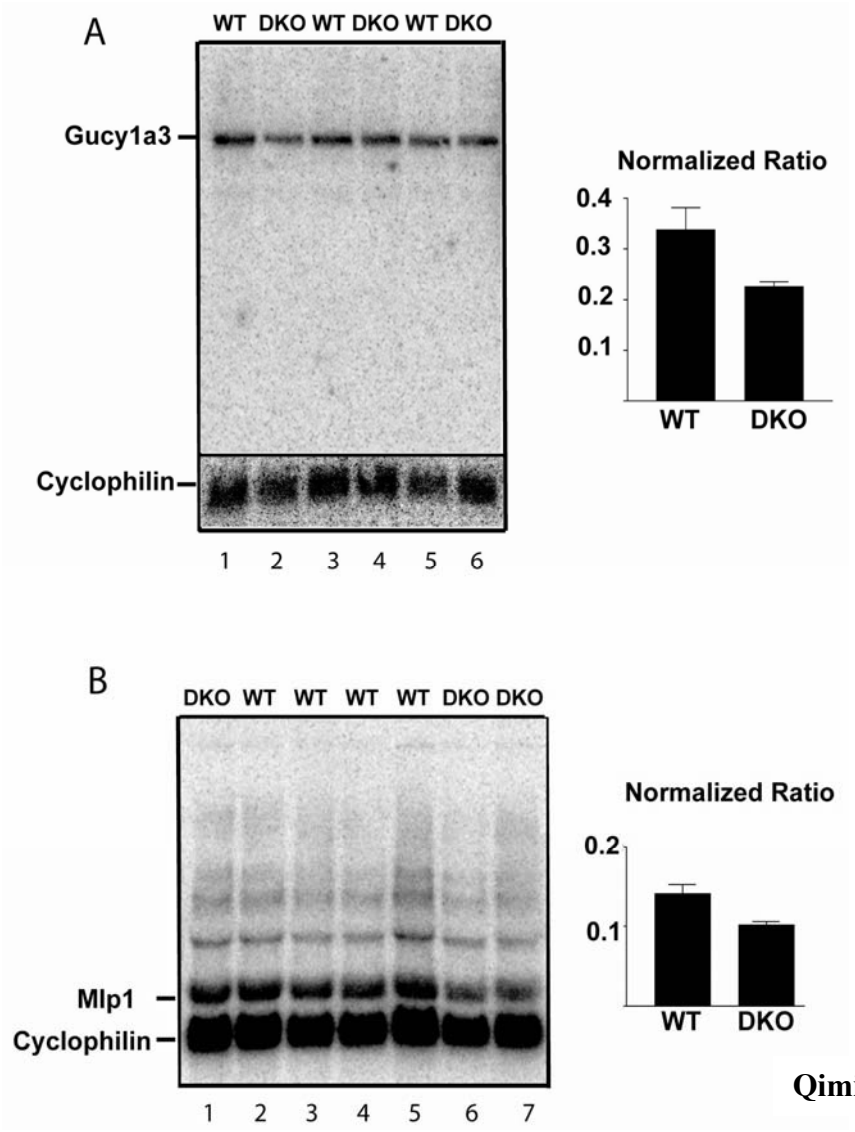


Figure 2. 4. Northern Blot analysis for Gucyla3 and Mlp1.

(A).Gucyla3 mRNA migrated at ~5.5 kbp, Lane 2,4 and 6 are from double knockout brains and Lane 1,3 and 5 are from wild-type brains. (B). Mlp1 mRNA migrated at ~1.5 kbp. Lane 1,6 and 7 are from double knockout brains and Lane 2,3,4 and 5 are from wild-type brains.

SUMMARY AND DISCUSSIONS

We have observed extremely high rate of early postnatal lethality in APP/APLP2 DKO mice in our husbandry, contrary to the ~25% escape observed by other groups [DKO in Dr. Hui Zheng's group at Baylor College of Medicine, (personal communication) and (von Koch et al., 1997)], even though all these mice are on C57BL/6 genetic background.

Global protein quantification analysis from the mice brain showed significant upregulation of Fe65 family of proteins, which point to an important role of physiological function of APP in related with Fe65 family. Mounting evidences showed the transcription function of Fe65, in either positive or negative regulation of various genes, with or without interaction with AICD and Tip60 [recently reviewed (McLoughlin and Miller, 2008)]; meanwhile, APP and Fe65 interaction, together with Mena, an actin cytoskeleton regulatory protein, have been implicated in regulating cell motility and neurite growth (Ikin et al., 2007; Sabo et al., 2001; Sabo et al., 2003). Furthermore, similar phenotypes were observed in triple knockout of APP/APLP1/APLP2 (Herms et al., 2004), and double knockout of Fe65/Fe65 L1 (Guenette et al., 2006), as well as Mena knockout mice (Lanier et al., 1999), strongly indicated the functional interaction among three families on neuronal development in vivo.

In order to investigate Fe65 targeted genes and the influence on gene expression by APP, we are collaborating with Dr. Yi (Eve) Sun's lab (UCLA, CA) to identify DNA sequences that bind to Fe65 family of proteins using ChIP-chip technology (Appendex A).

Obviously, growing evidences show that APP family of proteins has multiple physiological roles inside the cell, from axonal transportation, synaptogenesis, to transcriptional regulation, in resemblance to a growing family of multifunctional cytoskeletal regulators that can exert transcription effects as well. These proteins include Basal cell carcinoma-entiched gene 4 (BEG4)/Missing in metastasis (MIM) (Callahan et al., 2004; Gonzalez-Quevedo et al., 2005), Wnt pathway regulator β-catenin (Moon et al., 2002), and plakoglobin (Maeda et al., 2004). The one of the difficulties in deciphering physiological functions of APP is that we are still clueless about what signals APP may respond to during cellular processes.

CHAPTER THREE
GENERATION AND ANALYSIS OF SAPPB-FLAG KNOCKIN MICE

INTRODUCTION

APP gene has 19 exons, among which, alternative splicing of exon 7, 8, and 15 were detected and all eight isoforms of APP were observed (Sandbrink et al., 1994). Different isoforms have different expression patterns, with isoform APP695, missing both exon 7 and 8, has the highest expression in the neurons (Tanzi et al., 1988). All these isoforms have identical linker region, transmembrane region, and cytosolic region (AICD), with difference exists only in the ectodomain of the protein. The ectodomain of APP composites more than 85% of the total molecular weight of the holoAPP, and was reported to function as cell surface receptor, cell adhesion molecule, and most consistently, neurotrophic and synaptogenic factor as reviewed by Zheng and Koo (Zheng and Koo, 2006).

Among all the proposed physiological functions of APP, part of them seemed to be carried out solely by the α-form of the ectodomain while AICD was not needed. For example, (1) Keratinocytes of APP/APLP2 DKO mice exhibited reduced proliferation rates both in vivo and in vitro, which could be completely rescued by exogenously added recombinant sAPPalpha (Siemes et al., 2006), suggesting the role of sAPPα as an essential epidermal growth factor. (2) In *Caenorhabditis elegans*, neuronal expression of extracellular domain of APL-1 can rescue the lethality caused by apl-1 knockout (Hornsten et al., 2007). (3)

SAPPα-knockin mice rescued many minor phenotypes displayed by the APP-knockout mice, such as decreased body and brain weights, weak grip force, altered circadian locomotor activity, and impaired spatial learning ability and long-term potentiation (Ring et al., 2007).

However, there are also functions of APP that are depended on the existence of AICD. For instance, embryonic lethality of APPb-MO injection in zebrafish can only be rescued by expression of full lengh wild type APPb or human APP in a specific amount, but not be rescued by expression of ectodomain of the protein alone (Dr. Pimplikar, presented at satellite meeting of 2007 annual meeting of SFN, no publication yet). In mice, where utero electroporation of shRNA to knockdown APP in developing cortex, and thus causes impairment in neuronal migration, it was shown that expression of both extracellular domain and AICD are required for efficient rescue (Young-Pearse et al., 2007).

We were also interested in studying the extent of functions covered by sAPP fragment of the protein. Instead of generating sAPPα form as Dr. Müller's group did (Ring et al., 2007), we chose to create sAPPβ-FLAG knockin mice to investigate whether sAPPβ carries out any unique functions.

MATERIAL AND METHOD

Generation and maintenance of APPs-KI mice

A genomic clone containing exon 15, 16, and 17 of APP gene was used to construct the targeting vector for homologous recombination. Basically, a 4.8 kb loxP flanked fragment was inserted at the intron region 310 bp upstream of exon16. Inside the loxP flanked region, there was another wild type exon16 truncated right after residue KM (Swedish mutation site, 595 and 596 of 695 form, or 670 and 671 of full length form) followed by two repeats of FLAG sequence (DYKDDDDK DYKDDDDK) then stop codon; poly A signal region (474 bps) from human growth hormone (hGH) was constructed after stop condon, followed by two repeats of PGKneo-cassette flanked by FRT sequences. Exon 16 and 17 after loxP flanked region were humanized, with Swedish and London mutation on exon 16 and 17, respectively. (Additional mutations constructed in exon 17 as described in chapter 5). Diphtheria toxin gene (DT) was constructed to the end of homologous sequence (for negative selection). Linearized targeting vector was electroporated into embryonic R1 stem cells (Nagy et al., 1993), and cell clones resistant to both positive (G418) and negative selection (DT) were screened by Southern blot to identify clones with correct homologous recombination event. Out of 480 clones screened, there were 27 were corrected targeted according to Southern blot analysis. Two clones were used to inject into C57BL/6 blastocysts.

Chimeric offspring were bred to produce homozygous knock-in mice that were viable, fertile and had no obvious phenotype. The initial knock-in line was crossed with FLP transgenic mice to remove PGK-neo cassette. Genotyping of mice was performed by PCR using primer HL033 (GTAATGCCTGTGTGGCCAAACACATGCCA) and HL037(AAGTAATGGATTTGTTCTCCCAGGTCG), both resided in the intron region upstream of exon 16. Wild type allele would produce a 230 bp PCR product, while the knock-in allele would give a 270 bp product due to the insertion of restriction enzyme site (SseI) and LoxP sequence. Mice were bred and maintained using standard mouse husbandry procedures.

Southern blot analysis of APPs-KI

Southern blot was performed using DNA prepared from ES cells or tail DNA digested with BamH I, which would cut genomic DNA outside of the targeting vector. Digested mixtures were separated by 0.8% agarose gel electrophoresis. Hybridization was done using Random Primed DNA labeling Kit (Roche Applied Science, Indianapolis, IN) with both 5' and 3' probes. The 5'-probe was a 500 bp PCR product from genomic clone using primers HL020 (TCACCCCCACTAAATGGCA) and HL021 (CCCTTTTGGTAAGCATTTG). In order to generate 3'- probe, a 3.4 kb Kpn I/BamH I fragment from genome clone containing 8 kb region after exon 17 was subcloned into pBlusecript II KS

vector (Stratagene, La Jolla, CA). A 462 bp Xho I fragment from this construct, which resided 518 bp inside the 3'-BamH I site, was used as the 3'-probe for Southern screen.

Co-immunoprecipitation (Co-IP) experiments

For FLAG-pull down experiments looking for potential interaction partners of sAPPβ: Adult knockin mice and wild type their littermates (~ 2 month old) were sacrificed and brain tissues were homogenized in PBS with 5 mM EDTA, 1 mM PMSF, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, and 1 μg/ml of Pepstatin A. Up to 1% Triton X-100 was added to homogenates, and each tube of brain homogenate was incubated for 30 min at 4º on a vertical rotator. Total brain lysates were clarified by centrifugation at 20, 000 x g for 10 min. The supernatants were incubated with M2 beads at 4º for 2 hours with rotation. Then the beads were collected by centrifugation and washed five times in PBS with 5 mM EDTA and 0.5% Triton X-100. Proteins bound to the beads were first eluted by 3xFLAG peptide and then by adding 2x SDS-PAGE sample loading buffer directly to the beads. Polyacrylamide gel was stained by Sypro-Ruby protein stain solution after the electrophoresis, and protein band of interest was cut from the gel and submitted for LC/MS/MS protein identification. For protein interaction verification experiments: Brains from wild type and knock mice were collected, homogenized, and supernatant of the brain lysates were prepared the same mentioned as above. IP with M2 beads or Myc-beads was done by adding to

lysates and incubated at 2 – 3 hours at 4º before washing. IP with other primary antibodies was done by first adding the primary to lysates for 2 hour incubation at 4º, then adding protein A beads for addition 1 hour incubation. All the incubations in 4º were done with constant rotation on a vertical rotator.

Lentiviral Infection Assays

Recombinant lentiviruses were produced by transfecting human embryonic kidney 293T cells (HEK 293) with three plasmids using FuGENE 6 reagent for each infection. The three plasmids are: the plasmid with genes of interest listed below, together with VSVg and CMVΔ8.9 plasmids encoding the elements essential for packing of viral particles. pFUGW is used as the backbone vector encoding the gene of interest and containing recombination arms for incorporating into mammalian genome.

For verification of sAPPβ expression level, four pFUGW plasmids were made expressing (1) sAPPβ, (2) sAPPβ-FLAG, (3) full length APP, and (4) APP-frm, which is full length APP with frameshift mutation at C-tail. Production of this plasmid has frameshift mutation after residue ^{731}I (numbering as APP770 isoform). The resulting mutant protein is 14-residue shorter than the wild type of the same isoform, and does not have the correct sequence of the last 39 residues of the wild type. pFUGW containing the enhanced green fluorescent protein (EGFP) was used as control plasmid.

Viruses produced by HEK 293 cells were harvested 48 – 60 hours after transfection by collecting the medium from transfected cells, and spin down aro 2000 x g for 5 minutes to remove cellular debris. Neurons were cultured on 6-well plates, with 3 ml medium per well. Infections were done by adding 750 µl of virus containing HEK 293 cell medium to each well of high-density neurons at 1 DIV. Neurons were infected for 24 h for expression, and media were exchanged back to normal growth media and sustained until 13–15 DIV for biochemical analyses.

RESULTS

3.1 Generation of sAPPβ-knock-in mice:

In order to check whether the functions of APP are depended only on the production of the secreted extracellular domain, we generated knock-in mice which express a truncated version of APP at BACE1 cleavage site instead of the full length APP. SAPPβ knock-in targeting vector was constructed using genomic clones containing exon 16 of APP gene (Figure 3.1). In the targeting vector, a truncated exon 16 tagged with FLAG sequence at C-terminus, followed by a stop codon, poly A signal region, and double PGKneo-cassette was flanked by lox P sites to allow excision by cre recombinase. (The double neo-cassette was surrounded by flp sites to allow the removal of neomycin gene.) This loxP

flanked region was inserted into the intron region in front of exon 16, which was humanized and with Swedish mutations (exon16**). The resulting knock-in mice were crossed with mice with FLP transgene to remove the PGKneo-cassette.

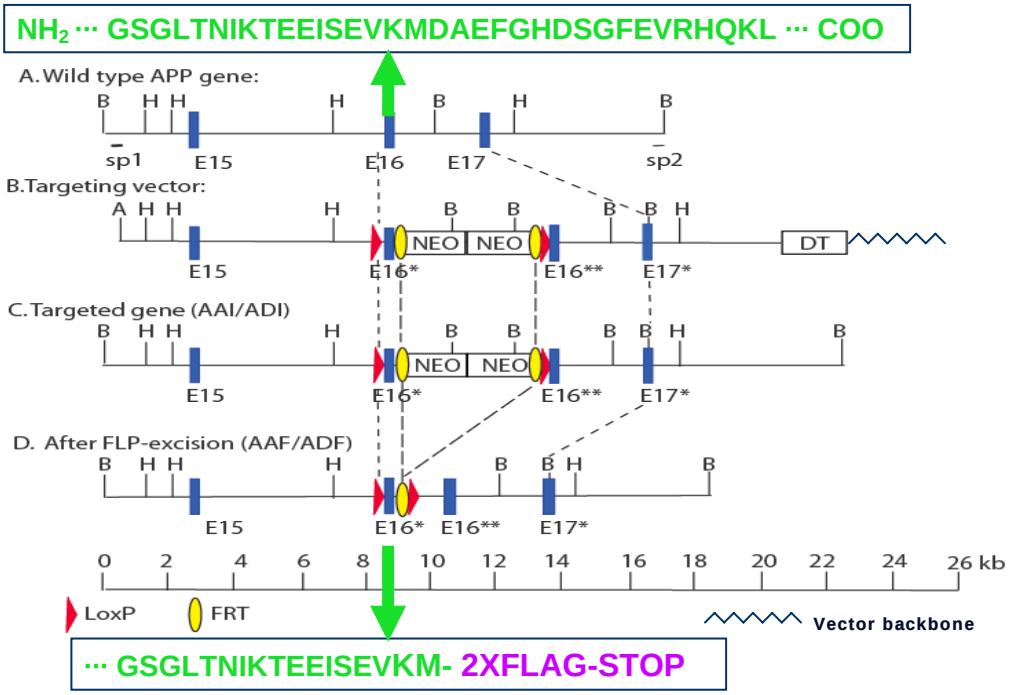


Figure 3. 1. Generation of sAPPβ-FLAG knock-in mice.

Diagram of sAPPβ knock-in strategy, showing APP wild type gene around exon 15, 16, 17, targeting vector, knock-in gene after homologous recombination, and FLP-excised gene where neomycin-resistant cassettes were removed. Positions of both 5'- probe (sp1) and 3'-probe (sp2) used to screen ES cells by southern blots are indicated. A, Asc I; B, BamH I; H, Hind III; E15, E16, E17, exon 15, 16, 17, respectively; E16*, truncated exon 16 with FLAG tag, stop codon, and polyA signal; E16**, humanized exon 16 with Swedish mutation; E17*, humanized exon17 with London mutation and either Arctic or Dutch mutation.

3.2 Analysis of APPs-KI Mice.

Southern blot analysis was used to verify the correct gene construct in the mutant mice (Figure 3.2 a). The 5' probe recognizes the wild type allele at 10.2 kbp, where the knockin allele is 10.8 kbp. In order to separate these two bands successfully, agarose gel electrophoresis for BamH I digested genome DNA was run at 20V for two days before it was transferred to the hybridization membrane.

sAPPβ-FLAG knockin mice are viable, fertile, with no other obvious phenotypes. Basically, the homozygous knockin mice are indistinguishable from their wild type littermates by visual observation. Western blot analysis was used in order to validate the knock-in fusion protein is truly sAPPβ-FLAG (Figure 3.2 b). Western blot using M2 mAb (Sigma-Aldrich Inc. St. Louis, MO) was able to detect FLAG tagged fusion protein around ~110 kDa from the knockin mouse's brain lysate, but not from the wild type littermate. Monoclonal antibody raised against sAPP (Chemicon, Temecula CA) confirmed that the band detected by M2 antibody is truly APPs, and only one band corresponding to the fusion protein was detected in the homozygous knockin mouse by this antibody, whereas there are three bands in the wild type lane, corresponding to the immature form of APP, sAPP, and the full length mature APP. Polyclonal antibody raised against carboxyl-terminus of APP in our lab showed that the full length APP was not detected in homozygous knock-in mice. These results confirmed that the knock-

in mice were expressing truncated sAPPβ-FLAG fusion protein instead of full length APP

The sAPPβ-FLAG fusion protein would be expected to be expressed in a variety of tissues in the knock-in mice as the full length APP would do in the wild type. Immunoblots of various tissue homogenates such as adrenal gland, brain, heart, kidney, liver, lungs, skeletal muscle, spleen and testis, all showed the expression of sAPPβ-FLAG probing by M2 monoclonal antibody (Figure 3.2 c**)**. .

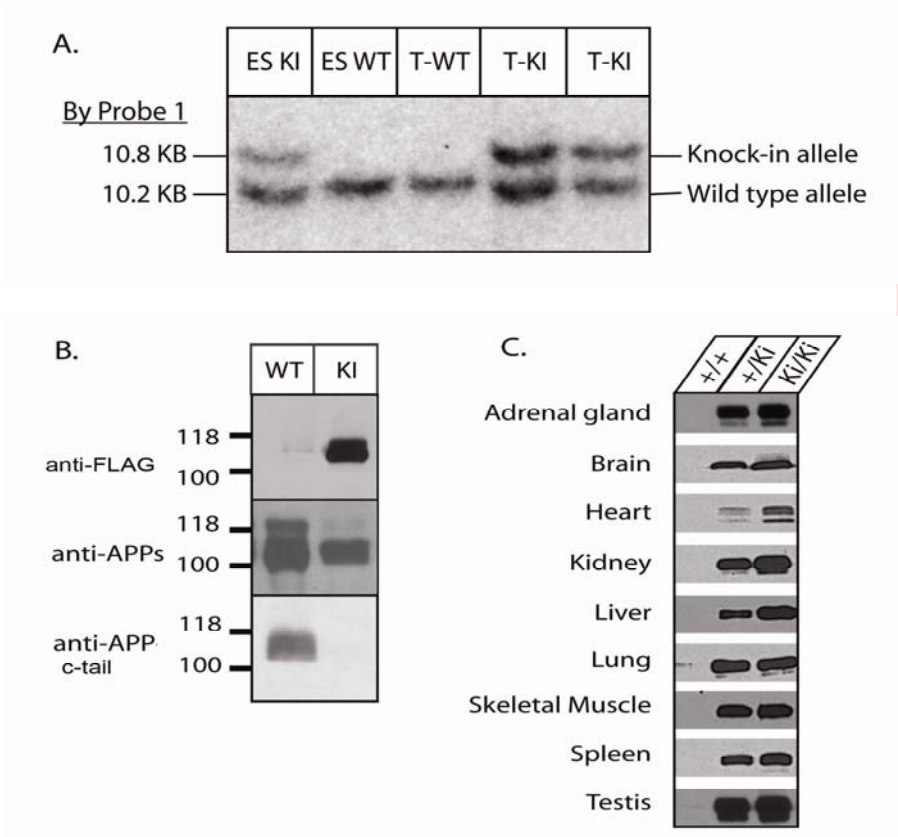


Figure 3. 2. Validations of the expression of knock-in protein.

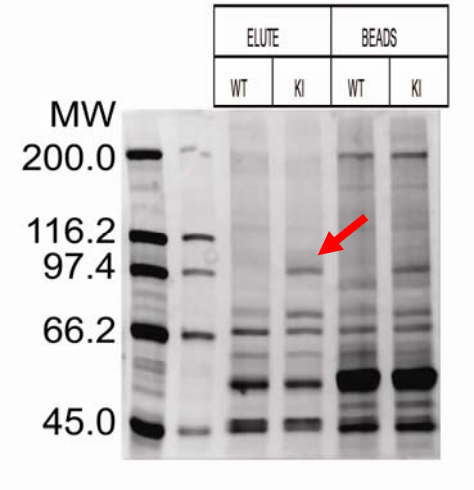
(A) Southern blots of representative ES cells and knock-in mice. Lane 1 and 2, DNA from positive ES cells (ES-KI) and wild type ES cells (ES-WT), respectively; Lane 3, tail DNA

from wild type mice (T-WT); Lane 4 and 5, tail DNA from two lines of heterozygous knock-in mice (T-KI). (B) Immuno-blot of whole brain lysates from wild type mice and homozygous knock-in mice by M2 (anti-FLAG mAb), mAb 348 (against APPs mAb), and rabbit pAb U955 that was raised against C-terminus of APP (anti-APP c-tail). (C) Western blots to detect expression of knock-in sAPPβ-FLAG in various tissue types from knock-in mice and their litter mate control by M2 mAb.

3.3 Co-immunoprecipitation of sAPPβ-FLAG and Bip/Grp78 in KI mice

We did immuno-precipitation using M2-beads to pull-down sAPPβ-FLAG and its potential partners from the brain lysate of the knockin mice. The most prominent protein band running around 97 kd on the 8% gel was identified by tandem mass spectrometry with 32 peptide hits, subsequent sequence analysis showed it is heat shock protein Bip/Grp78. Thus raised the question whether knockin fusion protein sAPPβ-FLAG was unfolded and thus elicited unfolded protein response (UPR), or there was genuine interaction between sAPPβ and Bip/Grp78 as part of the physiological functions of APP.

IP with M2 beads from KI brain lysate



Protein Name	Score	Pep. No.	MW (kDa)
GRP78/BiP [Mus musculus]	4094	32	72.4 kDa
beta-amyloid protein	267	5	78.5 kDa
Vesicle-fusing ATPase (Vesicular-fusion protein NSF) (N-ethylmaleimide sensitive fusion protein)	261	11	82.5 kDa
Myotubularin-related protein 1 [Mus musculus]	148	6	75.3 kDa
Hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A thiolase/enoyl-Coenzyme A hydratase	142	3	82.6 kDa

Figure 3. 3. Co-immunoprecipitation of Grp78/Bip with sAPPβ-FLAG from brain lysates of the knock-in mice.

Left panel is image of the gel, with the red arrow indicates the banc submitted for LC/MS/MS protein identification. Right panel is the summary for results from mass spectroscopy analysis.

3.4 Verification of sAPPβ-FLAG and Grp78/Bip interaction

Observation of Grp78/Bip co-IP with sAPPβ-FLAG from brains of knockin mice can be explained by several possibilities: (a) Grp78/Bip is a bona fide interacting partner of sAPPβ; (b) Grp78/Bip binds tightly to the fusion protein because FLAG epitope tag elicited UPR and thus caused ER-retention of the knock-in protein; (c) Grp78/Bip is an abundant protein that is easily detected by MS protein identification. Even though we suspect possibility (c) is what happening here, we did experiments to carefully investigate the earlier two possibilities. Different research groups have reported observation of Grp78/Bip

expressed on the plasma membrane, as well as released into culture medium and be present in human peripheral circulation (Delpino and Castelli, 2002; Delpino et al., 1998; Shin et al., 2003). Thus we can not exclude the possibility of sAPPβ and Grp78/Bip binding together to exert unknown functions.

In this section, we described two approaches we took to examine whether Grp78/Bip co-IP with sAPPβ in wild type situations:

(1) COS cell co-transfection experiments, followed by IP by myc-beads or protein A beads:

pCMV-myc vector was used to construct plasmid expressing Grp78/Bip with myc fused at c-terminus. The rest of plasmids of sAPP, APP, and BACE were provided by Dr. Qiming Li. We co-tranfected COS cells with following combinations of plasmids, and conditioned medium as well as total cell lysates were collected and clarified for myc-beads immunoprecipitation experiments.

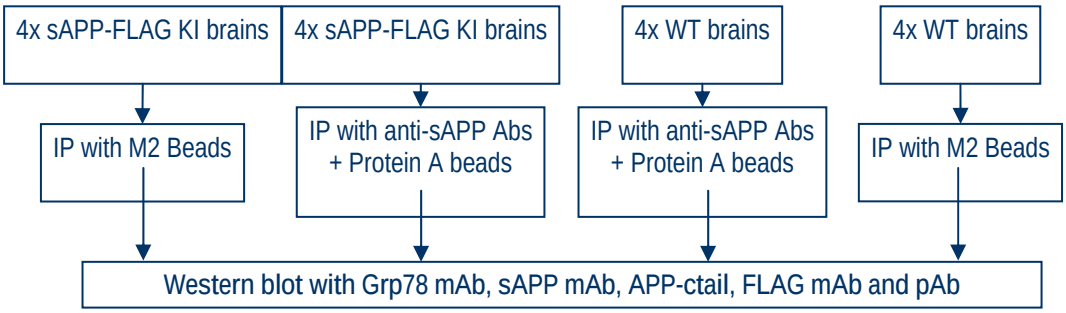
- Grp78-myc & sAPP-Ig (1:1 ratio);
- Grp78-myc & APP-Ig (1:1 ratio);
- Grp78-myc & APP (1:1 ratio);
- Grp78-myc & APP & BACE (1:1:0.25 ratio);

All results from these sets of experiments were negative (data not shown), demostrating that Grp78/Bip-myc does not form any stable complex with sAPPβ in COS-co transfection experiments.

(2) IP from brain lysates of wild type mice

We then tried a series of direct sAPP immunoprecipitation from wild type brains using sAPP antibodies we can find. These are: mAb 348 (Chemicon), sAPP pAb (164C, Südhof lab), sAPPβ-specific pAb (Covance), APP-ctail pAb (U955, Südhof lab). Figure 3.4 showed the flow chart of the experimental design, and a typical result done with mAb 348 antibodies.

(A)



(B)

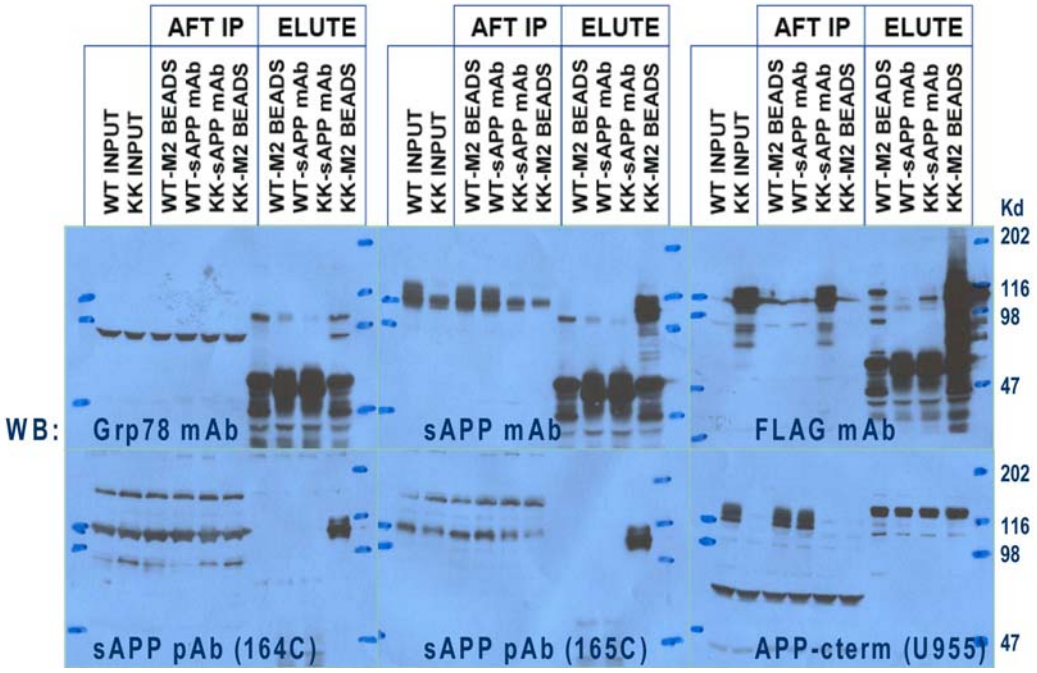


Figure 3. 4. co-IP experiment using sAPP mAb 348.

(A) Flow chart of the experimental design. Four sets of IP were carried out simultaneously, with M2 beads IP from homozygous knockin brains as the positive control, and M2 beads IP from wild type brains as the negative control. (B) Western blot of the IP. Antibodies used for western blot were indicated on the image of each gel. Input brain lysates, flow-through after IP (AFT-IP), and elution portions from the beads (ELUTE) were run on the gel as indicated in the columns above gel images.

In short, none of the antibodies we tried worked well for IP experiments. This is indicated by facts that there no significant reductions of sAPP amount in the lysates before and after IP, and no obvious enrichment of sAPP in beads elution portions, except the positive control. This was the case with all the

antibodees we tried. So, co-IP approach from wild type brains wasn't successful to give us any conclusive results.

(3) IP from lentiviral infected neurons with either sAPPβ-FLAG or sAPPβ-myc.

In order to nail down whether FLAG epitope was the reason for sAPPβ-FLAG bound to Grp78 in the knockin brains, we constructed lentiviral vectors of sAPPβ-FLAG and sAPPβ-myc. High-density rat cortical neuronal culture of more than ten 6-well plates were produced in each rounds of experiment, and both conditioned medium and total cell lysates were collected, quickly frozen by liquid nitrogen, and currently stored in -80º for the co-IP experiments with M2 and myc beads.

3.5 Verification of sAPP secretion level.

In order to find out whether the knockin protein sAPPβ-FLAG is functionally secreted out of the cell, we adopted two experimental approaches: (1) Comparison of sAPP secretion level in neuronal cultures infected with lentiviral vectors expressing sAPPβ, sAPPβ-FLAG, full length APP, APP-frm, pFUGW with EGFP protein as the infection control. (2) Verify sAPP expression in the dissociated neuronal culture from homozygous knockin mice in comparison with their heterozygous littermates. In the preliminary experiment, we also observed sAPP secreted well from both cultures to conditioned medium.

(1) Lentiviral infection assays of wild type cortical culture:

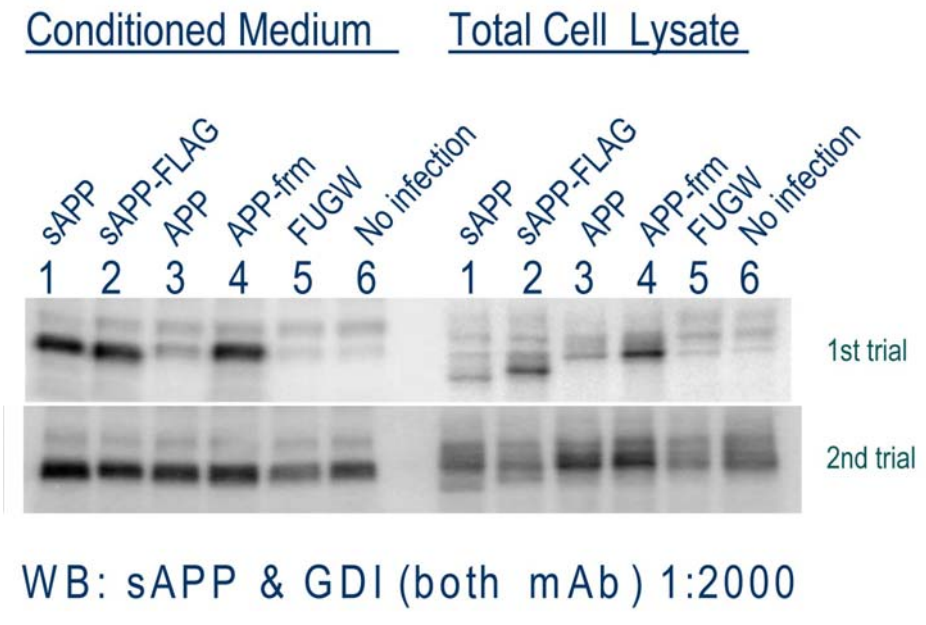
High-density cortical cultures growing on 6-well plates were infected with following lentivirus on DIV 1 for 24 hours, with one well for each infection condition, plus an untouched well as no-infection control. sAPP

- sAPP-FLAG
- APP
- APP-frameshift
- GFP-control

Conditioned medium and total cell lysates were collected on DIV 14, and subjected to SDS-PAGE followed by quantitative immunoblotting analysis using ^{125}I-labled 2nd antibodies as described in the chapter 2.

We have repeated this experiments many times. Figure 3.5 (A) showed two of the repeats. We kept the volume of the total cell lysate as 1/10 of the volume of conditioned medium. And the same volume was loaded on 8% SDS-PAGE gel. Thus, quantification of sAPP secretion level was calculated by the ratio of band density of conditioned medium times 10 versus band density of the corresponding total cell lysates. Four round of quantification results were shown on Figure 3.5 (B).

(A)


Conditioned Medium
Total Cell Lysate
sAPP
sAPP-FLAG
APP
APP-frm
FUGW
No infection
1 2 3 4 5 6
1st trial
2nd trial
WB: sAPP & GDI (both mAb) 1:2000

(B)

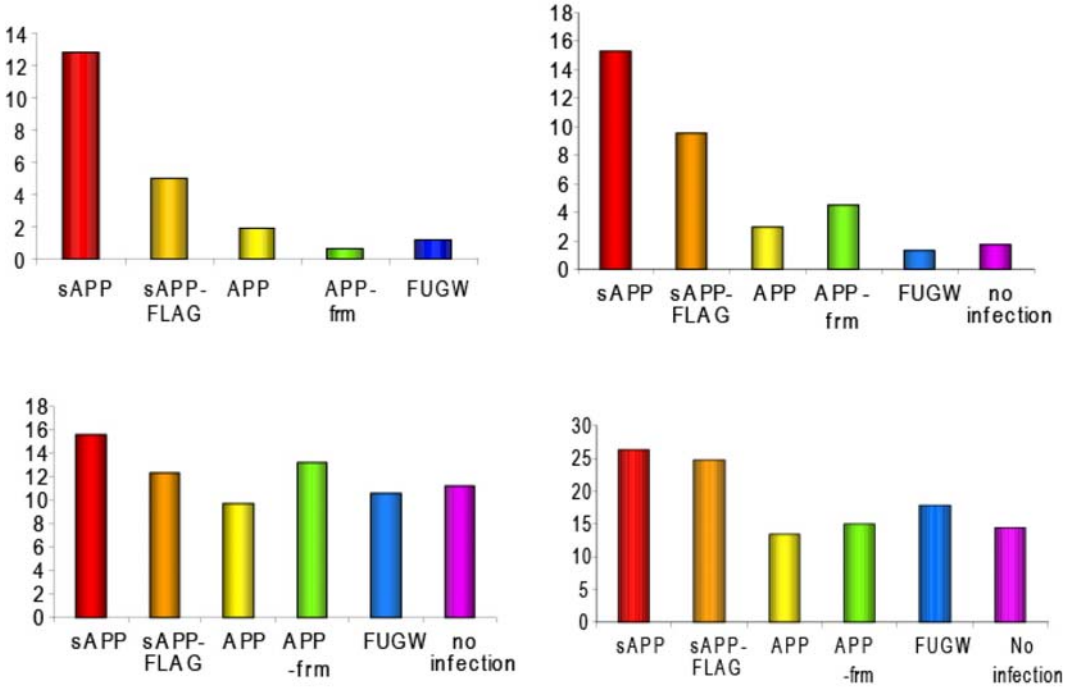


Figure 3. 5. Quantification of sAPP secretion level in lentiviral infected assays.

(A) Quantitative immunoblot images from two rounds of the assay. GDI used as loading control (image not shown). (B) Quantification results from four rounds of test of band density ratio: CM * 10 / TCL.

In all rounds of the tests, we consistently observed sAPPβ-FLAG secreted more than full lenth APP infection. The problem is that there are huge variations on the exact secretion amounts batch from batch, which might due to the difference of the neuronal culture and virus production in each round of experiment. All the experiments presented in Figure 3.5 were done on one or two 6-well plates with each well had different infection. In order to decrease the

batch-to-batch variation to obtain more consistent number for secretion level, we plan to increase the number of 6-well plates for each round of experiments to 6 – 8 plates each time. Then the variation in each well might be averaged by the increased number of plates. However, it is impractical to do the large scale experiments using brains from mice pups, because we are unlikely to obtain so many pups at one time for one batch of large scale culture. Thus, we switch to using rat cortical cultures for the large scale lentiviral infection assays.

(2) Cortical Culture from sAPPβ-FLAG knockin mice

We also quantified the sAPP secretion level on cortical cultures from the homozygous knockin pups in comparison with their heterozygous littermates. Brains from three pairs of littermates were collected to make cortical culture. Conditioned medium and total cell lysate were collected at DIV 14 for quantitative immunoblot analysis. As shown by Figure 3.6, sAPP mAb recognizes a non-specific band on top of the sAPP band in conditioned medium (Upper panel of gel images), which was not seen in total cell lysate of the cortical culture, or total brain lysate (comparing with Figure 3.4 (B)). We quantified the lower bands in the conditioned medium section of the gel, which corresponding to the sAPP secreted, with either the sAPP amount in total cell lysate (lower band alone in the total lysate section) or the total APP amount inside the cell (all three bands combined in the TCL section). Both quantifications showed that cortical

culture from homozygous pups secreted similar amount of sAPP in comparison with their heterozygous littermates.

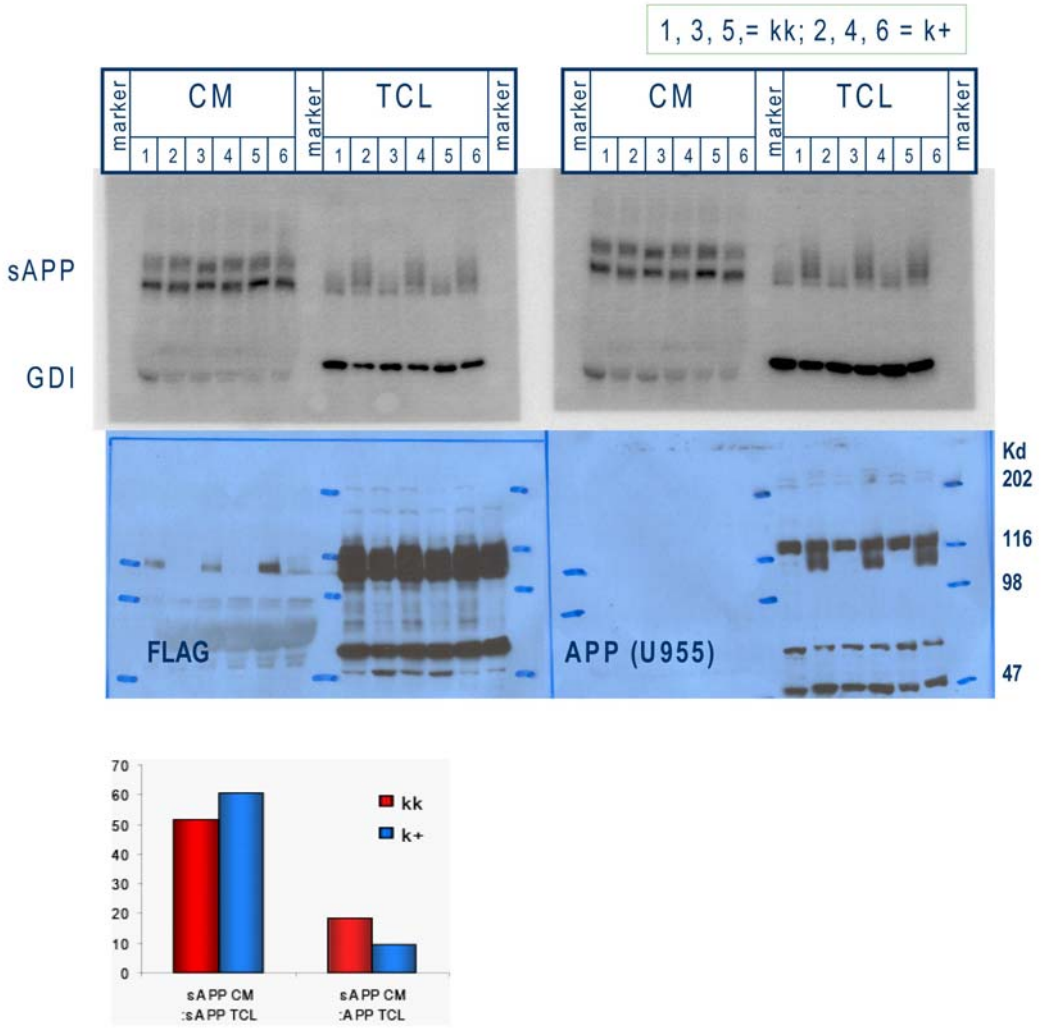


Figure 3. 6 Quantification of sAPP secretion level from cortical culture of the sAPPβ-FLAG knockin pups.

Upper panel, quantitative immunoblotting images from phosphor-imager using sAPP mAb, GDI mAb (loading control) followed by 125I-labeled 2nd antibodies. Middle panel, Enhanced

Chemiluminescence (ECL) images of immunoblotting using FLAG mAb (M2) and APP-ctail pAb antibody (U955). Bottom panel, quantification data of sAPP secretion calculated either by comparing the amount of sAPP in conditioned medium with the amount in total cell lysate (sAPP CM : sAPP TCL), or comparing sAPP in conditioned medium with all the APP (sAPP and full length APP) in total cell lysate (sAPP CM : APP TCL).

SUMMARY AND DISCUSSIONS

The sAPPβ-FLAG knockin mice we generated express truncated sAPPβ with FLAG tag instead of full length APP. The tissue expression pattern of the knockin protein is as wide spread as wild type APP, as far as we observed.

Knockin mice are viable and fertile, and do not have any obvious phenotypes. We measured body weight at different age points for this knockin line; however, since it wasn't recorded systematically, we didn't include the data here. No difference in body weight was observed in the preliminary data.

Co-IP of Grp78/Bip with knockin protein is an unexpected result that we still tying to understand with more assays. We can not simply ignore results as an artifact of the IP – protein identification, even though other projects had encountered similar situation (personal communication). There is an earlier publication showed that Grp78 interacted with APP and inhibited Aβ40 and Aβ42 secretion in HEK293 co-transfection experiments (Yang et al., 1998). In these experiments, cells were labeled with [^{35}S]methionine and [^{35}S]cysteine for 4 h after co-transfection of Grp78 and APP constructs. Immunoprecipitation was

done on cell lysate and radiolabeled proteins were detected by fluorography. Since authors were looking at the possible interplay between APP and Grp78 in ER, they didn't perform immunoprecipitation from the conditioned medium. However, even though Grp 78 is a well know ER markers, its presence has been observed in plasma membrane and in human peripheral circulation (Delpino and Castelli, 2002; Delpino et al., 1998; Shin et al., 2003), so we cannot excluded the possibility that ectodomain of APP might be interacting with Grp 78 on membranes other than ER, and/or in extracellular space. Besides, familial Alzheimer's disease-linked presenilin-1 (PS1) mutation downregulates the unfolded protein response and leads to vulnerability to ER stress, which implicated the role of ER stress in the neuronal death of AD [reviewed (Katayama et al., 2004)]. Since PS1 is one of the key components of γ-secretase in processing of APP, there might also be endogenous interactions between APP and Grp78 for the normal physiological functions. Unfortunately, the assays we tried so far still unable to give us conclusive results whether the co-IP of Grp78 and sAPPβ-FLAG is due to the FLAG epitope tag or through the interaction with the sAPP region. We are conducting further experiments to answer the question.

Next important question for our knockin mice is how well the sAPPβ was secreted. Does the FLAG-tag cause any ER retention as implicated by the IP results? Two approaches were taken to illuminate the issue: (1) A series of lentivial plasmids were constructed. Infected neurons with different constructs

would allow us to compare whether FLAG tagged sAPPβ exhibit ER retention, thus have less secreted level in conditioned medium in comparison with other non-FLAG tagged constructs. Multiple rounds of experiments all showed that there are FLAG-tagged sAPP have comparable or even more secretion level than wild type full length APP. (2) Quantify sAPP secretion from the cortical culture made from knockin mice. We see that homozygous knockin culture secreted similar amount of sAPPβ in comparison with heterozygous culture. All these results suggesting that the knockin mice line we created have normal secretion of sAPPβ, thus, can be used in study of physiological functions of sAPP in future study.

CHAPTER FOUR
CHARACTERIZATION OF sAPPβ-FLAG KNOCKIN/APLP2 KNOCKOUT MICE

INTRODUCTION

The most prominent phenomenon of APP knockout is that, when it is combined with APLP2 knockout, they can lead to early postnatal lethality. sAPPα gene-targeted mice were shown to be able to rescue most of the minor abnormalities observed in APP-deficient mice (Ring et al., 2007) when APLP2 wild type gene was uninterrupted. We would like to know whether the sAPPβ-FLAG knockin mice we created can rescue the postnatal lethality in the APP/APLP2 DKO mice, thus, our study presented in these chapter is focused on analyzing the sAPPβ-FLAG/APLP2 KIKO line.

MATERIAL AND METHODS

Generation of combined mutant and survival analysis

Homozygous sAPPβ-FLAG knock-in mice were bred with APP/APLP2 compound knock-out mice ($APP^{+/-}$ $APLP2^{-/-}$), and offspring with ($APP^{ki/-}$ $APLP2^{+/-}$) genotype were selected. Breeding pairs of ($APP^{ki/-}$ $APLP2^{+/-}$) genotype, as well as a few pairs of ($APP^{ki/+}$ $APLP2^{+/-}$) x ($APP^{ki/-}$ $APLP2^{+/-}$) were used to produce offspring for survival analysis.

Quantitative Western blot analysis

Three littermate pups were sacrificed at P1, and brain tissue was collected and tail was used for genotyping. Brain tissue from the selected genotyping pups were homogenized in PBS with 5 mM EDTA, 1 mM PMSF, 10 µg/ml Leupeptin, 10 µg/ml Aprotinin, and 1 µg/ml of Pepstatin A. The whole brain lysates (25 ug per lane) were analyzed by SDS/PAGE. Quantitative immunoblotting was carried out by using ^{125}I-labeled secondary antibodies and Storm 860/ImageQuant (GE Healthcare, Piscataway, NJ) for detection with GDP-dissociation inhibitor (GDI) or vasolin-containing protein (VCP) as internal standard. Most antibodies that were used were described previously (Rosahl et al., 1995, Schoch et al., 2002).

Generation Antibodies against Fe65 family of proteins force-plate analyses

Adult littermate mouse were placed on a force plate (28 x 28 cm) for 6 min, and force-plate experiments were done as described previously (Fowler et al., 2001). Ataxia index is calculated as the area over the distance traveled on the force plate.

RESULTS

4.1 Generation and Analysis of sAPPβ KI – APLP2 KO (KI/KO) mice.

It is well known that mice deficient of both APP and APLP2 are postnatal lethal (Heber et al., 2000; von Koch et al., 1997). If major functions of APP only depend on its secreted ectodomain without requirement of presence of cytosolic tail, we would expect sAPP knock-in allele would behave like a full lengh APP,

and thus, when breeding to APLP2 knockout background, the resulting knockin/knockout (KIKO) should be able to rescue the postnatal lethality of DKO pups. In order to see whether the expression of sAPPβ-FLAG would have any effect on early postnatal lethal phenotype APP/APLP2 DKO, we crossed homozygous knock-in mice (wild type for APLP2) with $APP^{+/-}$ $APLP2^{-/-}$ mice to generate mice expressing sAPPβ-FLAG over APLP2 deficient background. Survival analysis showed that expression of sAPPβ-FLAG could not fully rescue the lethal phenotype of APP/APLP2 DKO. However, we did observe 6 mice out 251 with $APP^{ki/-}$ $APLP2^{-/-}$ genotype survived till at least P19 (~11% of expected Mendelian segregation). Survival study on new born pups the day after birth (**Figure 4.1**) showed expression of sAPPβ-FLAG was able to extend the life span of new born pups to a limited degree. It was suggested that this life-extended phenomenon is unlikely caused by the expression of the knockin protein, but rather the ES-cell electroporation that introduced R1 genetic background to the C57BL/6 mouse line. Dr. Muller (University of Heidelberg, Heidelberg, Germany) had experienced similar phenomenon before (personal communication). Thus, we conclude that expression of sAPPβ-FLAG is not sufficient in rescue the postnatal lethality of APP/APLP2 DKO, indicating importance of AICD in physiological functions of APP.

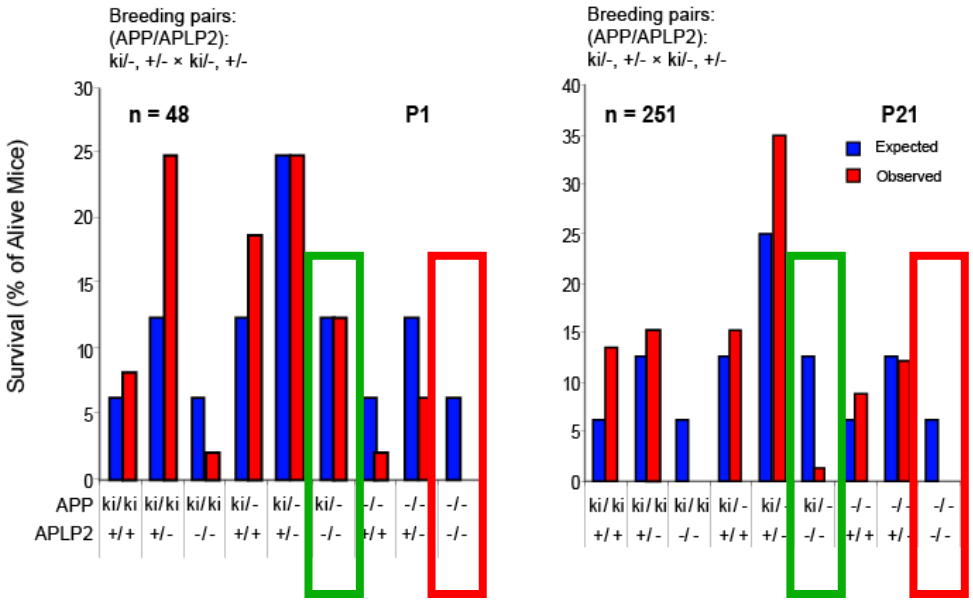


Figure 4. 1. Survival analysis of APPs knock-in mice with APLP2 knockout background. Left panel, genotypes of 48 offspring from mating of $APP^{ki/-}$ $APLP2^{+/-}$ mice were analyzed at birth. Right panal, genotypes of 251 adult offspring from mating of $APP^{ki/-}$ $APLP2^{+/-}$ mice were analyzed. Data shown are percentages of expected Mendelian segregation (white bars) and observed genotypes (grey bars) over the total number.

4.2 Quantification Fe65 expression level in KIKO mice line

Since we observed increasing protein level of Fe65 family of proteins in brain lysates of APP/APLP2 DKO pups, we would like to know whether the same effect would happen to the knock-in pups on APLP2 knockout. From quantitative western blot assay, the initial test failed to demonstrate that Fe65 expression level have any statistically significant increase in the KIKO pups (data not shown). This result is quite unexpected, and recollected the three litters of offspring of

KIKO breeding, run the brain lysate samples on the same gel with three pair littermates from APP/APLP2 DKO pups. Again, quantification showed clearly that Fe65 is upregulated in the APP/APLP2 DKO pups, but not in the KIKO line of mice (**Figure 4.2**).

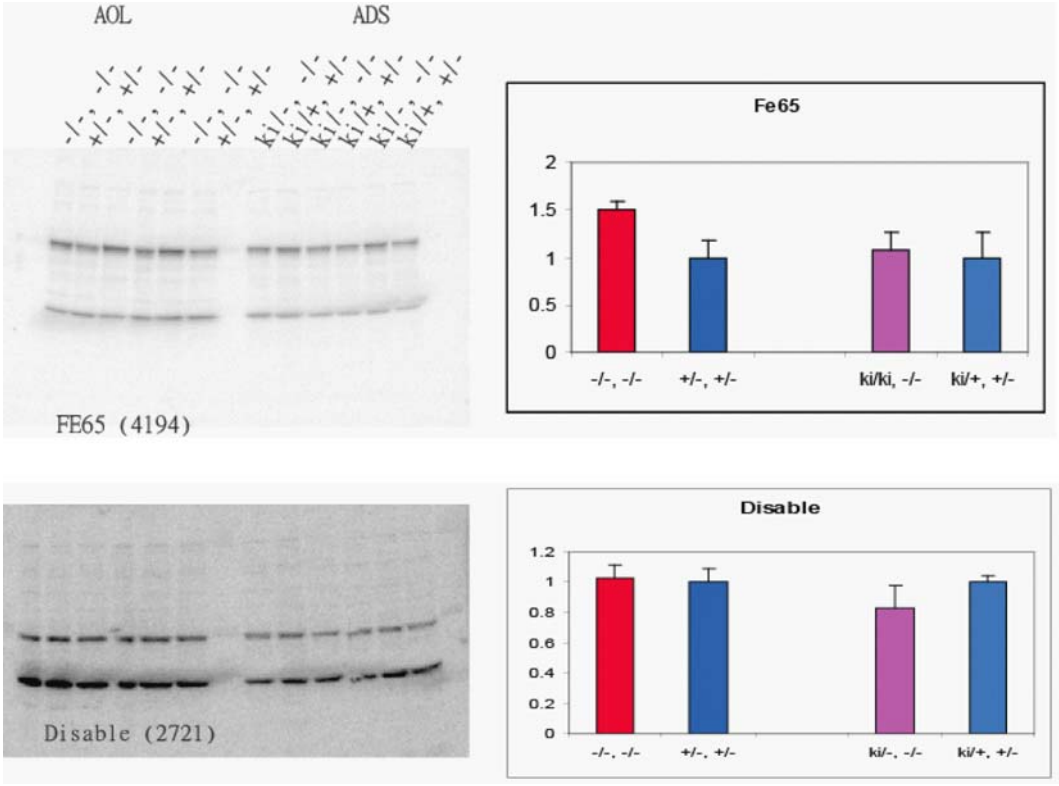


Figure 4. 2. Expression levels of Fe65 and Disable in brain of the KI/KO P1 mice. Left panel, quantitative western blot using ^{125}I-labled 2nd antibodies; right panel, bar-diagram showing the quantification results of the immunoblots. AOL is the nickname we gave to APP/APLP2 DKO mice and ADS is what we called the sAPPβ-FLAG/APLP2 KIKO line. (APP, APLP2) genotype of each lane was indicated on the top panel. Antibodies used were marked on the gel images. GDI, GDP-dissociation inhibitor was used as internal standard (the lower-molecular-weight set of bands on each image). Data

shown are means ± SEMs from three sets of independent litter mates normalized to internal standard.

We have been quite puzzled by this result. Expression of sAPPβ-FLAG was not able to rescue the lethal phenotype of DKO, but by some unknown mechanisms, it rescued the Fe65-upregulation phenotype. Since we are still in the dark on by what pathway(s) lead to the increase of Fe65 in the APP/APLP2 DKO brain, we are even more perplexed by how the effect can be compensated by the expression of sAPPβ-FLAG. After all, the two proteins do not reside in the same cellular compartments. We can only speculate the ectodamain of APP can signal through another unidentified factor to affect the protein level of Fe65.

4.3 Abnormal Motor Behavior in KIKO survival mice.

A few number of homozygous KIKO mice escaped the postnatal lethality and survived till adulthood. However, these mice all exhibited obvious abnormality in at least two ways: (1) body size and the body weight of the escaped KIKO are much smaller than their compound heterozygous littermates. They can be easily identified from just visual observation. (2) Escaped KIKO mice exhibit abnormal motor behavior, as recorded in the analysis using force plate actometer (Fowler et al., 2001) in Figure 4.3.

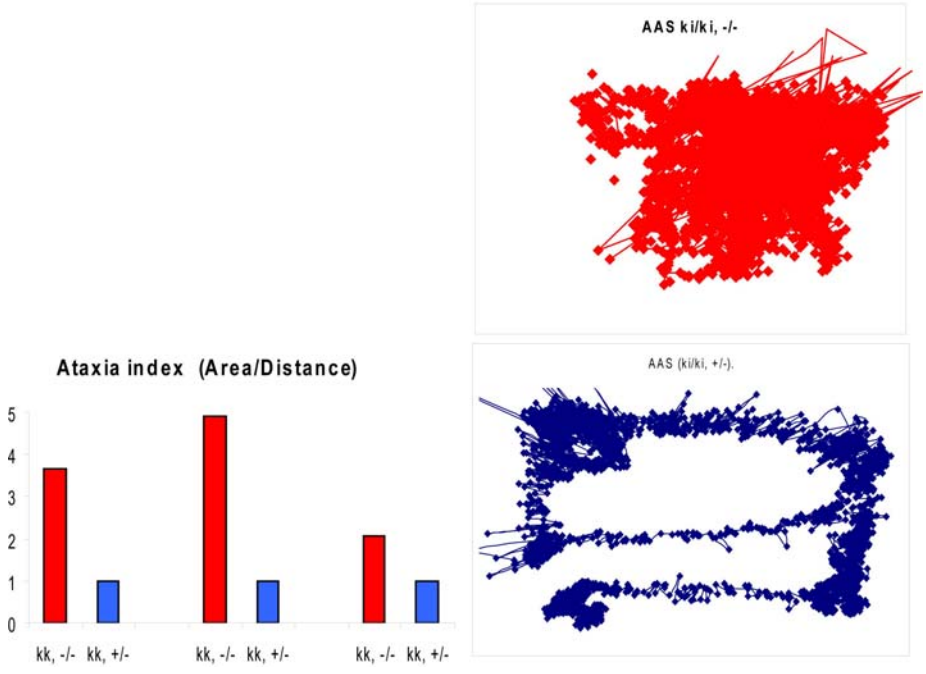


Figure 4. 3. Behavior abnormalities of KIKO mice that escaped lethality to adulthood, as observed by force plate test.

Three double homozygous KIKO mice that escape the lethality to adulthood were tested on force plate, in comparison with their compound heterozygous KIKO littermates. Right panel showed the locomotor sample trace of ($APP^{ki/ki}$, $APLP2^{-/-}$) in red and ($APP^{ki/ki}$, $APLP2^{+/-}$) in blue. Left panel showed that bar diagram of Ataxia index analysis. For each littermate pair, the homozygous KIKO has high ataxia index as measured by Area/distance.

Three littermate pairs from the KIKO line were used in force plate analysis. Each mouse was placed and recorded for 6-min session. We also video taped the first 30 second of each session for the record. The homozygous KIKO mice all like to take the center of the stage and turn around and around, while their heterozygous littermates prefer to huddle on the corners and explore the side

of the platform, as shown by the right panel of Figure 4.3. In the 6-min session of each analysis, the location of the mice was recorded every 0.2 second. The area measure for each data point of the trace is the area formed by three successive points travelled by the mouse. Thus, the mouse with normal motor behavior would tend to leave relative straight traces that result in smaller total area measurements, while the abnormal wobbly mice will leave more zigzagged traces that greatly increase the accumulative measurement of area. Left panel of Figure 4.3 showed that homozygous KIKO mice have much higher ataxia index (Area/Distance travelled) than their heterozygous littermates.

SUMMARY AND DISCUSSIONS

Breeding the sAPPβ-FLAG knockin mice to APLP2 knockout background showed that expression of sAPPβ-FLAG does not rescue the lethal phenotype of the APP/APLP2 DKO. This is different from the preliminary result reported by Dr. Müller (University of Heidelberg, Heidelberg, Germany) at last year's annual SFN meeting, that expression of sAPPα at APLP2 knockout background was able to rescue the postnatal lethality to ~ 60%. If this is the case, then we need to investigate how sAPPα form can make such a big difference functionally than the sAPPβ form, with only 17 amino acid difference between the two forms at C-terminus.

The few homozygous KIKO mice that escaped postnatal lethality all have much smaller body sizes and body weights than their less mutant littermates; and all these "escapers" exhibit motor behavior abnormality. We don't have enough homozygous APP/APLP2 DKO mice escaped to adulthood for comparison with "escapers" from the KIKO line. Nonetheless, communication with Dr. Hui Zheng's group (Baylor College of Medicine, Houston, TX) indicates that what we observed on KIKO is quite similar with what they have seen in the DKO "escapers", where there is a higher rate of survival in their mice husbandry. So, it suggested that expression of sAPPβ-FLAG has no effect on either the postnatal survival, growth restriction, or the motor behavior abnormality.

The only difference the knockin protein made, as we observed, seemed to be the restore the "normal" level of expression of Fe65 from the upregulation of the DKO mice. This indicates that besides the direction interaction of APP with FE65 through its intracellular domain AICD, APP has other indirect route to be connected to Fe65 through its extracellular domain and other unknown factor(s).

Physiological functions of APP are turning out to be a much complicated system than we had originally expected. Expression of fragments of APP might be able to substitute some of the functions of APP, but not all. There are physiological processes that require only the ectodomain of APP, as showed in *C. elegans*' case (Hornsten et al., 2007) and the publication on sAPPα knockin mice (Ring et al., 2007); meanwhile, there are also processes that the AICD is sufficient

to do the job, as shown in the most recent publication that Dr. Muller also co-authored (Schrenk-Siemens et al., 2008). The abnormal reduction of protein and mRNA level of vesicular glutamate transporter (VGLUT2) were observed in ES-cell derived glutamatergic neurons lacking both APP and APLP2; and this phenotype can be restored by expression of human AICD, suggested again that AICD is crucial for transcriptional regulations. There are also situations that existence of both extracellular and intracellular domains are required, as the study on APP's function on neuronal migration has showed (Young-Pearse et al., 2007).

In short, many physiological roles have been proposed for APP molecule, but we still unclear on how these functions are orchestrated in the normal cellular system.

CHAPTER FIVE
GENERATION AND ANALYSIS OF APP ARCTIC AND DUTCH KNOCKIN MICE LINES

MATERIAL AND METHODS

Generation of Arctic and Dutch Knockin Lines, breeding with APLP2 knockout line, and survival analysis.

Gene targeting strategy is illustrated in Figure 5.1, and targeting vector was described in Chapter Three. Exon 16** has Swedish mutation and humanized Aβ sequence. Exon 17* has humanized Aβ sequence and London mutation. For Arctic construct, exon 17* has additional Arctic mutation and BamHI site. For Dutch construct, exon 17* has additional Dutch mutation, AatII site and Bam HI site. There is an unexpected frameshift mutation happened after residue I731 (numbered by APP770 isoform) on Actic construct, resulting incorrect of C-tail sequence afterwards and stop codon at residue 756 (14 amino acid shorter than the wild type APP).

sAPPβ-FLAG knockin mice as described in Chapter Three were breed with Protamine-Cre recombinase transgenic mice (O'Gorman et al., 1997) to excise truncated exon16* flunked by LoxP sequences as shown in Figure 5.1. The F1 male offspring were selected to breed with wild type C57BL/6 mice in order to establish Arctic and Dutch knockin lines. Genotyping was done by two steps. First step is the same reaction described in Chapter 3. Second step is done by primer pair (HL4-21: gccgctaaaaccaagtcaacatttc) and (HL5-35:

ctgaatcatgtcggaattctgcatccagg). Excision allele gives PCR product: ~400 bp. No product from wild type allele or allele with neo cassette.

Mice homozygous for Arctic and Dutch knockin were bred to APP/APLP2 knockout line. Offspring with APP ki/+ or ki/- and APLP2 +/- were selected and breed to each other for survival analysis.

Immunohistochemistry

Adult mice were anaesthetized by Halothane and perfusion-fixed with 4% freshly prepared paraformaldehyde in 100 mM PB (85 mM Na_2HPO_4, 15 mM NaH_2PO_4). Brains were removed and post-fixed overnight at 4ºC in 4% paraformaldehyde before cryoprotection in 30% sucrose/PBS at 4ºC for more than 24 hours. Next, brains were frozen in Tissue-Tek O.C.T. compound immersed in dry ice-chilled 2-methyl butane and kept in - 80º C. 50 µm saggittal sections were permeabilized with 0.5% Triton X-100, blocked with 2% goat serum 0.1% Triton X-100, and incubated with primary antibodies overnight at 4°C. Sections were further washed and incubated with secondary antibodies for 1 hour room temperature, followed by incubated with PAP (Activity Select peroxidase – anti-peroxidase comples, Sternberger) for 1 hour room temperature. Brain sections were developed in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB) with 1% nickel chloride as a metal enhancement, prepared in 100 mM PB). Sections were mounted on micro slides, then air-dried overnight, dehydrated in 70%, 90% and 100% ethanol, cleared in xylene, and coverslipped with Permount.

ELISA

Aβ40 and Aβ42 ELISA kits were purchased from IBL Co., Ltd. Japan. 9 month old Arctic and Dutch knockin mice with their littermates were euthanized by Halothane. Brains were collected homogenized in PBS with 5 mM EDTA and protease inhibitors. Total brain lysates were centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant was termed "soluble fraction"; the pellet was solublized by RIPA buffer for 1 h at 4 °C, followed by centrifugation at 100,000 x g for 1 h at 4 °C. The supernatant from this centrifugation was called "membrane portion". The pellet from this step is considered as "insoluble fraction". The insoluble fraction was dissolved in 6M guanidine hydrochloride, and then the high salt was removed by using C18 tips. Aβ40 and Aβ42 quantification was done following company's protocol.

RESULTS

5.1 Generation and Initial Characterization of Arctic and Dutch knockin lines.

Knockin mice described in Chapter Three were crossed with transgenic mice carrying cre recombinase driven by Protamine promoter (O'Gorman et al., 1997). Male offspring were selected for genotyping analysis, and mice with

knockin allele were bred with wild type female C57BL/6 to establish the knockin line.

Homozygous Arctic and Dutch mice are viable, fertile, with no obvious phenotype.

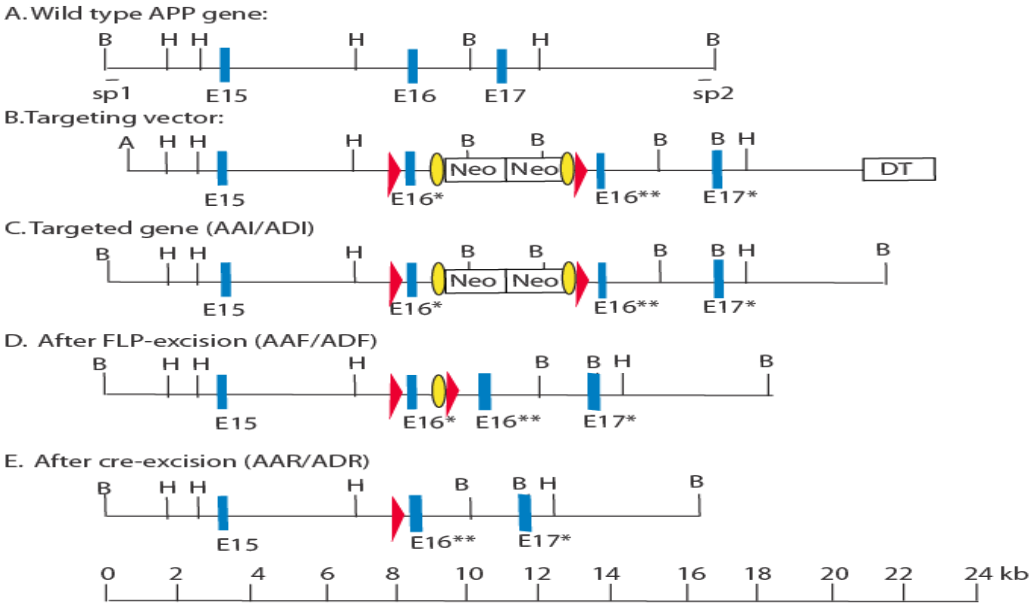


Figure 5. 1. Generation of sAPPβ-FLAG knock-in mice.

After cre-excision, exon 16** and exon 17* will be expressed. Exon 16** has Swedish mutation and humanized Aβ sequence. Exon 17* has humanaized Aβ sequence and London mutation. For Arctic construct, exon 17* has additional Arctic mutation and BamHI site. For Dutch construct, exon 17* has additional Dutch mutation, AatII site and Bam HI site.

5.2 No plaques observed from 9 month old knockin brains.

We have constructed Swedish mutation, London mutation, and humanized Aβ sequence, in addition to the Arctic and Dutch mutation in these two lines. We

were hoping all these mutations might have synergistic effect in facilitating plague formation.

Immunohistochemistry were done on 9-month old homozygous knockin mice, using 2 month old APPswePS1ΔE9 transgenic mice (JAX# 4462) as positive control. Primary antibody is rabbit polyclonal antibody raised against Aβ oligomers, produced in our lab. Unfortunately, none of the homozygous knockin mice have any plague formation even to 9 month old. We now accumulated more aged mice up to 15 month old and we will check for the plague formation again. Meanwhile, presenilin 1 M146L knockin mice were provided by Dr. Jie Shen's lab to be bred with our APP mutant mice. We expect APP/PS1 double knockin mice will produce plaques at much early age.

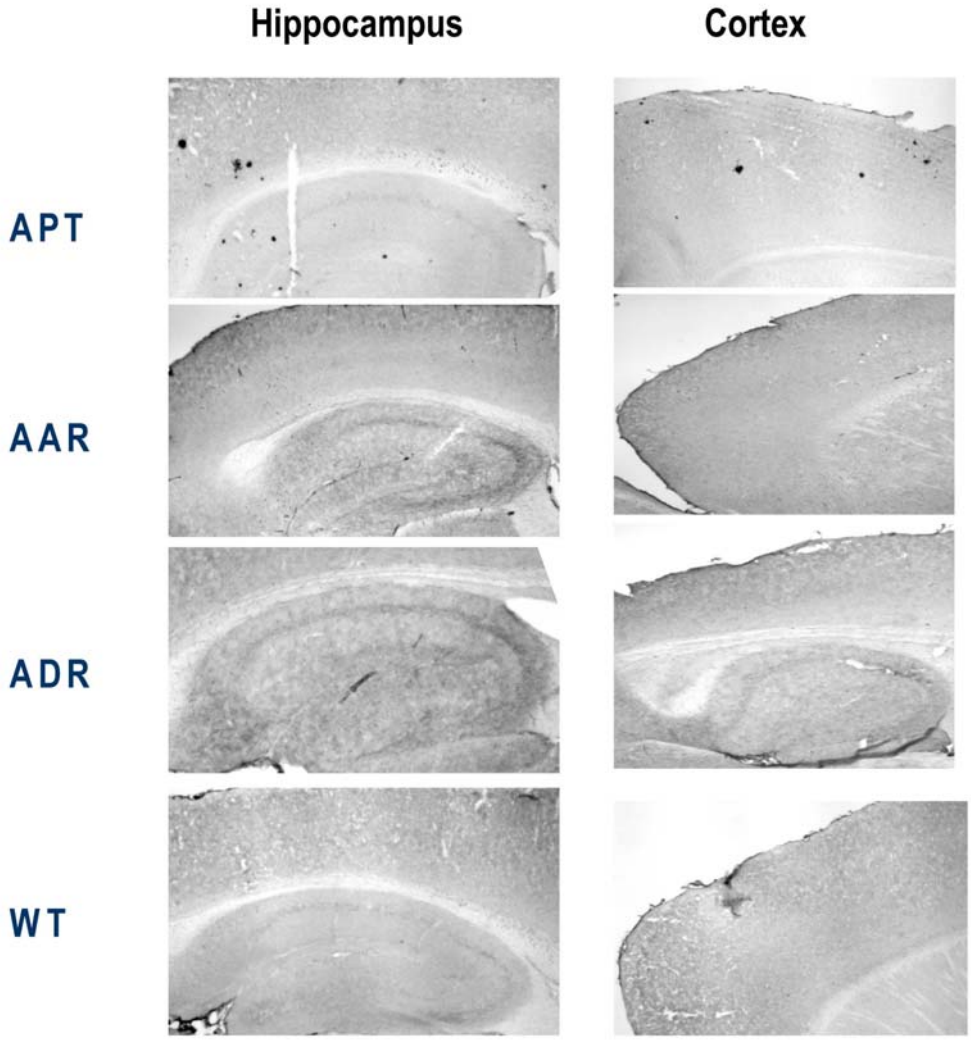


Figure 5. 2. Arctic and Dutch knockin lines have no plaques developed in the brain up to 9 month old.

Antibody against Aβ was used to detect any possible plague formation in immunohistochemistry. APT: APPswePSdE9 transgenic mice, used as positive control; AAR: Arctic knockin mice; ADR: Dutch knockin mice; WT: wild type negative control. No plague was detected in either Arctic line or Dutch line up to 9 month of age.

5.3 Quantification of Aβ40 and Aβ42

In order to monitor the Aβ40 and Aβ42 level in these mice at 9-month old time point, we sacrificed homozygous knockin mice together with their heterozygous littermates. Homozygous sAPPβ-FLAG knockin mouse (ADS on Figure 5.3) was used as negative control because it did not have Aβ region. APPswe-PS1ΔE9 double transgenic mouse (APT) at six month of age was used as positive control. Brains homogenates from these mice were fractionated into soluble fraction, membrane fraction and insoluble fraction. All three fractions were assayed using ELISA kit.

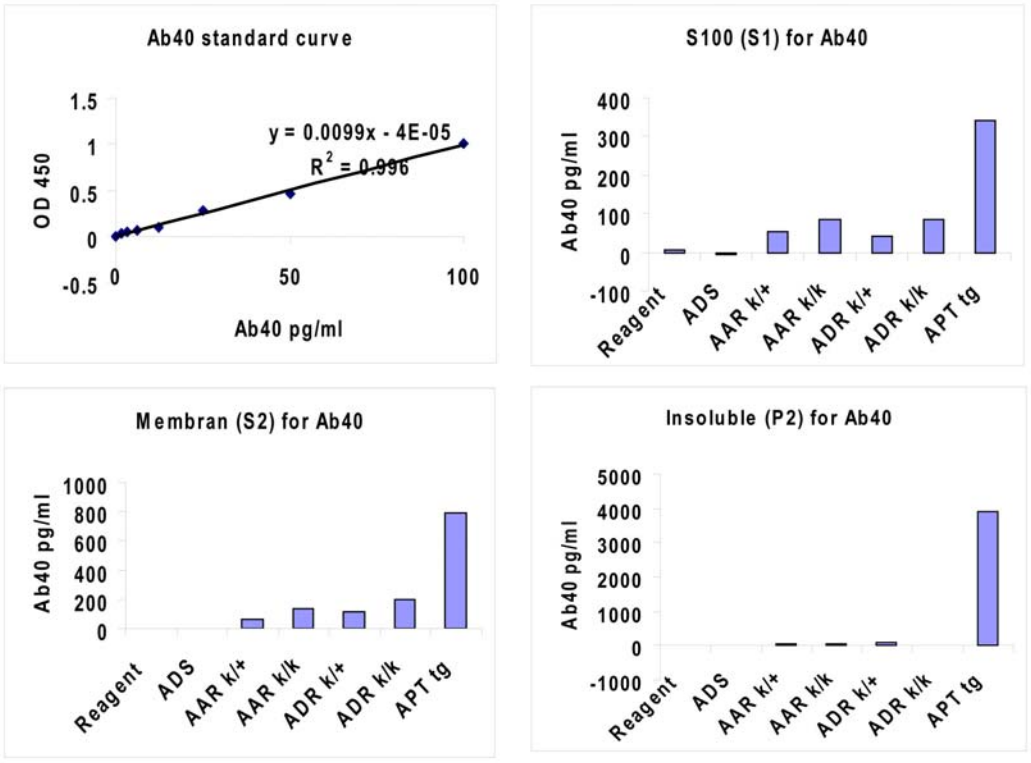


Figure 5. 3. ELISA assay for Aβ40 quantification for mice at 9-month old.

Brain homogenates were fractioned into soluble (S100, S1), membrane (S2), and insoluble (P2) fractions. All three fractions were subject to Aβ40 quantification using ELISA kit. ADS: homozygous sAPPβ-FLAG knockin mouse; AAR: Arctic knockin mouse; ADR: Dutch knockin mouse; APT: APPswe – PS1ΔE9 transgenic mouse.

We see a nice allele-depended Aβ40 abundance in both soluble and membrane fraction for both Arctic (AAR) and Dutch (ADR) knockin lines. No Aβ40 was detected in the insoluble fraction, which correlated with the fact that no plague was observed in these mice.

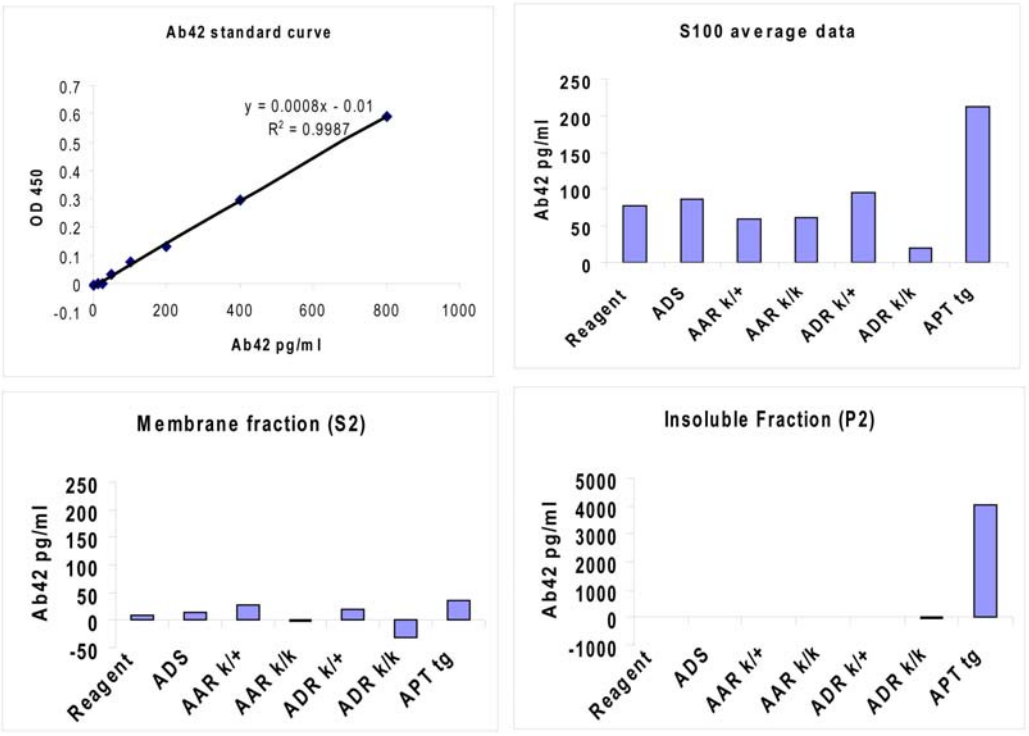


Figure 5. 4. ELISA assay for Aβ42 quantification for mice at 9-month old.

Aβ42 ELISA results (Figure 5.4) showed that there were no detectable Aβ42 in these knockin lines.

With the initial immunohistochemistry and ELISA results, we decided to age these knockin lines to more than 15 months before we collected brain tissue.

5.4 Survival Analysis of Arctic/Dutch knockin with APLP2 knockout.

Both Arctic and Dutch knockin lines were breed to APLP2 knockout mice. We fully expected to see full rescue, because the mutant full length APP should be able to perform most of the functions of APP. In fact, this is exactly what we observed in Dutch KI/APLP2 KO breeding. We stopped this type of breeding when we observed 11 KIKO survival out of 13 expected (Figure 5.5).

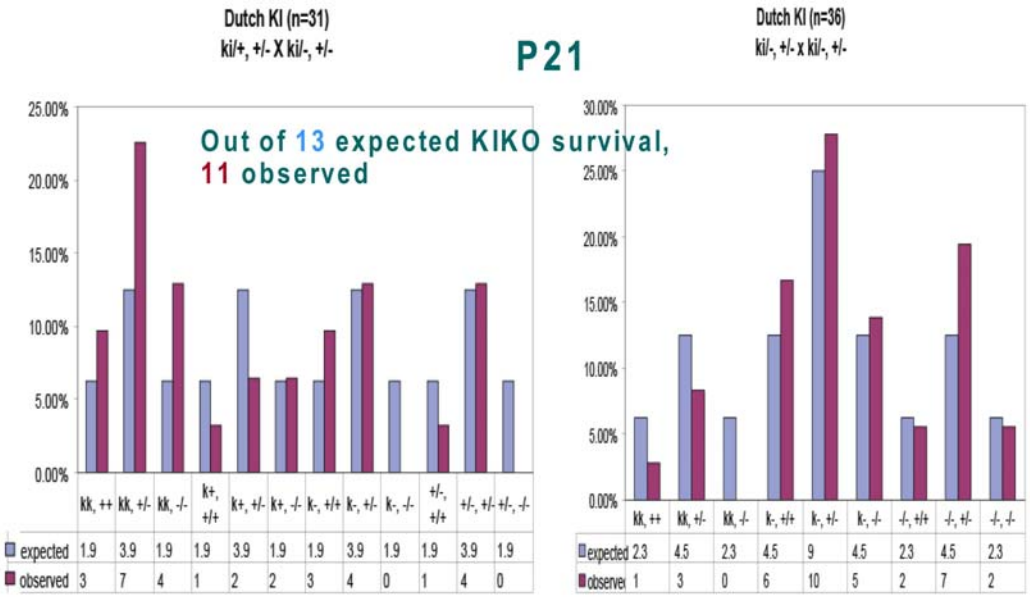


Figure 5. 5. Survival study for Dutch knockin and APLP2 knockout mice.

Results from Arctic knockin/APLP2 breeding, on the other hand, give quite unexpected results. The survival rate is abnormally low for this KIKO line. As shown in Figure 5.6, breeding of (APP, APLP2) (ki/-, +/-) pairs, we only find five mice with mutant APP and homozygous knockout of APLP2 survived till adulthood while 21 were expected according to Mendelian ratio. The survived mice are fertile. One male survivor was able to produce offsprings, but there is no KIKO survivor in the litter (right bottom panel of Figure 5.6).

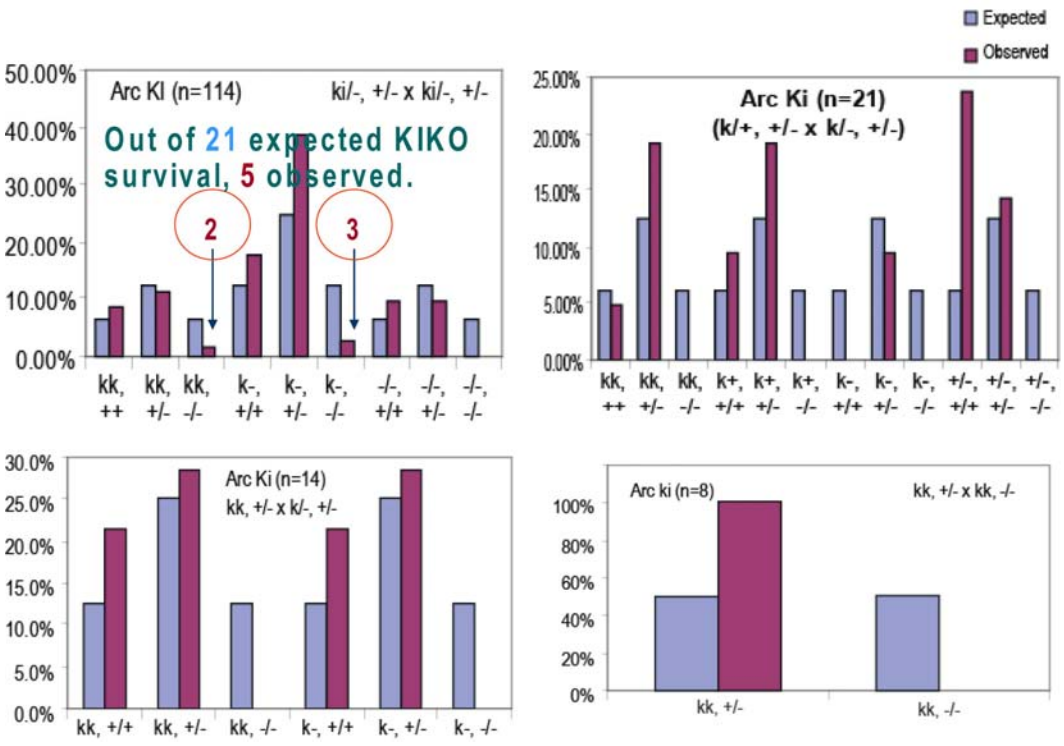


Figure 5. 6. Survival study for Arctic knockin and APLP2 knockout mice.

5.5 Frameshift mutation in Arctic knockin line.

Bewildered by above results, we felt it is unlikely that Arctic construct would normally acting as APP null allele. Since we already observed Aβ40 production from EILSA experiments, we look further at the expression of C-tail of the protein in the knockin line.

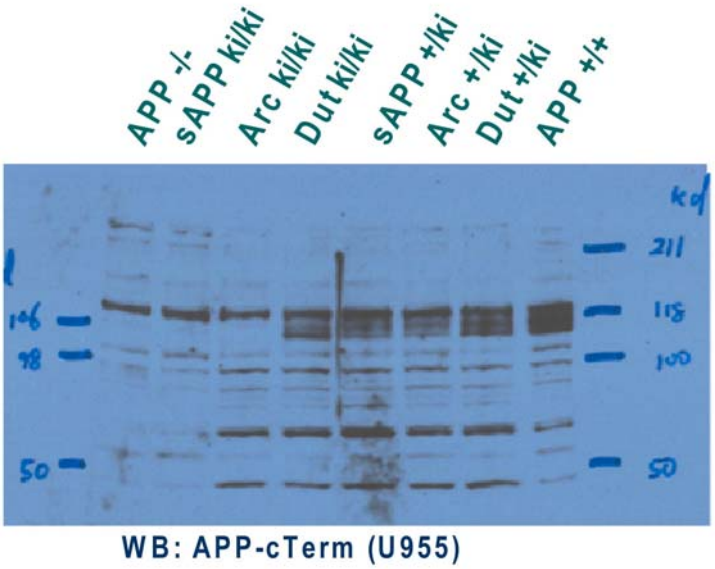
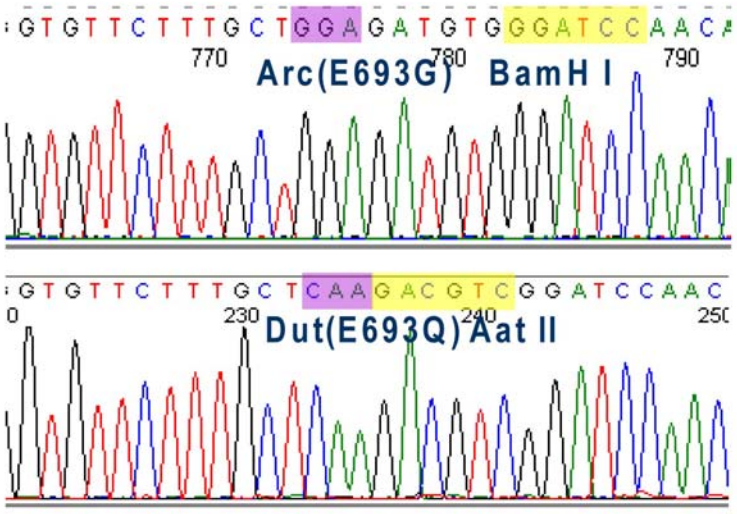


Figure 5. 7. Arctic knockin line does not have the same c-terminal region as the wild type APP

Brain lysates from homozygous Arctic and Dutch knockin mice and their littermates were subjected to western blot analysis using antibody against c-terminus of APP. Brains from APP -/- and sAPPβ ki/ki were used as negative control, and wild type tissue was used as positive control.

Western blot analysis, as shown in Figure 5.7, illustrated that mutant APP produced in Arctic knockin line can not be recognized by our APP C-tail antibody, where Dutch knockin line behave as expected.

We decided to sequence those exons encoding AICD region in both knockin lines. As it turned out, gene mutations we designed for the construct are all well in places. Arctic mutation (E693G), BamHI restriction site, and London mutation (V717I) for Arctic line, as well as Dutch mutation (E693Q), AatII and BamHI sites, and London mutation (V717I) for Dutch line, all of these have been confirmed by the TA cloning of the PCR product of exon 17 (upper two panel of Figure 5.8). However, close to at the end of exon 17, 14-residue away downstream of London mutation, we found a frameshift mutation in the Arctic knockin line (lower two panel of Figure 5.8). Thus, the mutant APP in the Arctic knockin line does not have the correct sequence of the last 38 residues of the wild type APP, including the highly conserved NPTY motif. Instead of the last 38 residue, it expresses 24 nonsense residues then encountered stop codon.



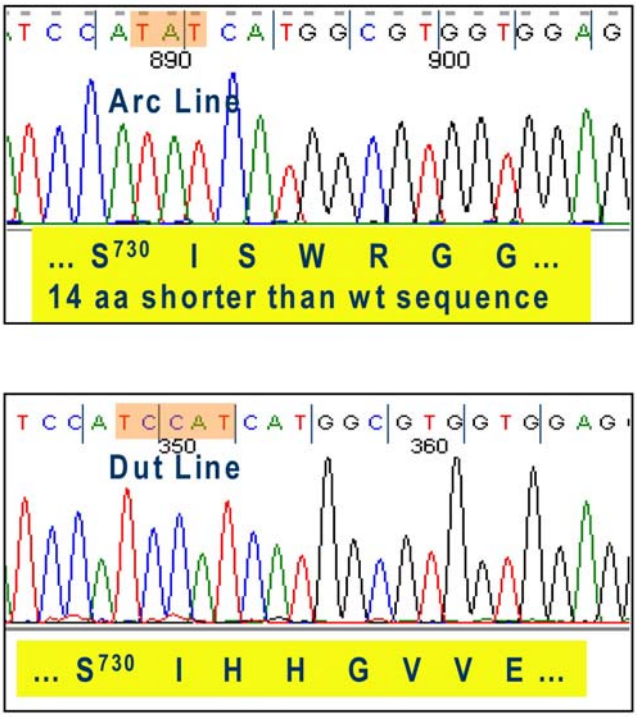


Figure 5. 8. Sequencing analysis revealed that Arctic knockin line harbored unexpected frameshift mutation after residue I^{731}.

5.6 Quantification of sAPP and Fe65 in Arctic knockin/APLP2 knockout mice.

This unforeseen frameshift mutation disrupt our original research plan of the Arctic and Dutch constructs, however, it offered a good control for our sAPPβ-FLAG knockin mice. Results presented in chapter three and four were continuously raising doubts in our mind, whether the sAPP expression was compromised by the FLAG tag. Here, frameshifted Arctic line offers production of sAPPβ, Aβ40, and un-functional intracellular region. It is already shown from

the survival study that the mutant APP without the AICD region failed to rescue postnatal lethality of APP/APLP2 DKO. Next, we examine the secretion of sAPP in this knockin line and the expression level of Fe65 in the Arctic KIKO mice.

Three pairs of littermates of the Arctic mice were sacrificed at 2-month of age, and the brains were homogenized in the 0.32 M sucrose solution (made in PBS with EDTA and protease inhibitors) to preserve vesicles. S100 preparation was done by centrifuge the total brain lysate at 100,000xg for 1 hour. sAPP presented in the S100 portion can be rougly considered as the secreted portion. As showed in Figure 5.9, there is no significant difference between either the total sAPP amount, or the secreted amount as observed from S100 prep.

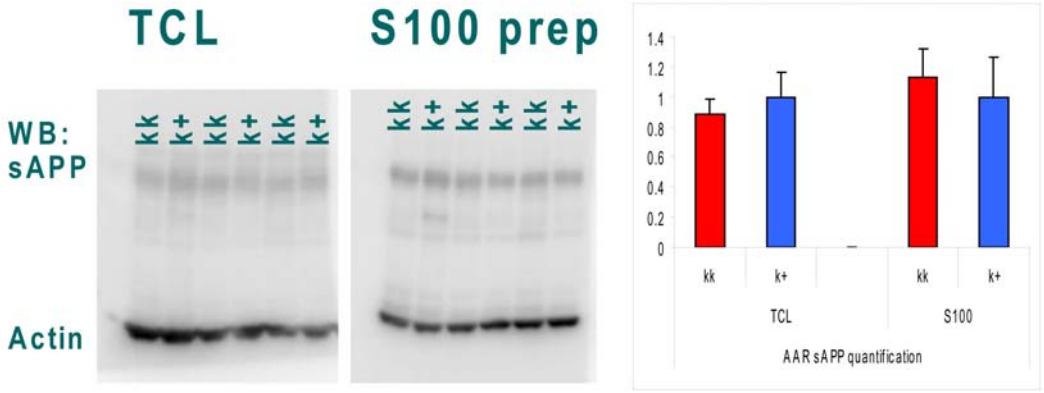


Figure 5. 9. Quantification of sAPP secretion level in the frameshift Arctic knockin line

Next, expression of Fe65 and Fe65 L2 were quantified in the brains of Arctic KI/APLP2 KO mice (Figure 5.10). Again, the result is similar to what we observed with sAPPβ-FLAG KIKO, that Fe65 is no longer upregulated in these

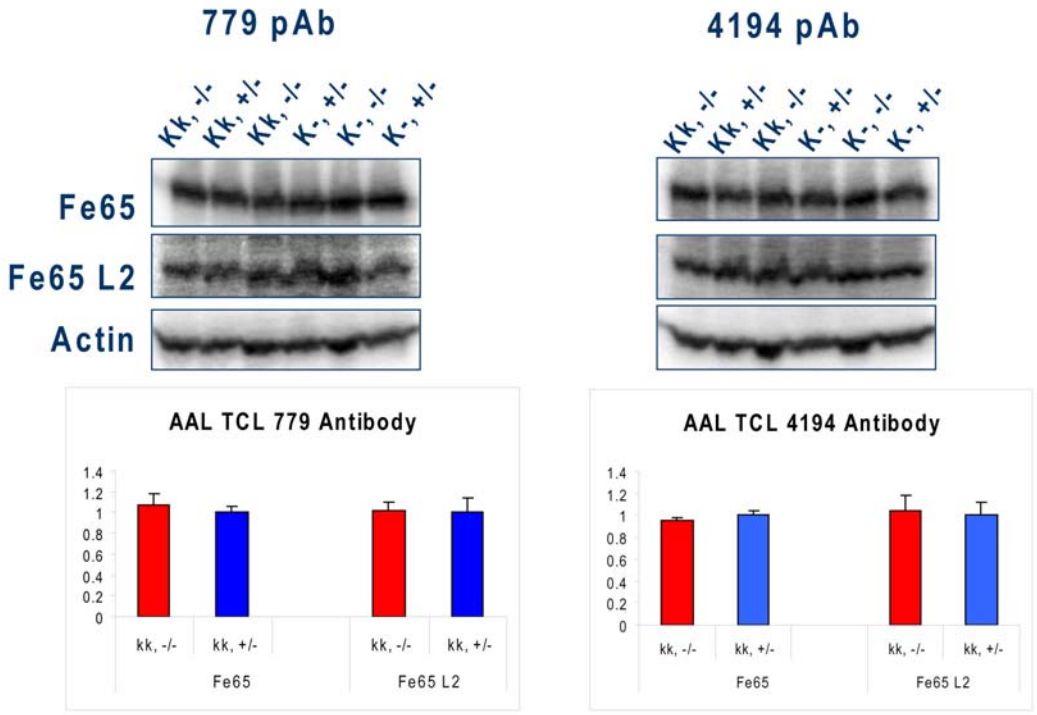


Figure 5. 10. Quantification of Fe65 and Fe65 L2 expression level in the Arctic/APLP2 KIKO mice.

SUMMARY AND DISCUSSION

Our original research plan in generating the Arctic and Dutch knockin mice was to study how single amino acid substitution can cause quite different phenotype in the brain, with Arctic mutation, it results in heavy parenchymal Aβ deposit and cause severe AD, whereas Dutch mutation produces sever vascular amyloidosis. Our mice model would allow us to study the difference between the two in the same genetic background. Unfortunately, with frameshift mutation in

the AICD region of the Arctic construct, it can no longer serve as a good control for the Dutch construct. We will still investigate the production of Aβ in these mice, and the double knockin with PS1. It would be interested to see whether the messed-up intracellular domain would change the production of Aβ in anyway. We just need to be very cautious with any possible conclusions we want to draw, because we don't have the "perfect" control for the frameshift Arctic line.

In a twisted sense, it is fortunate for us to have the frameshift Arctic line that can provide us more definitive answers to the questions we had on the sAPPβ-FLAG project. Specifically, survival analysis from APP-Arctic/APLP2 KIKO breeding has provided further proof that AICD is crucial for APP functions.

We have observed various phenomena regarding the physiological functions of APP using the mice we generated. However, we haven't found any underline mechanisms yet to explain or link these phenomena into a complete picture. So further effort is needed to forward our understanding in why APP is been processed and how all the observed physiological functions coordinate together.

APPENDIX A

ChIP-chip Results of Fe65-binding Sequences

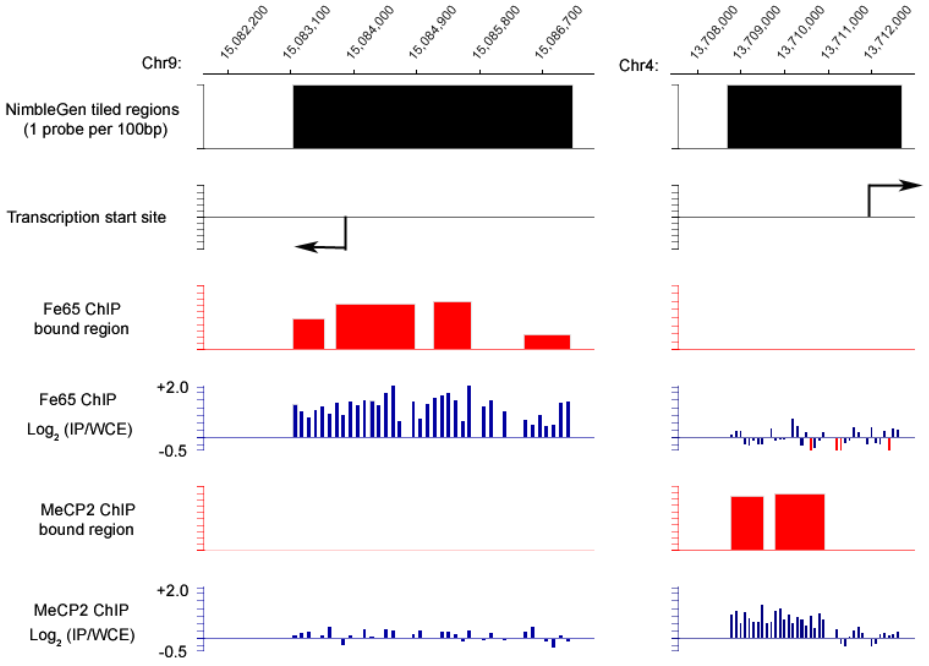
ChIP-on-chip (or ChIP-chip) is the method that joins chromatin immunoprecipitation (ChIP) with micro array (chip) to isolate from the whole genome and identify DNA sequences that bind to a specific protein.

In collaboration with Dr. Yi-Sun's lab in UCLA, we are using ChIP-on-chip technique to identify DNA sequences that bind to Fe65 family of proteins. We would also investigate whether APP expression would alter these identified Fe65 targets.

The first round of test was done using brains from adult wild type mice, just to see what genes have their promoter bind to Fe65, thus, might be influenced by this family of proteins. The antibody used in the first round of test is #4194, a rabbit polyclonal antibody generated in our lab (details in Dr. Xinwei Cao's Ph. D. thesis). ChIP experiments were carried out by Dr. Hao Wu in Dr. Yi (Eve) Sun's lab in UCLA. The micro array assay was submitted to Roche NimbleGen Inc. Dr. Hao Wu analysis the raw data and send us the result from the first round of study:

(1) At the first glance, it is already obvious that Fe65 enriched a different pool of promoters in comparison of DNA sequences enriched by MeCP2 (methyl CpG binding protein 2 (Rett syndrome), used here as control).

"Representative raw enrichment of one Fe65 target in wild-type mouse cortex identified by NimbleGen customized promoter tiling array (covers -3250 to +750 bp of ~20,000 protein coding transcript and ~400 microRNA promoters). ChIP-chip in cerebellum using polyclonal antibodies against MeCP2 is shown here as controls." - HaoWu



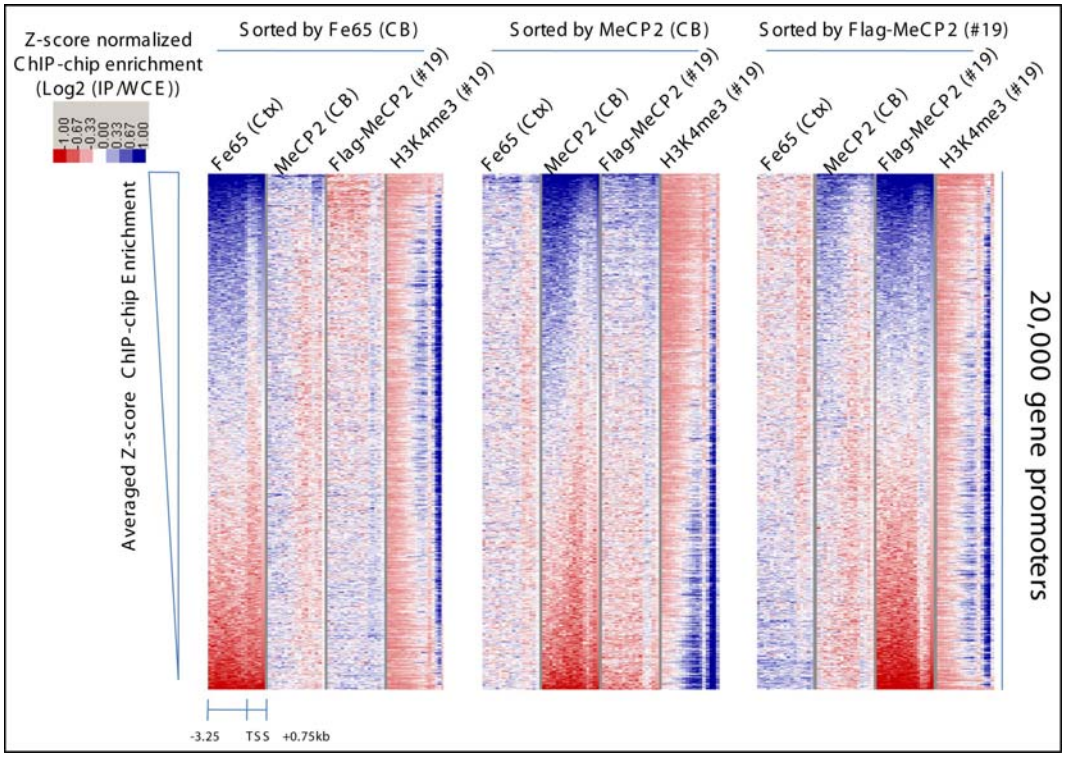
(2) Sorting DNA sequences from high to low affinities to Fe65-IP showed that the pattern is quite different from results obtained by MeCP2-IP from mice brain, FLAG-IP of Flag-tagged MeCP2 from MeCP2 KO mouse ESC-derived neurons, or H3K27me3-IP. While results from MeCP2- IP and FLAG-IP of Flag-tagged MeCP2 correlate very well with each other, and MeCP2-IP has some correlation

with H3K27me3-IP. These further prove that the chip results are specific for Fe65-binding regions, which are quite different from other specific DNA binding proteins. H3K27me3 is involved in homeotic gene silencing and marks the inactive X chromosome (Kouzarides, 2007).

Log_2(IP/WCE) results come from the fact that ChIP-chip is typically done using two-channel micro array, with the IP channel measures the relative concentration of the DNA fragments binds to the protein of interest, while the whole cell extract channel (WCE) measure the relative concentration of the DNA sequence in the total chromatin preparation, or IP by an non-specific control antibody. The higher the normalized Log_2(IP/WCE) score (Z-score), the more reliable that the identified sequence might be the genuine binding site for the protein of interest.

Below is the sorting comparison provided by Dr. Hao Wu:

"Global comparison between Fe65, MeCP2 and H3K27me3 ChIP-chip in mouse brain tissues (Ctx and CB) and/or mouse ESC-derived neuorns. Fe65 occupancy is not specifically correlated with MeCP2 occupancy, but endogenous MeCP2 occupancy is correlated with Flag-tagged MeCP2 occupancy in MeCP2 KO mouse ESC-derived neurons (#19)." - Hao Wu



(3) 1st round of ChIP-chip assay of Fe65 using pAb 4194 were summarized by Dr. Hao Wu. It is rather interesting that we see a large number of sequences corresponding to genes of olfactory receptors. We don't know whether this has any relation with the fact that olfactory dysfunctions are observed a number of neuropsychiatric conditions, including AD and Parkinson's disease [recent review, (Berg, 2008; Doty, 2008)]. In general, we see Fe65 binds to a wide spectrum of sequences that related to synapse functions, cell adhesion, apoptosis, and other factors that regulate transcription and translation. Dr. Hao Wu's summaries are listed below:

"To further elucidate the potential biological functions of Fe65 occupied targets, I examined the gene ontology terms overrepresented in the ~1,000 Fe65 occupied protein coding transcripts. Here is an incomplete list of top candidate GO terms:

GO terms	Target #	P-value
G-protein coupled receptor activity	264	2.4E-72
Olfactory receptor activity	209	7.4E-69
Translation factor activity	12	2.6E-2
Synaptic transmission	15	4.9E-2
Cell adhesion	26	7.1E-1
Anti-apoptosis	10	1.5E-2

GOTERM_BP_ALL:synapse transmission	
GENBANK_ACCESSION	**Gene Name**
NM_009670, NM_170687	**ankyrin 3, epithelial**
NM_008073, NM_177408	**gamma-aminobutyric acid (gaba-a) receptor, subunit gamma 2**
NM_001038701,	**gamma-aminobutyric acid (gaba-a) receptor,**

NM_008071	**subunit beta 3**
NM_007471	**amyloid beta (a4) precursor protein**
NM_021789	**trafficking protein particle complex 4**
NM_009498	**vesicle-associated membrane protein 3**
NM_008171	**glutamate receptor, ionotropic, nmda2b (epsilon 2)**
NM_020492	**glycine receptor, alpha 1 subunit**
NM_008069	**gamma-aminobutyric acid (gaba-a) receptor, subunit beta 1**
NM_008066	**gamma-aminobutyric acid (gaba-a) receptor, subunit alpha 2**
NM_009306	**synaptotagmin i**
NM_177639	**expressed sequence bb075781**
NM_028736, NM_130891	**glutamate receptor interacting protein 1**
NM_020253	**neurexin ii**
NM_010250	**gamma-aminobutyric acid (gaba-a) receptor, subunit alpha 1**
NM_020274	**5-hydroxytryptamine (serotonin) receptor 3b**
NM_011807	**discs, large homolog 2 (drosophila)**

GOTERM_MF_ALL:translation factor activity, nucleic acid binding	
GENBANK_ACCESSION	**Gene Name**
NM_017368, NM_198683	cug triplet repeat, rna binding protein 1
NM_019702	hbs1-like (s. cerevisiae)
NM_175374	mitochondrial translational release factor 1-like
NM_001013824	similar to eukaryotic translation initiation factor 1a (eif-1a) (eif-4c)
NM_144958	eukaryotic translation initiation factor 4a, pseudogene 4
NM_033618	suppressor of ty 16 homolog (s. cerevisiae)
NM_145625	eukaryotic translation initiation factor 4b
NM_198303	cdna sequence bc018347
NM_153402	eukaryotic translation initiation factor 2c, 3
NM_018796	eukaryotic translation elongation factor 1 beta 2
NM_201256	eukaryotic translation initiation factor 4e binding protein 3
NM_001039519,	general transcription factor ii a, 2

NM_199151	

GOTERM_BP_ALL:cell adhesion	
GENBANK_ACCESSION	Gene Name
NM_009347	tectorin alpha
NM_001024708	similar to cadherin-11 precursor (osteoblast-cadherin) (ob-cadherin) (osf-4)
NM_023115	protocadherin 15
NM_008000	fer (fms/fps related) protein kinase, testis specific 2
NM_145452	ras p21 protein activator 1
NM_014194, NM_053150	killer cell lectin-like receptor, subfamily a, member 7
NM_011169	prolactin receptor
NM_007471	amyloid beta (a4) precursor protein
NM_001034962, NM_001034963	sorbin and sh3 domain containing 1
NM_177863	fras1 related extracellular matrix protein 1
NM_172853	cadherin 7, type 2

NM_007766	**protocadherin alpha 4**
NM_145519	**ferm, rhogef and pleckstrin domain protein 2**
NM_172862	**fras1 related extracellular matrix protein 2**
NM_010954	**neural cell adhesion molecule 2**
NM_007643	**cd36 antigen**
NM_013504	**desmocollin 1**
NM_016743	**nel-like 2 homolog (chicken)**
NM_053135	**protocadherin beta 10**
NM_013505	**desmocollin 2**
NM_172823	**leishmanolysin-like (metallopeptidase m8 family)**
NM_010233	**fibronectin 1**
NM_001013389	**glycosylphosphatidylinositol specific phospholipase d1**
NM_001008420	**cadherin 12**
NM_001039148, NM_001039149, NM_178687	**cd226 antigen**
NM_011800	**cadherin 20**

GOTERM_BP_ALL:anti-apoptosis	
GENBANK_ACCESSION	**Gene Name**
NM_007642	**cd28 antigen**
NM_010275	**glial cell line derived neurotrophic factor**
NM_007540	**brain derived neurotrophic factor**
NM_145452	**ras p21 protein activator 1**
NM_008943	**presenilin 1**
NM_145736	**proviral integration site 2**
NM_011169	**prolactin receptor**
NM_172858	**p21 (cdkn1a)-activated kinase 7**
NM_139299	**interleukin 31 receptor a**
NM_013479	**bcl2-like 10**

- Hao Wu

(4) Our collaboration is still on-going and the second round of experiments were done on APP transgenic mice with the wild type litter mates, in order to (1) verify

the result from the 1st round; and (2) see whether the over production of AICD will in any way influence the pool of genes that bind to Fe65.

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