Role of the 39-kDa receptor-associated protein (RAP) in Alzheimer's disease

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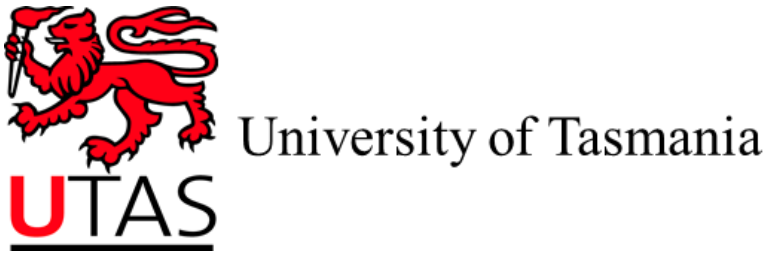

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Summary

Alzheimer's disease (AD) is the most common form of dementia that gradually worsens over time and leads to death. AD is characterized by an accumulation of β-amyloid protein (Aβ) in the brain. Recently, the 39-kDa receptor-associated protein (RAP) has been implicated in the AD pathology. RAP is found mainly in endoplasmic reticulum (ER) and functions as a chaperone for the maturation and trafficking of the low density lipoprotein (LDL) receptor family. Polymorphisms in the RAP gene have been associated with an increased risk of AD. Two studies have shown that down-regulation of RAP expression exacerbates Aβ pathology in transgenic mouse models of AD, suggesting that RAP may have an important role in Aβ production and clearance. It has been also shown that RAP binds strongly to Aβ, leading to an inhibition of Aβ aggregation and neurotoxicity. Furthermore, a recent study has shown that the level of RAP expression is significantly decreased in the AD brain compared with healthy controls.

This study aimed to identify the region of RAP which binds to Aβ and the effect of RAP over-expression and treatment on APP metabolism and Aβ production. The possibility that Aβ can bind to RAP in the human CSF was also examined. To determine the Aβ-binding region on RAP, an *in-vitro* assay was established and validated to study Aβ-self association, based on the binding of biotin labelled Aβ42 to synthetic Aβ42 seeded in the wells of microplates. The Aβ-binding region on RAP was identified by measuring Aβ-self association in the presence of different RAP fragments. The results indicated that the Aβ-binding site was located between amino acid residues 206 and 216 of the RAP sequence in loop region between domains D2 and D3. To examine the effect of RAP on APP metabolism, two strategies were used. The first strategy involved over-expression of RAP in sweAPP-CHO

and sweAPP-SH-SY5Y cells, after which the effect of RAP on APP processing and Aβ production was analysed. As a second strategy, the effect of RAP on APP metabolism was examined in primary cortical neurons derived from Tg2576 transgenic mice. The results showed that over-expression or treatment of RAP had no significant effect on the level of total APP. However, the levels of sAPPβ, C99 and Aβ were decreased and the production of C83 was significantly increased in RAP-transfected cells. The results suggest that RAP could decrease Aβ production by decreasing β-cleavage. Therefore, a decreased level of RAP in the brain could contribute to the pathogenesis of AD. Attempts to determine if Aβ can bind with RAP in the human CSF were not successful; the preliminary results, however, suggested that Clusterin/CLU is the main Aβ-binding protein in the human CSF. Taken together, these data suggest that RAP is a protective factor against Alzheimer's disease.

Declaration

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of the my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

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Statement of Authority of Access

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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List of abbreviations

ABCA7	ATP-binding cassette sub-family member 7
ACT	Anti-chymotrypsin enzyme
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
Aph	Anterior pharynx complex
APLP	Amyloid precursor-like protein
ApoE	Apolipoprotein E
ApoER2	Apolipoprotein E receptor 2
ApoJ/CLU	Apolipoprotein J/Clusterin
APP	Amyloid precursor protein
Aβ	Amyloid abeta
Aβ40	Amyloid beta containing forty amino acids
Aβ42	Amyloid beta containing forty-two amino acids
BACE	β-site APP cleaving enzyme
BBB	Blood brain barrier
BIN1	Bridging integrator 1
BSA	Bovine serum albumin
C	C-terminus
C31	31-amino acid APP C-terminal fragment
C83	83-amino acid APP C-terminal fragment

C99	99-amino acid APP C-terminal fragment
CAA	Cerebral amyloid angiopathy
CALHM1	Calcium homeostasis modulator 1
CHO	Chinese hamster ovary cell
CNS	Central nervous system
CSF	Cerebrospinal fluid
D	Domain
DIV	Days *in vitro*
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
FAD	Familial Alzheimer's disease
FCS	Fetal calf serum
GWAS	Genome-wide association study
HRP	Horse radish peroxidase
HSPG	Heparan sulphate proteoglycan
IDE	Insulin-degrading enzyme
Ig	Immunoglobulin
IP3	Inositol-1.4.5- triphosphate
kDa	Kilodalton
KPI	Kunitz-type protease inhibitor domain
LRP1	Low density lipoprotein receptor-related protein-1
LRP2	Low density lipoprotein receptor-related protein-2 or megalin
LTP	Long term potentiation
Nct	Nicastrin
NEP	Neprilysin

NFT	Neurofibrillary tangles
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
PBS	Phosphate buffered saline
PCN	Primary cortical neuron
PDAPP	Platelet-derived growth factor APP
Pen-2	Presenilin enhancer-2
PICALM	Phosphatidylinositol binding clathrin assembly protein
PS	Presenilin
RAP	Receptor-associated protein
sAPPα	Secreted fragment of APP produced by α-secretase cleavage
sAPPβ	Secreted fragment of APP produced by β- secretase cleavage
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA	Sarco-endoplasmic reticulum adenosine triphosphatase
siRNA	Small interfering Ribonucleic acid
SorLA	Sortilin-related receptor with A-type repeat
SweAPP	Swedish familial APP mutation
TBS	Tris buffered saline
TEMED	N,N,N’,N’-Tetramethylenediamine
TFA	Trifluoroacetic acid
TFE	Trifluoroethanol
TMB	3,3’,5,5’-tetramethylbenzidine
VLDLR	Very low density lipoprotein receptor
A2M	Alpha-2 macroglogulin

Chapter 1: Introduction and literature review

1.1 Protein misfolding and neurodegenerative diseases

Neurodegenerative diseases are a group of disorders involving degenerative changes in the peripheral or central nervous system. In the central nervous system, neurodegenerative diseases may affect abstract thinking, movement, cognition and memory (Soto, 2003). Although neurodegenerative diseases differ in the rate of progression and clinical symptoms, they share some common pathological features, such as the presence of abnormal protein deposits, neuronal loss and synaptic loss. Accumulating evidence shows that protein misfolding and aggregation is the main cause of many neurodegenerative diseases, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), transmissible spongiform encephalophathies (TSE) and amyotrophic lateral sclerosis (ALS). Several common neurodegenerative diseases with protein deposits are listed in Table 1.1.

1.2 The neuropathology of Alzheimer's disease

Alzheimer's disease is the most common and best characterized form of dementia, accounting for 50-80% of all dementia cases (Alzheimer's Association, 2011). AD was first described in 1906 by Alois Alzheimer (Alzheimer, 1907). The disease is more common after the age of 60, however early-onset AD can also occur. The symptoms of AD, such as memory loss, become gradually worse as the disease progresses. Later symptoms include impaired judgment, behaviour changes, confusion and difficulty in speaking and walking. Damage occurs early in

Table 1.1. Neurodegenerative diseases with abnormal accumulation of proteinaceous deposits.

Neurodegenerative disease	Proteinaceous deposits	Main component of deposits	Location of deposits	References
Alzheimer's disease	Senile plaques Neurofibrillary tangles	β-amyloid Tau protein	Extracellular Intracellular	(Selkoe, 2001)
Parkinson's disease	Lewy bodies	α-synuclein	Intracellular	(Gitler et al., 2008)
Familial British Dementia	Amyloid plaques NFT	BRI2	Extracellular Intracellular	(Choi et al., 2004)
Familial amyloid polyneuropathy	Amyloid plaques	Transthyretin	Extracellular	(Sousa et al., 2001)
Prion diseases	Amyloid plaques	Prion protein	Extracellular	(Aguzzi and Calella, 2009)
Huntington's disease	Neuronal inclusions	Huntingtin	Intracellular	(Rong et al., 2006)
Dementia with lewy bodies	Lewy bodies	α-synuclein	Intracellular	(Luk et al., 2009)
Multiple system atrophy	Glial cell inclusions	α-synuclein	Intracellular	(Campbell et al., 2001)
Amyotrophic lateral sclerosis	Inclusion bodies	SOD1, TDP-43, FUS	Intracellular	(Rothstein, 2009)

BRI2 or ITM2B: integral membrane protein 2B; NFT: neurofibrillary tangle; SOD1: superoxide dismutase 1; FUS: RNA-binding protein FUS (fused in sarcoma); TDB 43: TAR-DNA binding protein 43.

regions of the brain such as the hippocampus, the forebrain and the entorhinal cortex, all of which are critical for memory formation. As the disease progresses, patients become bedridden lose awareness and normally die within 7-10 years of diagnosis (Alzheimer's Association, 2011). In the United States alone, there are more than 5 million people currently suffering from AD in 2011 and the number is expected to increase up to 11-16 million by 2050, producing a significant burden on society (Alzheimer's Association, 2011). Therefore, extensive research effort has been focussed on elucidating the underlying mechanisms of AD pathogenesis with the aim of developing therapeutic treatments for the disease.

AD is characterized by several pathological hallmarks, including the accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles (NFTs) (Figure 1.1). Amyloid plaques are primarily composed of different forms of Aβ. There are two forms of amyloid plaques in AD brain, senile plaques and diffuse plaques. Senile plaques or neuritic plaques are characterized by a dense core of proteinaceous material, surrounded by dystrophic neurites and glia (Selkoe, 2001). On the other hand, diffuse plaques have an amorphous structure and can be found in the same regions of the brain associated with neuritic plaques (Iwatsubo et al., 1994). Studies suggest that diffuse plaques may be an early stage in the formation of neuritic plaques (Iwatsubo et al., 1995, Lemere et al., 1996). NFTs contain mainly hyperphosphorylated filaments of the microtubule-associated protein tau (Ihara et al., 1986, Gendron and Petrucelli, 2009). Additionally, NFTs have also been found in the brains of patients with other neurodegenerative diseases, including Parkinson's disease (Burack et al., 2010) and frontotemporal lobar degeneration (FTLD) (Dickson et al., 2011). It is generally believed that Aβ is the cause of AD.

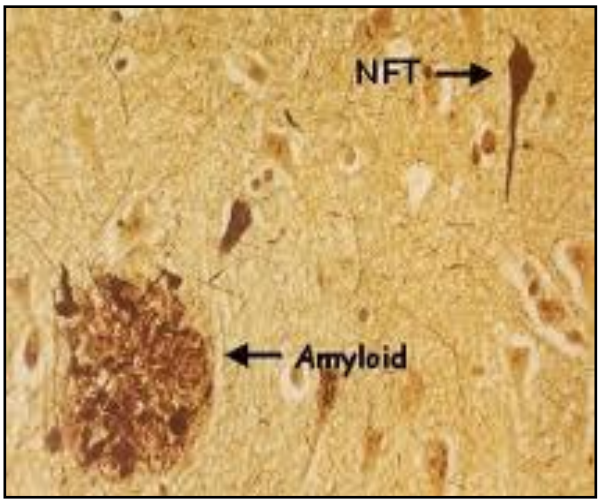

Figure 1.1. Neuropathology in a cortical slice taken from an AD patient. Alzheimer's disease is characterized by several pathological hallmarks, including the extracellular aggregation of Aβ into amyloid plaques, intracellular neurofibrillary tangles (NFTs), consisting of twisted hyperphosphorylated tau protein (image taken from http://med.kuleuven.be/legtegg/AD.html).

1.3 Proteolytic processing of amyloid precursor protein (APP) and Aβ production

Studies have shown that Aβ is the major constituent of amyloid plaques (Glenner and Wong, 1984, Masters et al., 1985, Selkoe et al., 1986). Subsequently, Aβ was found to derive from a large transmembrane protein called the β-amyloid precursor protein (APP) located on chromosome 21 (Kang et al., 1987). Alternative splicing of the human APP gene generates different APP mRNA that encodes several isoforms. The three major isoforms of APP contain 695, 751 and 770 amino acid residues (referred as APP695, APP751 and APP770, respectively). Both APP751 and 770 contain a Kunitz-type protease inhibitor (KPI) domain. In addition, APP770 has an extra Ox2 homologous domain in the extracellular region adjacent to the KPI domain. Unlike APP751 and 770, which are expressed in most cell types, the KPI-lacking APP695 isoform is expressed predominantly in neurons and is the major form of APP in brain (Rohan de Silva et al., 1997). There are some data showing that the expression of KPI-containing isoforms is increased in AD and that increased expression is associated with increased Aβ production (Siman et al., 1989, Zhan et al., 1995, Van Den Heuvel et al., 2000, Menendez-Gonzalez et al., 2005). Furthermore, it has been reported that there is a shift of expression from APP695 to KPI-containing APP isoforms in AD, suggesting that altered transcription of APP isoforms may contribute to AD pathogenesis (Matsui et al., 2007, Bordji et al., 2010). APP is synthesized in the endoplasmic reticulum (ER) and is transported via the Golgi apparatus, where it undergoes post-translational modifications. From the trans-Golgi network (TGN), APP can be delivered to the plasma membrane or to endosomes/lysosomes for degradation (Caporaso et al., 1994, Zhang et al., 2011). Several APP proteolytic processing pathways are known, including α-secretase, β-secretase and caspase cleavage pathways.

1.3.1 α-Secretase pathway

A major processing pathway of APP is the α-secretase pathway. Cleavage of APP by α-secretase within the Aβ sequence at a position between lys16 and leu17 releases a large extracellular domain of APP called sAPPα and an 83-amino-acid residue intracellular C-terminal fragment named C83. Further cleavage of C83 by γ-secretase produces the p3 fragment and the APP intracellular domain (AICD) (Fig.1.2) (Esch et al., 1990). Theoretically, this cleavage pathway precludes the formation of Aβ. Studies have suggested that α-secretase is a membrane-associated enzyme and cleaves APP mainly on the cell membrane (Sisodia, 1992). Three members of the disintegrin and metalloproteinase (ADAM) family: ADAM9, ADAM10 and ADAM17 (also known as tumor necrosis factor converting enzyme, TACE) have been found to possess α-secretase activity and may contribute to APP processing (Roberts et al., 1994, Asai et al., 2003).

Accumulating evidence suggests that ADAM10 is the main α-secretase. Over-expression of ADAM10 resulted in increased α-cleavage while inhibition of ADAM10 prevented α-cleavage of APP in various cell cultures (Lammich et al., 1999, Kuhn et al., 2010). An *in vitro* study has shown that over-expression of ADAM10 in APP transgenic mice led to increased sAPPα generation, decreased Aβ production and improved cognitive performance (Postina et al., 2004). In addition, conditional knockout of ADAM10 in neurons dramatically decreases sAPPα production (Jorissen et al., 2010). Moreover, the level of ADAM10 is reduced in platelets, correlating with a decreased level of sAPPα in the CSF of AD patients (Colciaghi et al., 2002). Taken together, these studies support the view that ADAM10 is the main α-

secretase and that decreased ADAM10 levels can reduce non-amyloidogenic processing of APP in AD.

The first evidence showing that ADAM9 or MDC9 has α-secretase activity came from studies where co-expression of ADAM9 with APP in COS cells was found to increase sAPPα production upon activation by phorbol ester (Koike et al., 1999, Hotoda et al., 2002). However, mice lacking ADAM9 showed no differences in the production of sAPPα and p3 compared with WT mice, arguing against a role for ADAM9 as an α-secretase (Weskamp et al., 2002). Knockdown of ADAM9 by siRNA in various cell cultures did not suppress the α-secretase cleavage of APP, further suggesting that ADAM9 is not important in APP processing (Kuhn et al., 2010).

ADAM17 or TACE has also been shown to possess α-secretase activity. Disruption of ADAM17 inhibited α- cleavage of APP in cell culture, indicating that ADAM17 may be an α-secretase and regulate APP cleavage (Buxbaum et al., 1998). Inhibition of ADAM17 prevented the production of sAPPα in neuron cultures (Blacker et al., 2002) and in the brain of Tg2576 mice without changing the level of Aβ (Kim et al., 2008). However, the level of ADAM17 is unchanged in AD patients (Skovronsky et al., 2001) , raising questions about the significance of ADAM17 for the pathogenesis of AD.

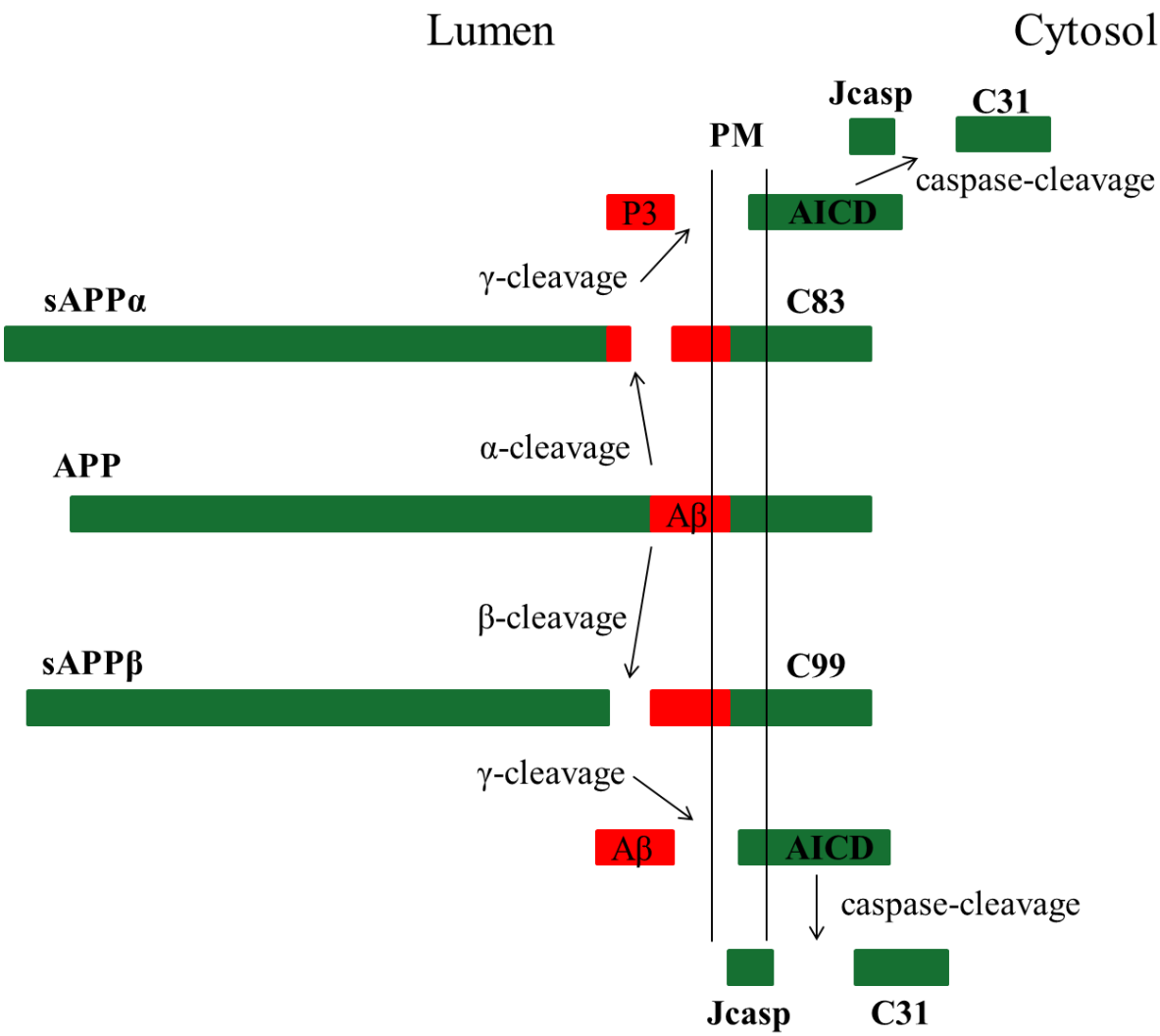

Figure 1.2. Diagrammatic representation of APP processing pathways. APP is a type I transmembrane protein, which contains the Aβ sequence, extending from the extracellular region to the transmembrane region of APP. There are three well characterised processing pathways of APP. The majority of APP is processed through α-cleavage pathway, which involves cleavage of APP within the Aβ sequence by α-secretase to release the extracellular domain of APP called sAPPα and the intracellular C-terminal fragment (C83). Further cleavage of C83 by the γ-secretase complex liberates the p3 fragment and the APP intracellular domain (AICD). Theoretically, this pathway precludes Aβ formation. In the second pathway, APP is first cleaved by β secretase to produce the sAPPβ fragment and the C-terminal fragment C99. Further cleavage of C99 by the γ-secretase complex generates Aβ and the AICD fragment. In addition, APP can be also cleaved by caspases at the cytoplasmic tail to produce the C31 and the Jcasp fragment.

1.3.2 β-Secretase pathway

Alternatively, APP can also be processed by sequential cleavage by β- and γ-secretases to produce Aβ. In this pathway, APP is first cleaved by β-secretase (BACE) to generate a soluble fragment (sAPPβ) and a 99-amino-acid residue intracellular fragment (C99). C99 can be further cleaved by the γ-secretase complex to produce Aβ and the APP intracellular domain (AICD) (Fig. 1.2) (Seubert et al., 1993). Depending on the specific cleavage sites by γ-secretase, different forms of Aβ, varying from 37- to 49-amino acid residues, are produced (Zhao et al., 2007). However, the two major species that are found in amyloid plaques are Aβ40 and Aβ42 (Selkoe and Wolfe, 2007). Studies show that Aβ42 can aggregate more readily and that it may be more neurotoxic than Aβ40, due to the presence of extra 2 amino acid residues in the C-terminus of Aβ42 (Zhang et al., 2002, Yan and Wang, 2006). Furthermore, small amounts of Aβ42 can seed the aggregation of Aβ40 (Jarrett et al., 1993). Although Aβ is central to the pathogenesis of AD, it is the product of normal APP processing (Shoji et al., 1992, Moghekar et al., 2011).

β-Secretase is a type I transmembrane aspartyl protease known as β-site APP cleaving enzyme (BACE1) (Cole and Vassar, 2008). The gene encoding human BACE1 is located on chromosome 11 (Saunders, 1999). There is some convincing evidence that BACE1 is the β-secretase and that BACE is the rate-limiting enzyme in the generation of Aβ from APP (Hunt and Turner, 2009). In addition to the generation of N-terminus of Aβ, cleavage within the Aβ sequence between Tyr10 and Glu11 by BACE1 can produce N-terminally truncated Aβ (Liu et al., 2002). Studies have shown that BACE requires an acidic environment for optimal activity, and as such, its activity is mainly found in the endosomes and lysosomes, although a

small amount of BACE1 can also be found on the cell surface (Huse et al., 2000, Stockley and O'Neill, 2008). Due to its important role in Aβ generation from APP, BACE1 has been explored as a therapeutic target for AD treatment. Other enzymes, such as β-site APP cleaving enzyme-2 (BACE2) and cathepsin B, have been also suggested as β-secretase, however they may only play minor roles in β-cleavage of APP, if any (Yan et al., 2001, Hook et al., 2009).

Studies have shown that the activity of γ-secretase complex requires four components: presenilin (PS), nicastrin (Nct), presenilin enhancer-2 (pen-2) and anterior pharynx complex (Aph), with deficiency in any of them leading to impaired γ-secretase activity (De Strooper, 2003, Edbauer et al., 2003, Kimberly et al., 2003, Luo et al., 2003, Shirotani et al., 2004). The stoichiometry of the γ-secretase complex is likely to be 1:1:1:1, based on estimated molecular mass (Sato et al., 2007, Osenkowski et al., 2009). In mammals, two members of the presenilin family, PS1 and PS2 have been identified as the active catalytic component of the γ-secretase complex (Capell et al., 1998, Sastre et al., 2001, Kim and Schekman, 2004). It has been shown that PS has two catalytically active asparate resisdues and mutations in these residues supress the activity of γ-secretase (Wolfe et al., 1999). At the cellular level, the γ-secretase complex can be found in the ER, Golgi network, endosomes and at the cell surface (Vetrivel et al., 2004, Chyung et al., 2005, Kaether et al., 2007). Apart from APP, more than 70 type-I membrane proteins have been shown to be cleaved by γ-secretase, including: Notch, ErB4, LRP1 and Delta (Ni et al., 2001, LaVoie and Selkoe, 2003, May et al., 2003, Lleo and Saura, 2011). Due to its essential role in producing Aβ, considerable effort has been put into the discovery and development of small-molecule inhibitors of γ-secretase as potential therapeutics for AD (Lleo and Saura, 2011, De Strooper et al., 2012). However, most of these

compounds interfere with Notch signalling, which results in immunosuppression and gastrointestinal toxicity (Wong et al., 2004). An alternative approach is to use small molecules which can modulate γ-secretase to alter Aβ production with varying degrees of selectivity with respect to Notch. For example, nonsteroidal antiinflammatory drugs (NSAIDs) can reduce the production of more neurotoxic Aβ42 and increase more soluble Aβ38 production (Weggen et al., 2001, Galasko et al., 2007). Other compounds such as kinase inhibitors are capable of reducing the cleavage of APP by γ-secretase while still allowing the enzyme to process Notch (Netzer et al., 2003, Fraering et al., 2005, He et al., 2010). Although, the efficacy and tolerance of some compounds in AD is poor in clinical trials, other selective compounds are currently at the various stages of development (De Strooper et al., 2012).

1.3.3 Caspase cleavage pathway

Mounting evidence suggests that APP is also a substrate for caspases and that caspase cleavage could be involved both in Aβ production and synaptic loss associated with AD pathology. Caspases belong to the cysteine protease family and cleave a subset of proteins with aspartate residues adjacent to cleavage site (Miller, 1997). It has been reported that apoptosis increases the level of Aβ production and the effect on Aβ can be attenuated by caspase inhibition (LeBlanc, 1995, Gervais et al., 1999). APP is cleaved by caspases (predominantly caspase-3) at Asp 664 (with respect to APP695 sequence) in the cytoplasmic domain, to generate a 31-amino acid fragment called C31. Further cleavage by γ-secretase produces a fragment named Jcasp (Fig. 1.2) (Gervais et al., 1999, Weidemann et al., 1999, Ayala-Grosso et al., 2002, Park et al., 2009). The level of caspase-3 is increased in dying neurons of AD brains and caspase-cleavage products of APP can be found in senile plaques

(Gervais et al., 1999, Uetsuki et al., 1999, Zhao et al., 2003). Moreover, caspase-3 and caspase-cleaved APP are co-localized in granules of AD brain (Su et al., 2002). However, it is unclear whether caspases cleave intact APP or APP-AICD fragments. Studies have shown that caspase cleavage products are toxic, although C31 has a more potent effect than Jcasp (Lu et al., 2000, Bertrand et al., 2001, Nishimura et al., 2002, Park et al., 2009). Blocking of caspase cleavage by mutation of Asp 664 completely prevents synaptic loss and behavioural deficits, in spite of unchanged levels of Aβ deposits in transgenic mice (Galvan et al., 2006). Similarly, inhibition of caspase-3 and caspase-9 activation by over-expression of Bcl-2 in triple transgenic mice prevents caspase cleavage of APP and reduced AD pathology (Rohn et al., 2008, Kumasaka et al., 2009), further indicating that caspase-cleaved products play an important role in the pathological and behavioural changes in AD transgenic mice. In support these findings, a recent study has shown that over-expression of APP induces caspase-6-dependent but Aβ-independent neuronal degeneration (Sivananthan et al., 2010). Overall, these studies suggest that activation of apoptotic pathways is likely to be an early event and that it may contribute to the underlying mechanism of AD.

It is unclear how caspase cleavage of APP is regulated. It was found that phosphorylation of APP at Thr668 supresses the cytoplasmic cleavage of APP by caspase-3 and caspase-8 (Taru et al., 2004). There are considerable data showing that activation of caspases increases Aβ production from APP , however the underlying mechanism is still poorly understood (Gervais et al., 1999, Stone et al., 2002, Takeda et al., 2004, Xie et al., 2007, Xiong et al., 2008, Zhang et al., 2008).

1.4 Genetics of Alzheimer's disease

There are two characterized forms of AD, early-onset familial AD (FAD) and late-onset AD. While FAD occurs before the age of 60, it accounts only for less than 5% of total AD cases. FAD can be caused by mutations in APP or presenilin genes, which lead to an increase in Aβ42 production and the overall level of Aβ. However, the majority AD cases are late onset AD and several risk factors have been implicated in late-onset AD.

1.4.1 Causative genes for early-onset or familial Alzheimer's disease

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1.4.1.1 APP mutations

More than 35 APP mutations associated with FAD have been identified (for an overview, see http://www.molgen.ua.ac.be/ADMutations/). Most of the APP mutations are centred around the regions of Aβ sequence and affect Aβ production (Wolfe and Guenette, 2007). Studies have shown that FAD mutations near the β-secretase cleavage site increase APP cleavage by β-secretase, and thus increase Aβ production (Reaume et al., 1996). FAD mutations within the Aβ sequence may increase Aβ aggregation, while FAD mutations near the γ-secretase cleavage site produce more of the neurotoxic Aβ42 than Aβ40 (Suzuki et al., 1994).

1.4.1.2 Presenilin mutations

Mutations in presenilin-1 (PS1) and presenilin-2 (PS2), which are located on chromosomes 14 (Sherrington et al., 1995) and 1 (Levy-Lahad et al., 1995), respectively, are responsible for

the remaining identified FAD cases. So far, 185 mutations in PS1 and 13 in PS2 have been reported (for an overview, see http://www.molgen.ua.ac.be/ADMutations/). While it is generally accepted that mutations in PS favour production of the more neurotoxic Aβ42 over Aβ40 (De Strooper et al., 1999), it is unclear how the mutations influence γ-secretase activity. It is hypothesized that mutations in PS1 and 2 result in partial loss of activity against APP (De Strooper, 2007). According to this hypothesis, cleavage of the C-terminus of Aβ region of APP by γ-secretase is a sequential process, starting from C-terminal upstream of Aβ sequence. Therefore, partial loss in PS activity may cause termination of APP at position 42 of Aβ, rather than position 40. This results in increased production of Aβ42 and decreased Aβ40 production (De Strooper, 2007).

1.4.2 Genetic risk factors for late onset Alzheimer's disease

1.4.2.1 Apolipoprotein ε4 allele

The most well characterized risk factor for late-onset AD is the ε4 allele of the apolipoprotein E gene, which is associated with 60-80% of all late-onset AD cases (Farrer et al., 1997). The ApoE gene is located on the chromosome 19 and encodes three isoforms: apoE2, apoE3 and apoE4, each of which contains 299 amino acid residues. The ε3 allele is the most common ApoE allele (70-80%), followed by the ε4 allele (10-15%) and the ε2 allele (5-10%)(Mahley, 1988). The sequences of the three ApoE isoforms differ at positions 112 and 158 with ApoE2 consisting of cys112 and cys158, ApoE3 consisting of cys112 and arg158 and ApoE4 consisting of arg112 and 158 arg. These differences significantly affect the structure and the biological function of the ApoE isoforms (Mahley et al., 2006). ApoE is highly expressed in

brain and liver and is the predominant apolipoprotein in the central nervous system (CNS). The primary function of ApoE is in trafficking and metabolism of lipids including cholesterol via interaction with members of LDL receptor family. These lipids can be used for synaptogenesis and synaptic maintenance (Pfrieger, 2003).

Numerous studies have demonstrated that ApoE4 is a strong risk factor for late-onset AD. ApoE4 may contribute to AD pathology through both an Aβ-dependent pathway by influencing Aβ production, Aβ aggregation and Aβ clearance: and an Aβ-independent pathway by inducing tau phosphorylation as well as causing cytoskeletal and mitochondrial disruption, leading to neuronal dysfunction and synaptic impairment (Kim et al., 2009, Huang, 2010). It has been shown that apoE4 has a dose-dependent effect on the age of onset and risk of sporadic AD. The risk for AD increases from 20% to 90% and the mean age of onset decreases from 84 to 68 years with an increasing number of ApoE4 alleles (Corder et al., 1993). Studies have shown that level of ApoE4 expression is increased in late-onset AD (Strittmatter et al., 1993a) and fibrillar Aβ burden is significantly associated with ApoE4 in a dose-dependent manner in cognitively normal patients (Reiman et al., 2009). ApoE immunoreactivity was found in the amyloid plaques, indicating that ApoE may bind to Aβ (Namba et al., 1991, Dickson et al., 1997, Thal et al., 2005). Subsequent data showed that ApoE can form complexes with Aβ (Naslund et al., 1995, Munson et al., 2000). It is generally believed that lipid-associated ApoE2 and ApoE3 form stable complexes with Aβ more efficiently than ApoE4 (Yang et al., 1997, Tokuda et al., 2000). The Aβ-binding region is located on the C-terminal of ApoE, which is also responsible for lipid binding (Tamamizu-Kato et al., 2008, Petrlova et al., 2011), while residues 12-28 of Aβ sequence appear to bind to ApoE and this region has been used for blocking the ApoE-Aβ interaction (Sadowski et al.,

2006). There are conflicting reports about the effect of ApoE on Aβ aggregation. Some studies have shown that all ApoE isoforms increase Aβ aggregation according to the order ApoE4>ApoE3>ApoE2, consistent with the increased Aβ deposition in AD patients with the ε4 allele (Castano et al., 1995, Bales et al., 1999, Holtzman et al., 2000, Cho et al., 2001, Manelli et al., 2004, Dolev and Michaelson, 2006). However, other studies reported that all ApoE isoforms can inhibit Aβ fibrilization by interfering with Aβ nucleation and that ApoE4 is the least efficient (Wood et al., 1996, Naiki et al., 1997, Beffert and Poirier, 1998) or by stabilizing Aβ oligomers with ApoE4 being most potent (Cerf et al., 2011).

There is increasing evidence suggesting that ApoE can influence APP processing and Aβ production. It has been shown that ApoE can promote APP processing and Aβ production in an isoform-specific manner that is dependent on ApoE receptors such as low density-lipoprotein receptor-related protein-1 (LRP1) and apolipoprotein E receptor-2 (ApoER2) or through a direct interaction with APP. Ye et al (2005) reported that ApoE4 promotes APP processing and Aβ production though a LRP1-dependent pathway more potently than ApoE3. Furthermore, abolishment of ApoE4 intra-molecular interaction by replacing arginine-61 with threonine or pre-treatment of ApoE4 with small molecules attenuated the difference in Aβ production, suggesting that ApoE4-induced Aβ production is mediated through both LRP1 and domain interaction (Ye et al., 2005). Similarly, another study has shown that ApoE promotes APP endocytosis and Aβ production though an ApoER2-mediated pathway with ApoE4 triggering Aβ production more than ApoE2 and ApoE3 (He et al., 2007). ApoE has also been found to promote Aβ production by directly interacting with APP (Hass et al., 1998, Hass et al., 2001) or through indirect binding to an ApoE-binding protein (TMCC2) (Hopkins et al., 2011) in an isoform-specific manner (ApoE4>ApoE3>ApoE2). However, other studies

have reported contradictory results where treatments with ApoE or ApoE mimetic peptides increase α-secretase processing and decrease Aβ production both *in vitro* and *in vivo* (Irizarry et al., 2004, Hoe et al., 2006a, Minami et al., 2010).

Two major pathways of Aβ clearance have been suggested. The first pathway involves receptor-mediated clearance by cellular uptake in the brain parenchyma, interstitial fluid (ISF) and blood-brain-barrier (BBB). The second pathway involves proteolytic cleavage by Aβ-degrading enzymes such as neprilysin (NEP) and insulin-degrading enzyme (IDE). Receptor-mediated Aβ clearance is likely to be promoted by ApoE. Due to a high affinity for Aβ, ApoE3 and ApoE2 are more efficient at clearing Aβ than ApoE4. This idea is supported by several transgenic mouse models showing that human ApoE2 and ApoE3-expressing mice develop fewer amyloid plaques than ApoE4 mice (Holtzman et al., 2000, Bales et al., 2009). Deane et al (2008) showed that ApoE disrupts Aβ clearance through the blood brain barrier (BBB) in an isoform-specific manner with ApoE4 having most disruptive effect. ApoE2-Aβ and ApoE3-Aβ complexes are reportedly cleared via the BBB through LRP1 and VLDLR at a faster rate than ApoE4-Aβ complexes (Deane et al., 2008). Furthermore, inhibition of NEP may result in increased Aβ42 oligomerization in ApoE4 mice to a greater extent than in ApoE3 mice and ApoE-deficient mice (Belinson et al., 2008, Belinson et al., 2010, Zepa et al., 2011). In terms of promoting Aβ cleavage, ApoE has recently been shown to facilitate proteolytic degradation of Aβ by NEP and IDE. The efficiency at promoting Aβ degradation depends on the isoform of ApoE and its lipidation status. Interestingly, ApoE4 shows an impaired ability to promote Aβ degradation compared with ApoE2 and ApoE3 (Jiang et al., 2008). The idea that ApoE can promote Aβ degradation by IDE has been challenged by

another study that showed ApoE4 can down-regulate the expression of IDE by activating the N-methyl-D-aspartic (NMDA) receptor in hippocampal neurons (Du et al., 2009).

A number of studies have also implicated the Aβ-independent pathway of ApoE in the pathogenesis of AD. ApoE4 is preferentially cleaved in AD brain and in neurons, resulting in the accumulation of ApoE (1-272) fragments (Brecht et al., 2004). Aβ treatment promotes the formation of ApoE4 (1-272) fragments and NFT-like inclusion in neuronal cells (Huang et al., 2001). These ApoE4 fragments have been shown to enhance tau phosphorylation and induce AD-like pathology *in vivo* (Harris et al., 2003, Bien-Ly et al., 2011). Moreover, ApoE4 (1-272) can also interact and disrupt mitochondrial functions, although the mechanism is still poorly understood (Chang et al., 2005, Nakamura et al., 2009). Several studies have also implicated ApoE4 in synaptic impairments (Levi and Michaelson, 2007, Dumanis et al., 2009, Chen et al., 2010) and neuroinflammation (Colton et al., 2004, Guo et al., 2004). Because ApoE may contribute to the development of AD through multiple mechanisms, it is clearly an important target for developing therapeutic strategies for AD.

1.4.2.2 Bridging integrator 1

Recent studies from genome-wide-association studies (GWASs) have recently ranked bridging integrator 1 gene (BIN1) as the second highest risk factor for AD (http://www.alzgene.org/TopResults.asp). Harold et al (2009) first reported that variants in BIN1 gene encoding for amphiphysin-2 as a late-onset AD susceptibility factor at $p \leq 1x10^{-5}$ (Harold et al., 2009). Subsequent data reported a genome-wide statistical significance of the BIN1 locus for AD and this finding was replicated in an independent population (Seshadri et

al., 2010). More recent studies have confirmed strong association of BIN1 gene with AD risk in different populations (Hollingworth et al., 2011, Hu et al., 2011, Lambert et al., 2011, Lee et al., 2011a). However, no association between BIN1 variants and CSF biomarkers (Aβ42 and ptau181 levels) has been detected (Kauwe et al., 2011).

The BIN1 gene is located on chromosome 2 and encodes several tissue-specific isoforms produced by alternate RNA splicing, including brain-specific isoforms (Negorev et al., 1996, Wechsler-Reya et al., 1997). The protein, originally known as a tumour suppressor, contains 3 domains: a Myc-interacting region, a SH3 and a BIN-amphiphysin-RSV domain, which can bind to lipid membranes (Sakamuro et al., 1996). BIN1 has been implicated in receptor-mediated endocytosis based on the fact that it can bind to dynamin (Wigge et al., 1997). Mutations in BIN1 have been shown to disrupt BIN1's interaction with dynamin-2 and to cause centronuclear myopathies (Nicot et al., 2007, Toussaint et al., 2011). Amphiphysin-1-knockout mice have a parallel significant reduction in BIN1 expression in the brain and these mice show defects in synaptic vesicle recycling associated with learning deficits (Di Paolo et al., 2002). Although studies from BIN1-knockout animal models showed that BIN1 deficiency does not inhibit endocytosis, abnormal localisation of proteins was found (Zelhof et al., 2001, Muller et al., 2003). It is noteworthy that treatment of Aβ leads to cell-surface accumulation of BIN1 and disrupts synaptic vesicle endocytosis in neuronal cell cultures (Kelly and Ferreira, 2007). In a recent study, it was shown that BIN1 is required for EHD1-mediated endocytic recycling (Pant et al., 2009). However, the functional role of BIN1 in AD is largely unexplored.

Table 1.2. Genes implicated as risk factors for late onset AD

Gene	Chromosome	Protein encoded	Function of the protein	References
APOE	19	Apolipoprotein E	Lipid and cholesterol transport	(Pfrieger, 2003)
BIN1	2	Bridging integrator	Endocytosis and intracellular trafficking	(Wigge et al., 1997)
CLU	8	Clusterin	Chaperone protein	(Calero et al., 2000)
ABCA7	19	ATP-binding cassette sub-family member 7	Transporter molecule	(Wang et al., 2003)
CR1	1	Complement component receptor 1	Processing and clearance complement complexes	(Molina et al., 1996)
PICALM	11	Phosphatidylinositol binding clathrin assembly protein	Clathrin-mediated endocytosis	(Rohde et al., 2002)
SORLA	11	Sortilin-related receptor with A-type repeats	Receptor-mediated endocytosis	(Nielsen et al., 2007)
LRP1	12	Low density lipoprotein receptor-related protein 1	Lipid metabolism and receptor-mediated endocytosis	(Lillis et al., 2008)

1.4.2.3 Clusterin

Clusterin (CLU) or apolipoprotein J (ApoJ) is a multifunctional glycoprotein that acts as a chaperone-like protein for the trafficking of a range of molecules. In the CNS, CLU is primarily expressed by glia and, together with ApoE, is one of the two most abundant apolipoproteins in the brain (Roheim et al., 1979, Liu et al., 1998, DeMattos et al., 2001). The CLU precursor protein contains 449-amino-acid residues and is cleaved into two subunits, α and β containing 222 and 205 amino acid residues, respectively. Secreted forms of CLU are 70-80 kDa highly glycosylated heterodimers which consist of two 40 kDa α and β subunits linked by disulfide bonds (Murphy et al., 1988, Kirszbaum et al., 1992). The gene encoding CLU is located on the chromosome 8 (Tobe et al., 1991). Various functions have been attributed to CLU, such as acting as an anti-apoptotic signal, protection against oxidative stress, transporting lipids and serving as a chaperone to prevent protein misfolding/aggregation (Calero et al., 2000).

Accumulating evidence has strongly implicated CLU in the pathogenesis of AD. Recent data from genome-wide association studies have shown that CLU is associated with late-onset AD (Harold et al., 2009, Lambert et al., 2009, Hu et al., 2011, Lee et al., 2011a, Naj et al., 2011, Schjeide et al., 2011). The level of CLU is significantly increased in plasma and CSF of AD patients, suggesting that CLU could be potentially used as a marker for AD (May et al., 1990, Sihlbom et al., 2008, Thambisetty et al., 2010, Schrijvers et al., 2011). A recent study has shown that healthy adults carrying the CLU gene variant associated with increased risk of AD have lower white matter integrity (Braskie et al., 2011). Other studies suggest that increased CLU expression in AD could be a neuroprotective response and does not precede the

development of the disease. Thus CLU may not be useful as an early marker for AD diagnosis. Furthermore, CLU is also associated with other dementia and cancers, suggesting that it may not be useful to differentiate AD from other diseases (Nuutinen et al., 2009, Schrijvers et al., 2011).

Studies have demonstrated that CLU can influence Aβ aggregation, Aβ clearance through the BBB, and Aβ cellular uptake. CLU is present in amyloid plaques with dystrophic neurites (May and Finch, 1992, Iwata et al., 2005, Martin-Rehrmann et al., 2005). Binding of CLU to Aβ has been suggested to influence Aβ aggregation and Aβ neurotoxicity, although conflicting results have been reported. It was initially shown that binding to CLU prevented Aβ fibrilization (Boggs et al., 1996, Matsubara et al., 1996, Hammad et al., 1997). Other studies later showed that CLU knockout mice had significantly fewer amyloid plaques and less neuritic dystrophy than mice expressing CLU, indicating that CLU increases plaque formation and neuritic toxicity in the brain (DeMattos et al., 2002, Martin-Rehrmann et al., 2005). However, another study demonstrated that CLU had a biphasic effect on Aβ aggregation, depending upon the Aβ:CLU ratio. When Aβ is present in large molar excess, binding of CLU to Aβ increases Aβ fibrilization. In contrast, when the level of CLU is higher, CLU inhibits Aβ aggregation (Yerbury et al., 2007) . Moreover, like ApoE, CLU has been proposed to increase Aβ clearance across the blood brain barrier via an LRP1 and megalin-mediated mechanism (Zlokovic et al., 1996, Bell et al., 2007). However, it remains unclear what the net effect of CLU is on Aβ deposition. A study showed that although ApoE or CLU single knockout PDAPP mice have much less fibrillar Aβ than PDAPP mice, ApoE and CLU double knockout PDAPP mice have greater levels of Aβ deposition than all other genotypes, suggesting the additive effects of ApoE and CLU on Aβ deposition. Furthermore,

the level of Aβ is elevated in the CSF of double knockout PDAPP mice, implicating both ApoE and CLU in Aβ clearance (DeMattos et al., 2004). Further studies are needed to examine the role of human CLU on Aβ deposition and neurotoxicity *in vivo*.

1.4.2.4 Sortilin-related receptor with A-type repeats (SORLA)

SORLA is a 250 kDa protein that belongs to the VSP10P-domain receptor family (Wasco et al., 1992). It is highly expressed in neurons in the cortex and hippocampus. Several lines of evidence have implicated SORLA as a risk factor for AD. The involvement of SORLA in AD was initially demonstrated by the finding that the level of SORLA expression is reduced in AD brain (Scherzer et al., 2004, Dodson et al., 2006, Lee et al., 2007b). Variants in two different clusters of intronic sequences that may regulate SORLA expression have been reported to be associated with sporadic AD (Rogaeva et al., 2007). Subsequent data have shown that SorLA influences APP trafficking by sequestering APP in the TGN, away from endosomes, hence decreasing Aβ production. SORLA can interact with APP and BACE1 through their intracellular domains (Spoelgen et al., 2006, Rogaeva et al., 2007). Over-expression of SORLA reportedly re-distributes APP to the Golgi apparatus and decreases Aβ production, while SORLA depletion switches APP from the recycling pathway to the endocytic pathway, which leads to a decrease in Aβ production (Andersen et al., 2006, Offe et al., 2006). SORLA is regulated by an interaction with the adaptor proteins, GGA and PACS-1 which are involved in protein transport from and to TGN (Nielsen et al., 2007). *In vivo* studies have shown that loss of SORLA increases APP processing and Aβ production in transgenic mouse brain (Dodson et al., 2008, Rohe et al., 2008). Recently, it has been shown that BDNF acts as a regulator of SORLA expression and reduces the amyloidogenic

processing of APP (Rohe et al., 2009). Furthermore, phosphorylation of SORLA has been suggested to enhance SORLA-mediated effects on APP processing and Aβ production (Herskowitz et al., 2011). Taken together, current data suggest that SORLA may act as a negative regulator of APP processing and could be an alternative target for AD treatments.

1.5 Aβ protein

1.5.1. Aβ neurotoxicity

Yankner et al (1989) first demonstrated that Aβ is neurotoxic in cell cultures. Subsequently, this result was confirmed in a numbers of other studies (Frautschy et al., 1991, Kowall et al., 1991, Pike et al., 1993, Harris et al., 1995). It was initially believed that Aβ plaque numbers were correlated with cognitive decline in aging brains (Roth et al., 1966). However, subsequent studies showed that Aβ-positive plaques could be formed in the aging human brain in absence of cognitive deficits and it was suggested that synaptic loss is a more relevant indicator of cognitive decline (Price et al., 1991, Terry et al., 1991). There is increasing evidence suggesting that toxic Aβ oligomers are primarily responsible for cognitive decline in AD. Aβ assemblies ranging from dimers to 50-mers are known as oligomers (Haass and Selkoe, 2007). Lambert et al (1998) showed that small diffuse Aβ oligomers (ADDLs) could potently cause neuronal cell death and inhibit long-term potentiation (LTP) in hippocampal slices (Lambert et al., 1998). Injection of Aβ oligomers isolated from cell culture medium into rat brains resulted in marked inhibition of LTP (Walsh et al., 2002). However, it was unclear if Aβ oligomers could aggregate into higher structures after injection. A study by Lesne et al (2006) has shown that memory deficits in the Tg2576

transgenic mice model of AD is caused by the extracellular accumulation of 56kDa Aβ oligomers termed Aβ*56. Purified Aβ*56 from mouse brain was found to disrupt memory in adult rats (Lesne et al., 2006). The study suggested that Aβ*56 impairs memory and contributes to cognitive decline in AD, independent of plaque formation and neuronal loss. Furthermore, in APP transgenic mice with the APP Arctic mutation (E693G) which show a marked increase in neuritic plaque formation, but a decrease in Aβ*56, memory impairment was more correlated to the level of Aβ*56 than to fibrillar Aβ deposits, suggesting that reducing fibrillar Aβ plaques at the cost of increasing Aβ oligomers could have detrimental effects (Cheng et al., 2007). Numerous studies have characterized Aβ oligomers and demonstrated these oligomeric species are the most toxic forms (Lauren et al., 2009, Decker et al., 2010, Barry et al., 2011, Hashimoto et al., 2011, Li et al., 2011, Rammes et al., 2011). Moreover, it has been shown that Aβ oligomers are increased in cortex and CSF of AD patients (Kuo et al., 1996, Gong et al., 2003, Georganopoulou et al., 2005, Shankar et al., 2008, Sokolow et al., 2011). However, the structure and mechanism of formation of Aβ oligomers are still unclear. It is also largely unknown which forms of Aβ oligomers are the most neurotoxic.

The neurotoxic effects of Aβ have been thoroughly covered in a number of reviews. The neurotoxic effects include synaptic dysfunction, production of free radicals, tau hyperphosphorylation, disruption of neuronal mitochondria, induction of microglial inflammatory response, activation of program cell death (Behl, 1997, Small et al., 2001, Klein, 2002, Smith et al., 2006, Chiang et al., 2008, Colell et al., 2009, Huang and Jiang, 2009, Price et al., 2009, Thathiah and De Strooper, 2009, Parihar and Brewer, 2010, Malchiodi-Albedi et al., 2011, Tougu et al., 2011). However, there is not yet a consensus on

how Aβ exerts its neurotoxic effects. Due to limited space, this section will only focus on an emerging hypothesis: that Aβ-induced calcium dysregulation is an underlying mechanism for neuronal dysfunction and neurodegeneration for AD.

Neuronal calcium signalling is fundamental for learning and memory (Gomez et al., 2001, Mattson and Chan, 2003). It has been known that fluctuations of Ca^{2+} level are crucial for controlling both information storage and erasure, which are two important aspects of normal cognition (Rosenzweig and Barnes, 2003). The basis of the calcium hypothesis is that amyloid-induced upregulation in Ca^{2+} may enhance the erasure of newly-formed memory thus resulting in memory loss (Samaja et al., 1989, Mattson et al., 2000, Green and LaFerla, 2008, Berridge, 2011). There is some good evidence showing that Ca^{2+} level is up-regulated in transgenic mouse models of AD. Using multiphoton imaging, it was shown that the neuronal Ca^{2+} level in neurites and spines is higher in neurons in close proximity to amyloid plaques and this may lead to disruption of neuronal networks (Kuchibhotla et al., 2008). In a similar study, Lopez et al (2008) reported that the intraneuronal resting Ca^{2+} level is significantly increased in neurons from triple transgenic mice (Lopez et al., 2008). Interestingly, a polymorphism in the CALHM1 gene encoding a highly Ca^{2+}-permeable channel is reportedly associated with the risk of AD (Dreses-Werringloer et al., 2008). The increased Ca^{2+} level in AD could result from an alternations in Ca^{2+} entry and release from intracellular stores. Extracellular Ca^{2+}can enter neurons though voltage-gated, ligand–gated or store-operated channels in the plasma membrane (Tojima et al., 2011). Meanwhile the release of Ca^{2+} from intracellular stores (mainly ER) is mediated though inositol triphosphate receptor (IP3R) and ryanodine receptor (RyR) (Supnet and Bezprozvanny, 2010). There are three major mechanisms by which Aβ could disrupt calcium homeostasis: activation of

Ca^{2+}channels, disruption of membrane lipid bilayers and formation of Ca^{2+}-permeable pores on cell membrane.

Several lines of evidence have shown that Aβ can activate some membrane-permeable Ca^{2+} channels, including glutamate receptors (NMDA and AMPA receptors) (Snyder et al., 2005, Hsieh et al., 2006, De Felice et al., 2007, Shankar et al., 2008, Li et al., 2009, Liu et al., 2010), voltage-gated Ca^{2+} channels (MacManus et al., 2000, Ho et al., 2001, Rovira et al., 2002), nicotinic acetylcholine receptor (Buckingham et al., 2009, Mehta et al., 2009) and serotonin receptor (Ju Yeon and Yeon Hee, 2005, Holm et al., 2010), leading to an increase in Ca^{2+} influx. The release of Ca^{2+} from intracellular stores has also been implicated in the aberrant calcium homeostasis in AD. A number of studies have shown that presenilin mutations affect intracellular Ca^{2+}stores by reducing the leak of Ca^{2+} (Tu et al., 2006) and possibly via an interaction with the IP3 receptor or SERCA pump (Tu et al., 2006, Cheung et al., 2008, Green et al., 2008, Cheung et al., 2010, Zhang et al., 2010). Furthermore, as a component of γ-secretase complex, presenilin may influence Ca^{2+} signalling through generating the AICD which may act as a regulator of RYR transcription (Chakroborty et al., 2009).

There is also some evidence showing that Aβ can directly disrupt lipid membrane integrity, leading to an increase in Ca^{2+} permeability. Aβ has been shown to bind to several membrane lipid components such as phosphatidylglycerol, phosphoinositides and phosphatidylchloline (D'Errico et al., 2008, Davis and Berkowitz, 2010). The interaction of Aβ with plasma membrane results in alternation of membrane fluidity and may be responsible for increasing Ca^{2+} permeability (McLaurin and Chakrabartty, 1996, Avdulov et al., 1997). In a recent study, it was shown that Aβ oligomers increased membrane conductance by lowering the dielectric

barrier and thinning the membrane (Sokolov et al., 2006). However, these findings were challenged by another study where the increased transmembrane currents were suggested to be due to the presence of residual hexaflouroisopropanol in the preparation of Aβ (Capone et al., 2009).

It has also been hypothesized that Aβ can form calcium-permeable pores on the plasma membrane. Several studies have shown that Aβ increases the permeability of artificial lipid membrane to cations and they suggest that Aβ could form channels in the membrane (Arispe et al., 1993, Pollard et al., 1993, Quist et al., 2005). This hypothesis has been supported by findings showing that Aβ can form pore-like structures *in vitro* (Lin et al., 2001, Jang et al., 2008), computer modelling (Jang et al., 2010) and electron microscopy (EM) (Lashuel et al., 2002). There is also some evidence showing the presence of Aβ channels in native cell membranes. Based on studies using high resolution transmission EM, it has recently been suggested that Aβ pores are present both on the neuronal cell membrane and in the membrane of mitochrondria-like structures in the AD brains but not in the healthy controls (Inoue, 2008). Furthermore, Aβ has been shown to induce perforation of neuronal membranes in a similar way to other pore-forming toxins, leading to increase in Ca^{2+}entry (Sepulveda et al., 2010). Indeed, a recent study has shown that two compounds that block Aβ channels can protect neurons from Aβ neurotoxic effects (Diaz et al., 2009). The three hypothetical mechanisms of Aβ-induced Ca^{2+}dysregulation are not mutually exclusive. Aβ may induce toxic effects by incorporating all three mechanisms leading to increase in intracellular Ca^{2+}.

1.5.2 Physiological function of Aβ

Because Aβ is produced in most cell types, it has been suggested that Aβ may have a physiological function (Seubert et al., 1992). There is some evidence indicating that Aβ might have a role in controlling synaptic activity. It was found that evoked activity of hippocampal neurons increased Aβ production by increasing APP processing through the β-secretase pathway. This may provide a negative feedback to prevent excitoxicity, since Aβ depresses synaptic activity (Kamenetz et al., 2003). This result is supported by another study, in which the stimulation of NMDA receptors increased APP and promoted Aβ production in Tg2576 mice (Lesne et al., 2005). Similarly, a recent study by Alley et al (2010) showed that treatment of cell cultures and APP/PS1 transgenic mice with memantine, an uncompetitive NMDA receptor antagonist that has been approved for AD treatment, resulted in a decrease in Aβ production (Alley et al., 2010). Taken together, these studies suggest that APP processing and Aβ production are in close association with synaptic activity and that they may control synaptic activity.

There are also some studies implicating Aβ in neuronal survival. Inhibition of Aβ production or immunodepletion of Aβ may cause neuronal death (Plant et al., 2003). However, neuronal death is reportedly restored by addition of physiological levels of Aβ. Aβ40 was found to be most effective. The mechanism for the protective role of Aβ in neuronal death is unclear, but some studies have proposed the involvement of K^+ channels. In this regard, K^+ channels have been known to play an important role in neuronal excitability and apoptosis (Shieh et al., 2000). Aβ has been suggested to act as a regulator of K^+ channels as physiological concentrations of Aβ significantly increase K^+ channel currents, while blocking Aβ

production decreases the K^+ channel current (Ramsden et al., 2001, Plant et al., 2006, Kerrigan et al., 2008, Ray et al., 2011). Other studies have suggested a physiological role of Aβ in the regulation of Ca^{2+} channels (Ramsden et al., 2002) and in the expression of APP, BACE and ApoE genes by binding to their promoters (Buggia-Prevot et al., 2008, Bailey et al., 2011, Guglielmotto et al., 2011, Maloney and Lahiri, 2011). However, transgenic mice lacking Aβ production do not show severe deficits in neurology and behaviour (Phinney et al., 1999, Luo et al., 2001). Therefore the idea that Aβ has physiological functions is still debated.

1.6. Physiological functions of APP

Although APP has been extensively studied since its discovery (Kang et al., 1987), little is known about its physiological function. Because APP can be processed through various proteolytic pathways to generate fragments which might have important physiological roles, the functions of APP may be a combination of the activities of its proteolytic products. Several physiological roles of APP have been reported in neurite outgrowth and synaptic formation, neurogenesis, axonal pruning and degeneration, cell signalling and gene transactivation, cell adhesion and apoptosis. However, all of these proposed functions need further investigation.

1.6.1 Expression and structural properties of the APP family

The APP gene is located on the chromosome 21 in the human genome. APP belongs to a small and highly conserved type I membrane protein family. Different APP orthologs have

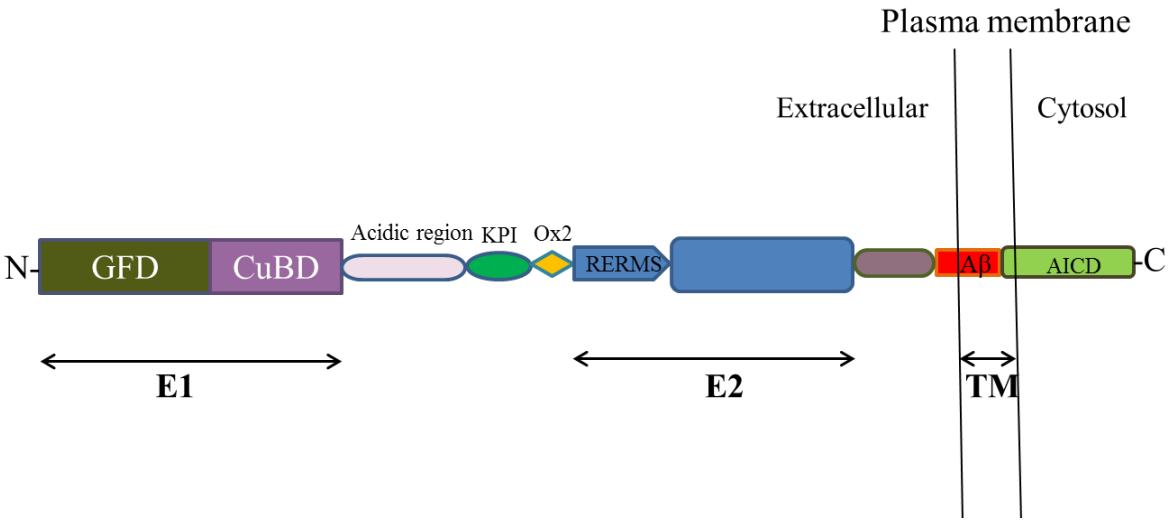

Figure 1.4. Schematic representation of APP770 structure. APP consists of a large ectodomain, a transmembrane region and a short cytoplasmic C-terminus. The ectodomain contains the E1 domains that consists of a putative growth factor domain (GFD) and a putative copper-binding domain (CuBD) linked to the E2 domain or central APP domain via an acidic region. The RERMS pentapeptide sequence which is suggested to possess neurotropic activity is located in the E2 domain. The E2 region is followed by the transmembane domain (TM), which contains part of the Aβ sequence, and the cytoplasmic domain AICD. Unlike APP695, APP751 and APP770 contain the Kunitz-type protease inhibitor (KPI) domain. The Ox2 region is only present in APP770. Abbreviations: N: N terminus; C: C terminus

been found in non-mammalian species, including APL-1 in *C. elegans* (Daigle and Li, 1993), APPa and APPb in *Zebrafish* (Musa et al., 2001, Lee and Cole, 2007, Joshi et al., 2009) and APPL in *Drosophila* (Rosen et al., 1989). In mammals, three APP homologs have been identified, APP, APLP1 (Wasco et al., 1992) and APLP2 (Wasco et al., 1993). APLP1 is only expressed in neurons (Slunt et al., 1994, Lorent et al., 1995), while APP and APLP2 expression is found in both neuronal and non-neuronal cells in the brain and the periphery. Despite the fact that all APP-like proteins undergo similar proteolytic processing, only APP contains the Aβ sequence and can therefore produce Aβ.

Similar to other members of the same family, APP contains a large extracellular ectodomain and a short cytoplasmic region (Kang et al., 1987). The extracellular ectodomain contains the E1 domains (consisting of a putative growth factor-like domain and a putative copper-binding domain) linked to the E2 domain or central APP domain via an acidic region. The E2 region is followed by the transmembane domain, which contains the Aβ sequence, and the cytoplasmic AICD domain. It is noteworthy that the cytoplasmic domain is highly conserved in all APP homologs (Wolfe and Guenette, 2007).

1.6.2 Does APP function as a cell-surface receptor?

Since its discovery (Kang et al., 1987), APP has been suggested to function as a cell-surface receptor, due to its receptor-like structure and similarities to other cell-surface receptors such as Notch, a membrane protein involved in cell signalling (Rand et al., 2000). Experiments have shown that APP may interact with several ligands, such as F-spondin (Ho and Sudhof,

2004), Aβ (Lorenzo et al., 2000), Nogo receptor (Park et al., 2006), glypican (Williamson et al., 1996), netrin (Lourenco et al., 2009), BRI2 and 3 (Matsuda et al., 2005), reelin (Hoe et al., 2009), and CD74 (Matsuda et al., 2009b). F-spondin, a secreted signalling molecule involved in neuronal development, can bind to E2 region of APP and decrease β-secretase cleavage (Ho and Sudhof, 2004). Binding of F-spondin to APP may result in increased neuronal survival (Peterziel et al., 2011). APP has also been shown to bind to Aβ and promote Aβ toxicity (Lorenzo et al., 2000, Lu et al., 2003, White et al., 2003, Sola Vigo et al., 2009, Kedikian et al., 2010). Other studies suggest that the Nogo receptor binds to APP near the BACE1 cleavage site and that it increases Aβ production (Park et al., 2006, Zhou et al., 2011). Deletion of Nogo receptor has been shown to decrease Aβ deposition and ameliorate neuropathology in AD transgenic mice (Masliah et al., 2010). Recently, it was reported that Netrin-1, a neurotropic factor, interacted with APP and decreased Aβ production (Lourenco et al., 2009). Although both BRI2 and BRI3 can bind to APP and inhibit Aβ production, they may act through different mechanisms (Fotinopoulou et al., 2005, Matsuda et al., 2005, Matsuda et al., 2008, Matsuda et al., 2009a, Matsuda et al., 2011, Tamayev et al., 2011).

Reelin, an extracellular matrix protein, has been shown to bind to E1 domain of APP, leading to decreased Aβ production and increased neurite outgrowth (Hoe et al., 2006b, Hoe et al., 2009). Consistent with these findings, Reelin levels are decreased in human AD brain and reduced reelin expression results in accelerated AD pathology in transgenic mice (Chin et al., 2007, Kocherhans et al., 2010). However, other studies found that level of both reelin protein and its mRNA was increased in the AD frontal cortex (Botella-Lopez et al., 2006) and in the entorhinal cortex (Santa-Maria et al., 2010). Over-expression of APP led to an increased level of reelin in Tg2576 transgenic mice, as well as in Aβ42-treated cell cultures, suggesting that

Aβ alters reelin expression and processing (Botella-Lopez et al., 2010). Further studies need to elucidate these discrepancies. Similarly, CD74, a component of the class II major histocompatibility complex was reported to interact with APP and to act as a negative regulator of Aβ production (Matsuda et al., 2009b). Other studies show that APP may dimerize and act as a receptor for sAPPα (Gralle et al., 2009). Furthermore, APP has been recently suggested to act as a H-ferritin-like ferroxidase increasing iron export from neurons, and this activity is inhibited by zinc (Duce et al., 2010). Although the number of APP-interacting proteins continues being identified, there is not yet a conclusive evidence that APP is a cell-surface receptor.

1.6.3 APP promotes neurite outgrowth and synaptogenesis

The fact that mice lacking all three APP family members have severe abnormalities in brain development and die shortly after birth (Herms et al., 2004) suggests that APP and its homologs play important roles in CNS. Furthermore, APP expression is robustly increased during neuronal differentiation (Yoshikawa et al., 1990, Hung et al., 1992) and during traumatic brain injury (Van Den Heuvel et al., 2000, Itoh et al., 2009), implying the possible involvement of APP in cell maturation and differentiation. The first evidence that APP has a function in cell growth came from a study on the effect of decreasing APP level by using antisense APP on the growth of fibroblasts (Saitoh et al., 1989). The study observed retardation on cell growth and this effect was rescued by addition of sAPP. Subsequent work mapped the active domain to a pentapeptide motif (RERMS) in the E2 domain (Ninomiya et al., 1993, Jin et al., 1994). Indeed, administration of either RERMS or sAPPα into rat cortices increased synaptic density and memory retention (Roch et al., 1994, Meziane et al., 1998).

These findings were supported in several studies in which sAPPα was implicated in increased neurite outgrowth and neuronal survival (Milward et al., 1992, Thornton et al., 2006, Young-Pearse et al., 2008, Hoe et al., 2009, Magdesian et al., 2011). Furthemore, sAPPα was shown to promote stem cell proliferation and differentiation (Caille et al., 2004, Ring et al., 2007, Freude et al., 2011). It is also interesting to note that the N-terminal region in the E1 domain of APP shows structural similarities with cysteine-rich growth factor, suggesting that it can function as a growth factor to promote neurite outgrowth and synaptic formation (Rossjohn et al., 1999).

1.6.4 Role of APP in cell adhesion and migration

Several lines of evidence support a role for APP in cell-cell and cell-substrate adhesion. The APP ectodomain, E1 and E2 regions, has been found to interact with several extracellular proteins and heparin sulphate proteoglycan (HSPG) (Small et al., 1992, Beher et al., 1996, Williamson et al., 1996, Clarris et al., 1997, Small et al., 1999). It is suggested that a RHDS regions, a RGD-homology sequence located in region within the Aβ peptide sequence, acts in an integrin-like fashion to promote cell adhesion (Ghiso et al., 1992). Other studies show that APP and integrin may be co-localized on the cell surface (Storey et al., 1996, Yamazaki et al., 1997, Sabo et al., 2001). Subsequent work has demonstrated that APP interacts with integrins (Young-Pearse et al., 2008). Deletion of APP by siRNA-knockdown resulted in defects in neuronal migration in rodent cortex, which is reversed by full-length APP (Young-Pearse et al., 2007).

APP dimerization has also been implicated in cell adhesion and migration. Using X-ray analysis, it was reported that the E2 domain of APP can reversibly form antiparallel dimers, raising the possibility that full-length APP may form similar dimers and hence promote cell adhesion (Wang and Ha, 2004). Subsequent work showed that APP as well as other APP protein family members (APLP1 and APLP2) can dimerize in a homo or heterotypic manner and that the formation of these complexes promotes trans-cell adhesion in vivo (Soba et al., 2005). Heparin was shown to bind to APP within the E1 domain (Small et al., 1994) and E2 domain (Multhaup et al., 1995) and promote APP dimerization (Gralle et al., 2006, Dahms et al., 2010, Lee et al., 2011b). It is noteworthy that the cellular localization and dimerization of APLP1 is markedly different from that of APP and APLP2, suggesting that members of this protein family may serve different functions (Kaden et al., 2009). Another region of APP that has been shown to promote APP dimerization is the GxxxG motif located between the juxtamembrane and transmembrane regions (Munter et al., 2007, Gorman et al., 2008, Kienlen-Campard et al., 2008, Sato et al., 2009). Although the mechanism and effect of APP dimerization are not fully understood, it appears to increase the amyloidogenic pathway (Gorman et al., 2008, Asada-Utsugi et al., 2011), which is inhibited by depleting the formation of APP dimers (Richter et al., 2010).

1.6.5 Role of APP and its fragments in axon pruning and cell death

While sAPPα has been suggested to possess neurotropic and synaptotropic activities, recent studies have also implicated sAPPβ in axonal pruning and degeneration. This is somehow surprising, given the fact that sAPPα has only 17 amino acid residues more than sAPPβ at the C-terminus and that they both contain the putative neurotropic RERMS domain. One of the

first lines of evidence for a function of sAPPβ came from a study in which sAPPβ production was increased in tropic-deprived condition, and sAPPβ was observed to undergo cleavage to produce a 35 kDa N-terminal fragment (N-APP). This N-APP fragnment was shown to bind to death receptor 6 (DR6) on the neuronal cell surface and to trigger degeneration via caspase 6 (Nikolaev et al., 2009). The study suggested that sAPPβ may induce neuronal-self destruction via DR6 and in concert with Aβ, contribute to AD pathogenesis. However, it is unclear from this study what mechanism increased sAPPβ and what enzyme cleaved sAPPβ to generate N-APP. Furthermore, it is important to determine if the level of N-APP is increased in the AD brain. Recently, a study by Li et al (2010) showed that sAPPβ is highly stable and is not normally cleaved to produce N-APP *in vivo*. Using transgenic mice expressing sAPPβ, the author also demonstrated that although sAPPβ regulates transthyretin and Klotho gene expression, it does not rescue a neuromuscular synapse defect in APP/APLP2-double KO mice (Li et al., 2010). Taken together, the biological functions of sAPPβ in vivo need further studies.

Several papers have proposed a role for the AICD fragment in cell death and in impairment of neurogenesis (Ma et al., 2008, Muller et al., 2008). It was shown that over-expression of C99 caused cytotoxicity in cell cultures and animal models, possibly mediated through the AICD fragment (Yankner et al., 1989, Oster-Granite et al., 1996). Other studies directly implicated AICD in cell toxicity in studies involving over-expression of AICD in vitro (Kinoshita et al., 2002a, Kim et al., 2003). It was demonstrated that over-expression of AICD led to tau hyperphosphorylation and abnormalities in the behaviour in animal models when co-expressed with Fe65 (Ghosal et al., 2009, Ghosal et al., 2010). In contrast to sAPPα, AICD was found to negatively regulate neurogenesis (Ma et al., 2008). In addition, there are a number of papers suggesting that C31 and Jcasp, products of caspase cleavage of APP, can also induce apoptosis. Cell toxicity was observed in cell cultures overexpressing C31 or Jcasp

(Bertrand et al., 2001). In comparison to C31, Jcasp seems to play a minor role in cell toxicity (Park et al., 2009). Importantly, an *in vivo* study using transgenic mice expressing APP with a mutation at the caspase-cleavage site (Asp-664) showed that while Aβ deposition was unchanged, the pathological and behavioural changes were prevented. Although some studies suggest that AICD activates the death receptor, p53, and induces cell apoptosis (Alves da Costa et al., 2006, Ozaki et al., 2006) , the mechanism of these toxic effects is unclear.

1.6.6 Role of AICD fragment in cellular signalling and gene activation

AICD has been reported to interact with more than 20 proteins, many of which contain a PTB domain and function in signal transduction and gene regulation, such as the Fe65 family, the X11 family, disabled-1, the Shc family and cJun N-terminal kinase interacting protein (Borg et al., 1996, Sabo et al., 2001, Slomnicki and Lesniak, 2008). Many of these interactions require a YENPTY motif in the AICD sequence (Muller et al., 2008). Notably, it was demonstrated that AICD can form a transcriptional complex with Fe65 and Tip60 (Cao and Sudhof, 2001, Gao and Pimplikar, 2001). Although Kinoshita et al (2003) demonstrated that nucleus translocation of AICD and Fe65 complex is required for transactivation (Kinoshita et al., 2002b), subsequent data from Cao et al (2004) showed that membrane-tethered AICD could indirectly activate gene transcription by acting on Fe65 (Cao and Sudhof, 2004). Besides Tip60, AICD and Fe65 can form a transcriptionally active complex with CP2 transcription factor (Zambrano et al., 1998) and this complex has been suggested to regulate GSK3-β expression (Kim et al., 2003). Although the AICD function in gene activation remains to be determined, the expression of several genes has been suggested to be regulated by AICD signalling, such as p53 (Ozaki et al., 2006), BACE and APP (von Rotz et al., 2004),

neprilysin (Eisele et al., 2007), LRP1 (Liu et al., 2007), EGF receptor (Zhang et al., 2007), KAl1 (Baek et al., 2002) and α2-actin and transgelin (Muller et al., 2007).

A role of APP in cytoskeletal dynamics and calcium signalling has also been proposed. In this regard, APP may exert the effect by interacting with protein binding partners or AICD-mediated activation of several genes involved in cytoskeletal transport (Muller et al., 2007) and calcium homeostasis (Hamid et al., 2007, Ha et al., 2010, Linde et al., 2011).

1.7. Transgenic mouse models of Alzheimer's disease

A number of transgenic animals that express the APP gene have been generated. Expression of human APP has been achieved in *C. elegans*, Drosophila, zebrafish, rat and mouse. The literature is too large to comprehensively review here. For more information, the reader is directed to a number of reviews (Woodruff-Pak, 2008, Crews et al., 2010, Iijima-Ando and Iijima, 2010, Obulesu and Rao, 2010). This section focuses on more common and relevant AD models, namely transgenic mouse models of AD.

Transgenic mouse models of AD have contributed significantly to our understanding of AD pathogenesis and are important for the development of therapeutic treatments. A number of AD transgenic mice have been created which recapitulate at least to some extent the pathology of AD, including amyloid deposition, synaptic loss and behavioural deficits. Although these mice exhibit many key pathological features of AD, they do not recapitulate all neuropathological changes found in AD patients, such as the presence of NFT and

abundance of neuronal cell death. Some possible explanations have been suggested: Either Aβ is not sufficient to cause the neurodegeneration observed in AD, or rodent models do not precisely mimic human brain. Nevertheless, transgenic mouse models of AD offer a useful tool for understanding AD physiopathology and preclinical drug testing. Transgenic mice with altered expression of genes associated with the risks of AD (such as ApoE, LRP1 and CLU) have also been generated, particularly when crossed with APP and tau transgenic mice. The selection of AD mouse models should be guided by particular questions to be addressed and methodology to be utilized. Detail descriptions of current AD transgenic mouse models can be found at http://www.alzforum.org/res/com/tra/default.asp.

Although transgenic mice expressing human wild-type APP exhibit a subtle increase in Aβ levels, no recognizable AD-related pathology is observed (Mucke et al., 2000). However, transgenic mice carrying human APP with familial mutations develop a robust age-dependent Aβ deposition and AD-like pathology. The promoters used to drive gene expression in AD mouse models are often neuron-specific such as platelet-derived growth factor-β (PDGF), Thy-1 and Thy-1.2 or primarily expressed in neurons such as the hamster prion (PrP) promoter. The PDAPP mouse was the first transgenic mice that develop significant Aβ deposits and amyloid pathology such as synaptic loss, astrocytosis and microgliosis. These mice carry human APP gene with Indiana mutation V717F encoding for three isoforms: APP750, 751 and 695 under the control of PDGF promoter (Games et al., 1995). Perhaps the most commonly studied transgenic mouse model of AD is the Tg2576 mouse which was developed by expressing a human APP transgene with the Swedish FAD double mutation (K670N/M671L) under the control of a PrP promoter (Hsiao et al., 1996). Tg2576 mice exhibit clear amyloid plaques at the age of 11-13 month old. Hyperphosphorylated tau (but

not NFT) is present. Memory and learning deficits can be seen as early as 6 months before the formation of amyloid deposition, implicating soluble Aβ in neuronal dysfunction. Recently, it was shown that a 56 kDa Aβ oligomeric species named Aβ*56 is the most closely correlated with memory deficit in Tg2576 mice (Lesne et al., 2006). Based on these two models, many other transgenic mice were developed by overexpressing APP transgene with one or more FAD mutations under the control of strong promoters. For example, APP23 mice overexpressing APP751 with a Swedish mutation under the control of Thy-1 promoter exhibit plaque formation at 6 months of age (Sturchler-Pierrat et al., 1997). Memory and learning impairments can be observed as early as 3 months. CAA and neuronal loss have also been documented (Calhoun et al., 1998). A more aggressive model of AD, the TgCRNG8 mouse, which overexpresses APP695 with Swedish and Indiana mutations under the control of the PrP promoter, develops amyloid plaques at the age of 3 months and has neuritic pathology from 5 months of age. Furthermore, cognitive impairment can be seen at 3 months and becomes more obvious at 5 months of age (Chishti et al., 2001).

Several double APP/PS transgenic mice overexpressing APP and PS mutants have also been produced. FAD mutations in PS1 often result in an increase in production of Aβ42 or Aβ42/Aβ40 ratio and accelerate Aβ deposition. Of these models, APPswe/PS1M146L transgenic mice, which overexpress APP Swedish double mutant and PS1 with M146L mutation, are commonly used. These mice have significantly increased Aβ42 in the brain. Amyloid pathology is dramatically increased and some memory impairment is present as early as 3-5 months (Holcomb et al., 1998). Another line of APP/PS transgenic mice are the APPSwe/PS1deltaE9 strain, which overexpress APPswe and mutant PS1 with a deletion of exon-9. These mice develop Aβ deposition at 6 months and older mice are impaired in all cognitive tasks. Memory deficits in these mice are correlated with Aβ load in the brain (Savonenko et al., 2005). Notably, a more aggressive double APP/PS1 transgenic model was

generated by Oakely et al (2006) called 5 X FAD. These mice possess 3 mutations in APP; the Swedish, Florida (I716V) and London (V717I) mutations and 2 mutations in PS1; M146L and L286V both under the control of Thy-1 promoters. These mice exclusively produce Aβ42 and intraneuronal Aβ42 accumulation can be seen at 1.5 months of age. Amyloid deposition occurs at approximately 2 months of age and is associated with gliosis and hyperphosphorylated tau. Neuronal loss and learning deficits are evident after 4-6 months (Oakley et al., 2006).

All of the transgenic mouse models of AD described above exhibit amyloid pathology but not NFT. Thus they are regarded as incomplete models of AD pathology. In order to generate a transgenic mouse model with amyloid and NFT pathology, several groups have crossed mice expressing mutant APP with mice carrying tau mutations to create APP/tau transgenic mice. One of the earlier mouse models was generated by Lewis et al (2001) in which mouse overexpressed APPswe and P301M tau mutants. These mice exhibit both amyloid plaques at the same age as Tg2576 mice as well as NFT in the cortex (Lewis et al., 2001). Similarly, another model was generated by crossing Tg2576 mice with mice carrying tau with three mutations: G272V, P301L, and R406W (Perez et al., 2005). These mice show increased amyloid deposition and NFT. Neural loss is found in some vulnerable areas of the brain and spatial memory impairments are seen at the age of 16 months. Recently, a triple transgenic mouse model has been developed by Oddo et al (2003). The mice were generated by microinjected APPswe and P301L tau transgenes into a single-cell embryo from homozygous PS1 M146L mice. These mice progressively develop plaques and tangles and show synaptic dysfunction before the onset of plaque and tangle pathology. Furthermore, synaptic dysfunction is found to be closely correlated with the accumulation of intraneuronal Aβ,

suggesting a role for intracellular Aβ in synaptic dysfunction (Oddo et al., 2003). However, there are some potential limitations of all of the above-mentioned animal models, as they express the rare FAD mutations (APP, PS1 and PS2), and mutant APP has been reportedly processed in a different way from wild-type APP (as in the sporadic AD) (Yamakawa et al., 2010, Griffiths et al., 2011). The presence of NFT in AD transgenic mice with *tau* mutations is also doubtful because *tau* mutations are present in other tauopathies such as FTD, but not in AD patients. Furthermore, although all of the mouse models can recapitulate many aspects of AD pathology, including amyloid plaques and NFT, there is still a need to be cautious in the use of the mouse models because they may not fully represent all features of human AD.

1.8. The receptor-associated protein (RAP) and AD

The receptor-associated protein (RAP) is a 39 kDa protein which is expressed in various cell lines and tissues, with highest expression found in brain and kidney (Coukos et al., 1994). Within the cell, RAP is found mainly in the ER and in Golgi complex (Strickland et al., 1991), but a small proportion can be found at the cell surface and in endosomes (Bu et al., 1994). RAP plays an important role in LDL receptor family function by (i) regulating the binding of the LDL receptors to their ligands (Herz et al., 1991) and (ii) acting as a chaperone for the maturation and trafficking of the LDL receptor family (Bu and Marzolo, 2000). Apart from the LDL receptor family, RAP has been known to interact with several other proteins, including members of the vacuolar sorting protein (VSP) gene family (sortilin, SORCS and SorLA) (Tauris et al., 1998), lipoprotein lipase (Page et al., 2006) and thyroglobulin (Marino et al., 2001). RAP can be actively transported through the BBB (Pan et al., 2004) and across the cell membrane (Kim et al., 2004, Prince et al., 2004). Polymorphisms in the RAP gene

have been associated with the risk of Alzheimer's disease (Sanchez et al., 2001, Pandey et al., 2008). Treatment of RAP in cell cultures expressing KPI-containing APP isoforms led to a decrease in Aβ secretion (Ulery et al., 2000, Goto and Tanzi, 2002). Two studies have shown that down-regulation of RAP exacerbates Aβ pathology in transgenic mouse models of AD, suggesting that RAP may have an important role in Aβ production and clearance (Van Uden et al., 2002, Xu et al., 2008). Furthermore, decreased RAP expression was found in the brain of AD compared with the healthy controls (Provias and Jeynes, 2010). Two recent studies have showed that RAP can bind directly to Aβ and attenuate its neurotoxcity (Kanekiyo and Bu, 2009, Kerr et al., 2010). Taken together, RAP may play a neuroprotective role in AD pathology.

1.8.1 Structure and expression

RAP was discovered by co-purification with LRP by ligand chromatography (Kristensen et al., 1990, Strickland et al., 1990, Orlando et al., 1992). It was originally identified as a C-terminal fragment of the LRP2/megalin receptor (Pietromonaco et al., 1990). However, subsequent work demonstrated that RAP is a distinct protein which primarily resides in the endoplasmic reticulum (ER) and functions as a chaperone for the LDL receptor family (Strickland et al., 1990, Biemesderfer et al., 1993, Bu and Rennke, 1996). RAP was found to be expressed in all tissues examined with the highest expression in kidney and many brain regions such as cortex, hippocampus, cerebellum and brainstem (Zheng et al., 1994, Lorent et al., 1995, Van Uden et al., 1999).

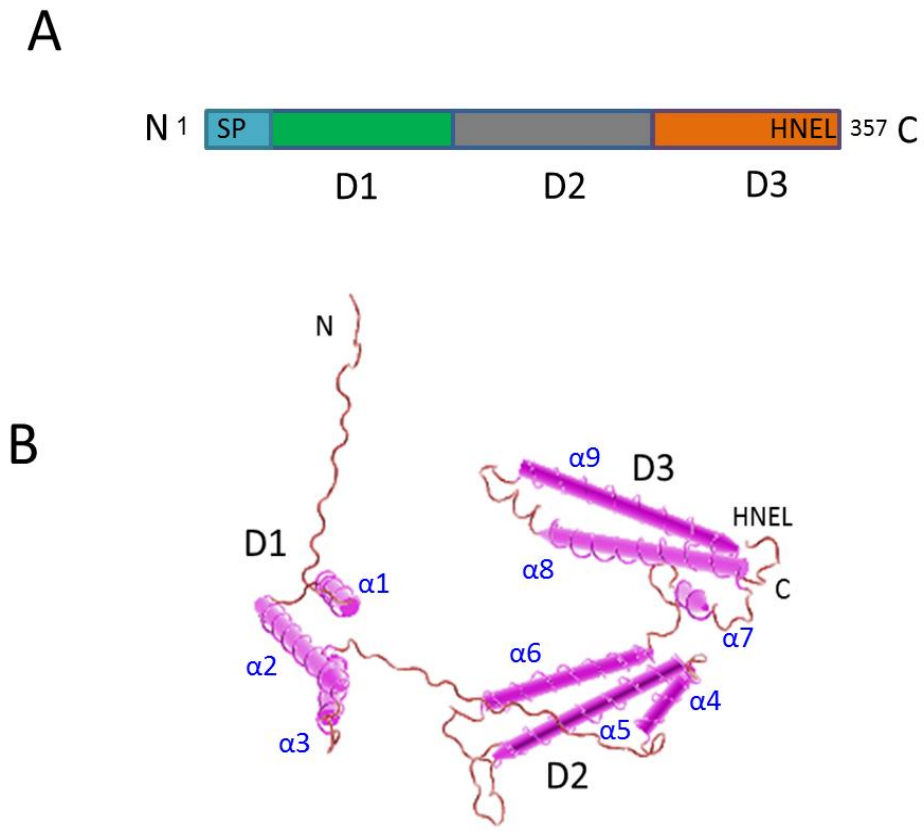

Figure 1.4. Schematic and 3-dimensional representation of the structure of RAP (adapted from Lee et al. 2007). (**A**) RAP is a 39kDa protein which consists of three independent binding domains termed: D1, D2 and D3. (**B**) Each domain contains about 100 amino acid residues and adopts a three-helix bundle structure. The three domains are linked together by flexible linkers, that allow the protein to adopt various conformations. SP: signal peptide; D1: domain 1; D2: domain 2; D3: domain 3; N: N-terminal; C: C-terminal.

RAP contains a putative signal peptide, an N-linked glycosylation site at arginine 234, a leucine motif and an HNEL C-terminal sequence, which is important for retrieval of RAP back to the ER (Strickland et al., 1991). A study using circular dichroism showed that RAP contains 38% α-helical structure (Strickland et al., 1991). Based on the triplication of the primary sequence, it was first predicted that RAP structurally consists of three domains (Bu et al., 1995). Later studies in which individual domains of RAP were expressed, demonstrated that the function of each domain is preserved (Ellgaard et al., 1997, Rall et al., 1998, Lazic et al., 2003). Using NMR spectroscopy and X-ray crystallography, the structures of three individual RAP domains (D1, D2 and D3) were solved. Each domain adopts a three-helix bundle structure and is connected by flexible linkers (Fig. 1.4). In RAP, linkers between the three domains play a very important role, allowing RAP to adopt different conformations.

The structure of D1 (residues 18-112) consists of three-distinct helices (H1-H3) arranged in up-down-up antiparallel topology with H1 (an amphipathic helix, residues 23-34), H2 (hydrophilic, residues 39-65) and H3 (residues 73-88) (Nielsen et al., 1997, Wu et al., 2004). The Heymann nephritis pathogenic epitope in RAP was mapped between residues 31-53 in the D1 (Kerjaschki et al., 1996). The D2 structure (residues 100-216) is also composed of 3 α-helices (H4-H6) consisting of the residues 117-217 (H4), 132-161 (H5) and 184-208 (H6). The linker between H5 and H6 is disordered and susceptible to protease digestion (Rall et al., 1998). The structure of D3 contains a short helix (H7, residues 222-230) followed by two longer helices (H8, residues 238-274 and H9, residues 281-315). The structure of D1 and D3 is stabilized by hydrophobic interactions within each structure (Lee et al., 2006). Using small-angle neuron scattering data and a novel simulated annealing protocol, the overall structure of RAP has been revealed (Lee et al., 2007a).

1.8.2 Functional role of RAP and phenotype of RAP transgenic mouse models

A study by Biemesderfer et al (1993) showed that RAP is associated with LRP2/megalin very early after synthesis and that the complex remains associated during folding, oligomerization and transport to the Golgi apparatus. Another study (Bu et al., 1995) demonstrated that RAP is an ER resident protein and that it is retained in the ER due to the presence of the HNEL ER retention signal. Pulse-chase analysis showed that RAP is transiently associated with LRP after synthesis and that it is dissociated from LRP at a lower pH later in the secretory pathway. This association ensures the proper folding and prevents the binding of other ligands to immature LRP. Over-expression of ApoE, a high affinity LRP binding ligand in RAP-deficient fibroblasts dramatically decreased the LRP level and this effect was rescued by co-expression of RAP The study suggested that RAP acts as a chaperone for LRP1 and regulates the binding to its ligands (Willnow et al., 1996). Over-expression of APP and ApoE in neurons does not affect LRP synthesis, maturation and distribution in the presence and absence of RAP, indicating that chaperone function is only attributable to RAP (Umans et al., 1999). Due to high binding affinity to LDL receptor family, RAP has been commonly used as an inhibitor to study the function of LDL receptor family (Deane et al., 2009, Cudaback et al., 2011, Fujiyoshi et al., 2011, Makarova et al., 2011, Rolyan et al., 2011).

To study the functional role of RAP in vivo, RAP was transferred by adenoviral vector into the livers of LDLR-/- and wild-type mice. A decrease in the activity of LRP was found by assessing the ability to clear its specific ligand, α2-macroglobulin, from the plasma in both mice. LDLR-/- mice that received the RAP gene had a significant increase in plasma cholesterol and plasma triglyceride concentration. This study suggested that RAP functions as

a receptor antagonist to block the removal of lipoproteins from the plasma by LRP and LDLR (Willnow et al., 1994). Although there is a 75% decrease in the level of mature LRP1 in the liver and brain in a RAP knockout mouse model, the mice are still viable and fertile (Willnow et al., 1995). The decrease in LRP activity was found by examining the rate of clearance of α2-macroglobulin and lipoproteins from the plasma. The study concluded that RAP plays an important role in stabilizing LRP within the secretory pathway. However, the level of LRP was unchanged in fibroblasts derived from RAP-KO mice, indicating that requirement of RAP for LRP maturation and trafficking is tissue and cell-type specific (Willnow et al., 1996). Another study showed that RAP is highly co-expressed with somatostatin in neocortical and hippocampal neurons (Van Uden et al., 1999). RAP-KO mice were found to be cognitively impaired in the Morris water maze test. The level of somatostatin was significantly decreased in neurons in the CA1 region of the hippocampus and there was a decrease in the number of somatostatin-expressing neurons which strongly correlated with cognitive deficits in the RAP-KO mice. It has been also shown that somatostastin promotes memory formation (Matsuoka et al., 1994, Matsuoka et al., 1995). Depletion of somatostatin caused memory impairment in rats and this effect was rescued by admistration of somatostatin. It is noteworthy that the level of somatostatin is decreased in cortex, hippocampus and CSF of AD patients (Gabriel et al., 1993, Molchan et al., 1993, Dournaud et al., 1994).

1.8.3 Interaction of RAP with other binding partners

RAP may interact with several other binding partners that do not belong to the LDL-receptor family. RAP may bind to HSPG and can be purified by heparin affinity chromatography (Furukawa et al., 1990, Orlando and Farquhar, 1993). Subsequent work using different

recombinant RAP fragments showed that the heparin-binding site is a highly positively charged region between residues 261 and 323 (Orlando and Farquhar, 1994). It was shown that exogenous RAP binds to HSPGs on human fibroblasts with low binding affinity but high capacity and that this binding is inhibited by addition of heparin and suramin (Vassiliou and Stanley, 1994). The study also showed that disruption of glycosaminoglycan chains on HSPGs with heparinase or chlolate did not affect the binding of RAP to cells, excluding a role for the glycosaminoglycan chains in RAP-cell binding. Another study demonstrated that three basic amino acid sequence motifs in the domain 3 are required for RAP binding to both heparin and LRP (Melman et al., 2001). It was shown in this study that the high affinity binding to heparin requires a $R^{282}VSR^{285}SR^{287}EK^{289}$ sequence motif on RAP, and that $R^{203}LR^{205}R^{206}$ and $R^{314}ISR^{317}AR^{319}$ motifs also contribute to this binding. This conclusion was supported by the evidence that treatment of heparin blocks the binding RAP to LRP. However, the biological significance of RAP-heparin binding is not clear. It is possible that HSPGs may serve as a mechanism to sequester extracellular RAP and to prevent any unwanted effect on the functions of LDL receptor family.

RAP has also been shown to bind to lipoprotein lipase (LPL) and it may function as a chaperone for LPL (van Vlijmen et al., 1999, Page et al., 2006). LPL is an enzyme produced by parenchymal cells of extrahepatic tissues and functions in the uptake of fatty acids and lipoprotein components. LPL is known to bind to LRP and HSPGs. Using mice lacking LDLR and LRP, it was demonstrated that RAP over-expression decreases the conversion of chylomicrons to remnant particles, an activity associated with LPL action, and increases the level of inactive LPL in the plasma (van Vlijmen et al., 1999). Using ELISA and surface plasmon resonance, it was demonstrated that RAP can bind to the C-terminal region of LPL

as a monomer (Page et al., 2006). Co-immunoprecipitation studies showed the formation of RAP-LPL complex in mixtures of purified RAP and LPL and in the lysates of adipocytes (Page et al., 2006). Binding of RAP to LPL was also demonstrated in vivo by cross-linking studies. Moreover, it was found that RAP-deficient adipocytes secreted LPL with specific activity that was lower than in the control cells. Taken together, these studies suggest that RAP may function as a chaperone for maturation and trafficking of LPL in the secretory pathway.

Marino et al (2001) has reported that RAP can bind to thyroglobulin (Tg), a precursor of thyroid hormones and a ligand for the megalin receptor. This study also showed that RAP is associated with Tg early in the secretory pathway (Marino et al., 2001). In vivo studies using RAP KO mice showed that there was a large decrease in the Tg aggregates in the thyroid follicles and a significant increase in serum Tg compared with the wild-type controls (Lisi et al., 2006). Taken together, it is possible that RAP plays a role in biosynthesis and storage of Tg. RAP has also been found to bind to the Gp95/sortilin receptor. It was first demonstrated that sortilin co-purified with RAP upon RAP affinity chromatography from brain extracts (Petersen et al., 1997). Subsequent work using surface plasmon resonance showed that the D3 domain of RAP binds to Vsp10 domain of sortilin (Tauris et al., 1998, Munck Petersen et al., 1999). Another study reported that endogenous RAP associates with 8 other ER proteins in various cell types, however, the biological significance of these interactions is unclear (Sarti et al., 2000).

1.8.4 High capacity of RAP to cross the cell membrane and the BBB

RAP can be highly internalized into cells by receptor-mediated endocytosis. Receptors involved in RAP uptake include LRP, megalin and HSPGs (Vassiliou and Stanley, 1994, Willnow and Herz, 1994, Czekay et al., 1997). RAP increases Aβ cellular uptake and this effect is blocked in the presence of heparin inhibitors, suggesting that HPSGs may mediate this process (Kanekiyo and Bu, 2009). A study showed that RAP conjugated to poly-D-lysine was highly effective as a gene delivery vector for transfection (Kim et al., 2004). In other studies, RAP fused to human lysosomal enzyme glucosidase or L-iduronidase was found to be efficiently taken up in cell culture (Prince et al., 2004). RAP covalently coupled to adenovirus particles mediated efficient targeting (Corjon et al., 2008). It has been shown that RAP can be rapidly cleared from the circulation after intravenous injection of the protein into rats, suggesting that there are efficient transport systems for this process (Warshawsky et al., 1993a).

Administration of RAP has been shown to be beneficial by rescuing the ApoE3-induced neurodegeneration or by blocking the disruptive effect of plasminogen activator (tPA) on the BBB (Veinbergs et al., 2001, Suzuki et al., 2009). RAP also has a high capacity to cross the BBB into the brain parenchyma though a saturable and efficient transport system and this process may be mediated by the megalin receptor (Pan et al., 2004). The inability of proteins and peptides to cross the BBB into the central nervous system normally makes it difficult to treat neurological disorders with the protein-based drugs. Therefore, high capacity transport of RAP through BBB could be potentially used as a carrier protein in protein-based delivery.

1.8.5 Association of RAP with AD

1.8.5.1 RAP as a genetic risk factor for AD

Analysis of the human RAP gene has identified 23 polymorphisms, mostly in introns. However, the biological significance of these is not clear (Van Leuven et al., 1998). Polymorphisms in intron 5 (insertion/deletion) of the RAP gene have been implicated as genetic risk factors for AD (Sanchez et al., 2001, Pandey et al., 2008). Sanchez *et al*. (2001) showed that the frequency of homozygotes for an insertion (II) in RAP was significantly lower in AD patients compared with controls and no patients below 75 years contained the II polymorphism. The study suggested that the II polymorphism may delay the onset of LOAD. The fact that this polymorphism is in a non-coding region, suggests that it may affect other biologically relevant polymorphisms in RAP or nearby genes (Benes et al., 2000, Sanchez et al., 2001). However, this finding has been disputed by Schutte et al (2003), who found no differences in allele or genotype frequencies in RAP by gender or by age at onset. In another study, the frequency of homozygotes for a deletion (DD) was higher in AD cases compared with controls (Pandey et al., 2008). However, all of these studies were conducted on a relatively small number of patients, therefore larger scale studies need to be carried out.

1.8.5.2 Effect of RAP treatment on Aβ production in cell cultures

Treatment of RAP results in increased levels of cell-surface APP and sAPPα and decreased level of Aβ in APP751-H4 human neuroglioma cells (Ulery et al., 2000). In this study, it was suggested that these effects might be the result of interaction between LRP and APP.

Blocking LRP activity by RAP could result in decreased levels of APP internalization and subsequently lower Aβ production. Using fluorescence resonance energy transfer (FRET), it was demonstrated that LRP can interact with APP both through LRP ectodomain/KPI-domain, which is RAP sensitive and through their intracellular cytoplasmic domains, which is RAP-insensitive (Kinoshita et al., 2001). In support with this contention, RAP is known to bind to the ectodomains (II, III and IV), but not to intracellular cytoplasmic domain of LRP. However, it is unclear whether LRP could influence the processing of APP695.

1.8.5.3 Effect of RAP knockout on Aβ deposition and neurodegeneration in transgenic mouse models of AD

Studies on APP transgenic, RAP-deficient mice provide have demostrated that RAP plays a important role in AD amyloidogenesis (Van Uden et al., 2002, Xu et al., 2008). Van Uden et al. (2002) crossed homozygous RAP-deficient mice with hAPP transgenic mice and found an increase in the amount of amyoid deposition in the RAP (-/-) APP mice compared with the normal APP mice. In addition, neurodegeneration was increased in the RAP (-/-) APP mice. The author suggested that reduced RAP level may decrease the levels of LRP and other lipoprotein receptors, leading to decreased Aβ clearance. These findings are in agreement with a more recent study by Xu et al. (2008) in which mice transgenic for APP695/PS1 and hemizygous for RAP were generated. The study showed a 50% reduction in RAP production and a small decline in the levels of mature LRP and SorLA, 20% and 15%, respectively, in the brain of the APP695/PS1/RAP-deficient mice compared with the APP695/PS1 mice. Importantly, partial reduction of RAP resulted in a 30-40% increase in Aβ deposition. The study concluded that RAP might modulate Aβ deposition by regulating the maturation of some proteins such as LRP and SorLA or by directly influencing APP processing and Aβ

metabolim. Consistent with these studies, it has been recently shown that RAP expression decreases in the AD brain compared with age-matched controls (Provias and Jeynes, 2010). It is noteworthy that oral administration of anti-aging Chinese medicine increases the level of RAP in brain in mice (Zhang et al., 2005), suggesting that RAP may play a protective role.

1.8.5.4 RAP-Aβ interaction and its effect on Aβ neurotoxicity

As reported by Xu et al. (2008), partial reduction in RAP production causes a small reduction in SorLA and LRP but it increases the amount of Aβ deposition. These findings raise a question as to whether RAP has more a direct effect on Aβ production and clearance other than that mediated by a member of the LDL receptor family. Recent studies examined the effect of RAP on Aβ-induced effects in cell cultures (Kanekiyo and Bu, 2009, Kerr et al., 2010) . It was shown that RAP and Aβ co-localized on the cell surface in a punctate fashion, but neither protein was associated with LRP (Kerr et al., 2010). Moreover, RAP was found to bind directly to Aβ, forming a RAP-Aβ complex which was stable in SDS and increased cellular uptake of Aβ. It was suggested that HSPG may mediate RAP-induced increase in cellular Aβ uptake (Kanekiyo and Bu, 2009). The effect of RAP on Aβ aggregation was also examined in vitro. RAP was shown to inhibit Aβ1-40 oligomerization and Aβ1-42 fibril formation. Co-injection of RAP with Aβ1-42 blocked an Aβ-induced increase in intracellular Ca2+ in SH-SY5Y cells and inhibited Aβ-induced memory consolidation in day-old chicks (Kerr et al., 2010). Taken together, these findings suggest that RAP can inhibit Aβ neurotoxicity and increase Aβ clearance by forming a RAP-Aβ complex. However, it is unclear how RAP interacts with Aβ.

The fact that most Aβ is found in the extracellular space in the AD brain, whereas RAP is primarily found in intracellular compartments (Strickland et al., 1991, Bu et al., 1994), raises the question about how RAP could interact with Aβ outside of cells. Studies have shown that significant amounts of RAP can be found at the cell surface of renal proximal tube cells (Orlando et al., 1992), rat carcinoma cell lines (Orlando and Farquhar, 1993) and fibroblasts (Birkenmeier et al., 1998). Although RAP has a putative signal peptide sequence (Strickland et al., 1991), RAP is not detected extracellularly under normal physiological conditions. The fact that RAP contains HNEL C-terminal sequence, which is an ER-retention sequence, has led to the suggestion that RAP could be retained in ER by KDEL receptors/ERD2 proteins (Strickland et al., 1991). Indeed, it has been demonstrated that RAP is retrieved by KDEL receptors/ERD2 proteins in the Golgi complex through the HNEL sequence and loss of this signal leads to secretion of RAP into the extracellular space and redistribution of RAP inside the cells (Bu et al., 1995, Bu et al., 1997). In addition, transient over-expression of RAP in mice results in secretion of RAP in the plasma of these mice, this could be the consequence of saturation of retention signal (Willnow et al., 1994). Furthermore, it was reported that expression of an LRP minireceptor construct also caused RAP secretion (Lee et al., 2006).

1.9. Hypothesis and aims of the study

There is evidence that RAP may play a role in the pathogenesis of AD. First, it has been reported that RAP polymorphisms were associated with AD (Sanchez et al., 2001, Pandey et al., 2008). RAP treatment decreased Aβ production and RAP expression was correlated with the amount of Aβ pathology (Ulery et al., 2000, Van Uden et al., 2002, Xu et al., 2008, Provias and Jeynes, 2010). Furthermore, RAP can bind tightly to Aβ, leading to reduced Aβ aggregation and neurotoxicity (Kanekiyo and Bu, 2009, Kerr et al., 2010). Taken together, these findings suggest that RAP may have a potential therapeutic value for the treatment of AD.

Hypothesis: RAP modulates APP processing to decrease Aβ production and to increase Aβ clearance *in vivo*. Therefore, intervention that increases RAP expression may have therapeutic benefits in AD.

The aims of the study were:

1. To identify the region of RAP which binds to Aβ;
2. To examine the effect of RAP overproduction on APP metabolism and Aβ production cell cultures;
3. To examine the effect of exogenously added RAP on APP metabolism and Aβ production in cortical neurons from APP transgenic mice (Tg2576);
4. To determine if Aβ can bind to RAP and other proteins in the human CSF.

This project aims to provide new insights into the role of RAP in the pathogenesis of AD.

Chapter 2: Development of an Aβ-self association assay to identify of the Aβ-binding domain on RAP

2.1 Introduction

Evidence of RAP binding to Aβ has come from the finding that treatment of RAP increased Aβ cellular uptake and RAP is associated with Aβ in the cell membrane (Kerr et al., 2010). Subsequent experiments using co-immunoprecipitation and western blot demonstrated that RAP could form a 46 kDa SDS stable complex with Aβ (Kerr et al., 2010). This complex may be the result of 1:1 ratio interaction between RAP and Aβ. Subsequent experiments showed that RAP can inhibit aggregation of both Aβ40 and Aβ42 (Kerr et al., 2010). These results are in agreement with another study by Kanekiyo et al (2009). By using gel filtration chromatography and cross-linking, Kanekiyo et al (2009) reported that RAP formed complexes with both Aβ40 and Aβ42 in a concentration-dependent manner. However, it was not clear in these studies how RAP binds to Aβ and which region of RAP might be involved in the interaction.

In this study, an *in vitro* Aβ-self association assay was first established and validated, based on the binding of biotin labelled Aβ42 to synthetic Aβ42 bound to in the wells of microplates. Next, the Aβ-binding region on RAP was investigated by measuring Aβ-oligomerization in the presence of various recombinant RAP fragments. The results showed that Aβ-binding site is most likely to reside in loop region between the D2 and D3 domains of RAP. This study

provided a new insight into RAP: Aβ interaction and may offer a potential to develop RAP-mimicking drugs to inhibit Aβ deposition and increase cellular Aβ uptake for AD.

2.2 Materials and methods

2.2.1 Materials

Human full-length RAP protein and different RAP fragments were kindly provided by Prof. Dudley K. Strickand (University of Maryland, USA). The synthesis and purification of the RAP fragments were described previously (Medved et al., 1999). Briefly, cDNA enconding for human full-length RAP, fragment 1-89, 89-216, 1-206 and 206-323 were cloned into pGex-2Tvector at *BamHI* and *EcoRII* cleavage sites, and then transformed into E.coli DH5 F'. The expression vector pGex-2T was designed to produce a fusion protein containing the RAP fragments and glutathione S-transferase (GST). Bacterial clones were selected and grown in LB broth containing ampicillin. Proteins were then purified and cleaved by thrombin to remove the GST. Aβ40, Aβ42 and scrambled Aβ42 were purchased from W. M. Keck Biotechnology Resource Laboratory (New Haven, CT, USA) and more than 95 % pure as judged by mass spectrometry and HPCL analysis. Biotin-Aβ42 was obtained from rPeptide (GA, USA). Trifluoroacetic acid (TFA) and trifluoroethanol (TFE) were purchased from Sigma (Sydney, Australia). Dimethyl sulfoxide (DMSO) was purchased from Merck (Melbourne, Australia). Twelve-well plates were purchased from Greiner Bio-one (VIC, Australia). Streptavidin-HRP and 3,3',5,5'- tetramethylbenzidine (TMB) solution were purchased from Invitrogen (Melbourne, Australia). Apolipoprotein 3 and 4 (ApoE3 and ApoE4) proteins were purchased from Protein science (Sydney, Australia). Anti-

chymotrypsin protein was purchased from Abcam (Sydney, Australia). Bovine serum albumin (BSA) was purchased from Invitrogen (Melbourne, Australia).

2.2.2 Preparation of Aβ

To prepare Aβ, the peptide was first dissolved in TFA at a concentration of 1 mg/ml (2 mM) and then incubated for 20 min at room temperature. The TFA was evaporated under a gentle stream of nitrogen gas, and then Aβ was twice redissolved in TFE at a concentration of 1 mg/ml. Each time, the solvent was evaporated under nitrogen gas. The TFA/TFE-treated Aβ was then stored at -20^{0}C. TFA/TFE treatments have been shown to reproducibly disaggregate Aβ (Jao et al., 1997).

2.2.3 Aβ-oligomerization assay

TFA/TFE-treated Aβ was dissolved in DMSO to yield a concentration of 10 mg/ml (2 mM) and then stored at -80^{0}C until use. To study Aβ-oligomerization, Aβ was diluted in miliQ-water to a concentration of 100 μg/ml and Aβ (100 μl) was added to each well of a 96-well plate. The plates were incubated with gentle agitation for 48 hr at room temperature. After washing three times with 300 μl of Tris-buffered saline (TBS) (50 mM Tris-HCl, 150 mM NaCl, pH 7.4), non-specific binding sites were blocked by incubating with 1% (w/v) BSA in 200 μl of TBS for 2 hr at room temperature. The plates were washed three times with 300 μl of TBS and then incubated with 50 nM biotin-Aβ42 (freshly prepared) in 100 μl TBS for 24 hr at room temperature with gentle agitation. After washing four times with 300 μl of TBS,

the plates were incubated with streptavidin-HRP (1:3000) in 100 μl of TBS for 2 hr. The plates were washed six times with 300 μl of TBS and then 100 μl per well of TMB substrate was added and the plate was incubated for 2 minutes. The reaction was stopped with 1 N HCl and measured by absorbance at 450 nm wavelength using a plate reader. The relative specific binding of biotin Aβ42 to Aβ in each well was calculated by subtracting to the absorbance of the control (without biotin-Aβ42).

2.2.4 Identification of Aβ-binding domain on RAP

To identify the Aβ-binding domain on RAP, the Aβ-binding assay was carried out in the presence of different recombinant RAP fragments (residues 1-100, 89-216, 1-206 and 206-323). RAP fragments were co-incubated with 50 nM biotin-Aβ42 in Aβ42-coated plates for 24 hr. The level of bound biotin-Aβ42 was determined by reaction with TMB and measured the absorbance at 450 nm wavelength.

2.2.5 Statistical analysis

All data are expressed as means ± SEM. Statistical significance was performed by a Student's t-test as well as by repeated-measured ANOVA test, and $p < 0.05$ was considered significant.

2.3 Results

2.3.1 Analysis of Aβ-oligomerization

To study Aβ aggregation, Aβ was immobilized on 96-well plates for 48 hr. The plates were blocked with 1% BSA in PBS for 2 hr and then biotin-Aβ42 was incubated at the concentration of 50 nM for 24 hr with gentle agitation at room temperature. The binding of soluble biotin-Aβ42 to the immobilized Aβ was measured by reading the absorbance at a wavelength of 450 nm. Non-specific binding of biotin-Aβ42 was determined as the binding to wells which were not coated with Aβ. Biotin-Aβ42 was found to bind to both substrate-bound Aβ40 and Aβ42. The binding to Aβ42 was greater than to Aβ40. Very little or no binding was observed to scrambled Aβ42, suggesting that binding of biotin Aβ42 to Aβ polymers was specific (Fig. 2.1).

Because it has been generally believed that Aβ42 is more neurotoxic and more readily able to aggregate than Aβ40 and as Aβ42 bound more strongly in the binding assay, Aβ42 was used in the assay for further experiments on Aβ aggregation. To study the time course of Aβ binding, biotin-Aβ42 was incubated on Aβ42-coated wells or control wells without Aβ42 for 0, 4, 24, 48 and 72 hr. The results showed that biotin-Aβ42 bound to substrate-bound Aβ42 in a time-dependent manner. Notably, biotin-Aβ42 was also bound significantly to wells without coating with Aβ42 (Fig. 2.2). It was also shown that biotin-Aβ42 had highest specific binding to substrate-bound Aβ42 after 24 hr of incubation (Fig. 2.2). Therefore, a 24 h incubation was used as the optimized time point for further studies.

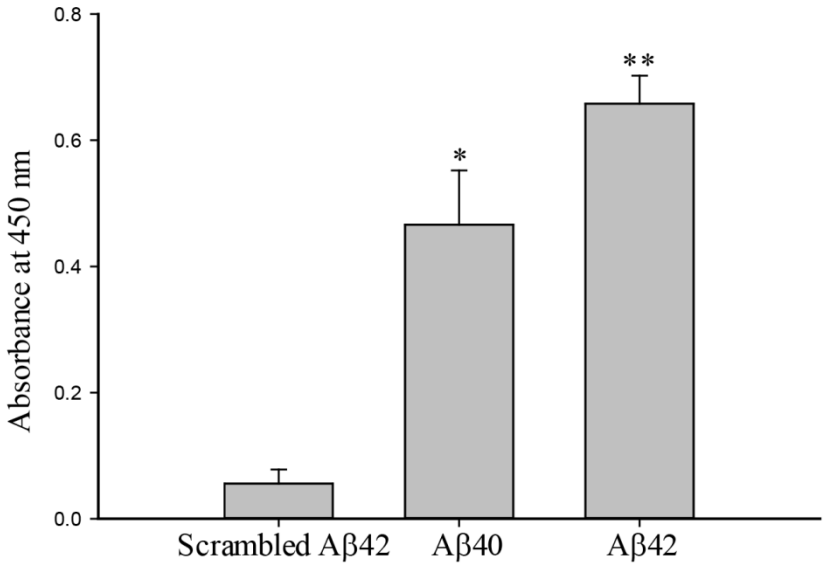

Figure 2.1. Binding of biotin-Aβ42 to Aβ-coated plate. Aβ (10μg per well) was seeded on 96-well plates and incubated with gentle agitation for 48 hr at room temperature. After washing 3 X 300μl TBS, plates were blocked with 1% BSA in 200 μl TBS for 2 hr. Plates were washed 3 X 300 μl TBS and incubated 50 nM biotin-Aβ42 in 100 μl TBS for 24 hr with gentle agitation. After washing 4 X 300 μl TBS, plates were incubated with streptavidin-HRP (1:3000) in 100 μl TBS for 2 hr. The level of bound biotin-Aβ was determined by reaction with TMB and measured the absorbance at 450 nm wavelength as described in methods. The specific binding of biotin Aβ42 to Aβ polymers in each well was calculated by subtracting to the control. Data are expressed as means ± SEM of n=4, * $p < 0002$, ** $p < 0.001$, Student's t-test.

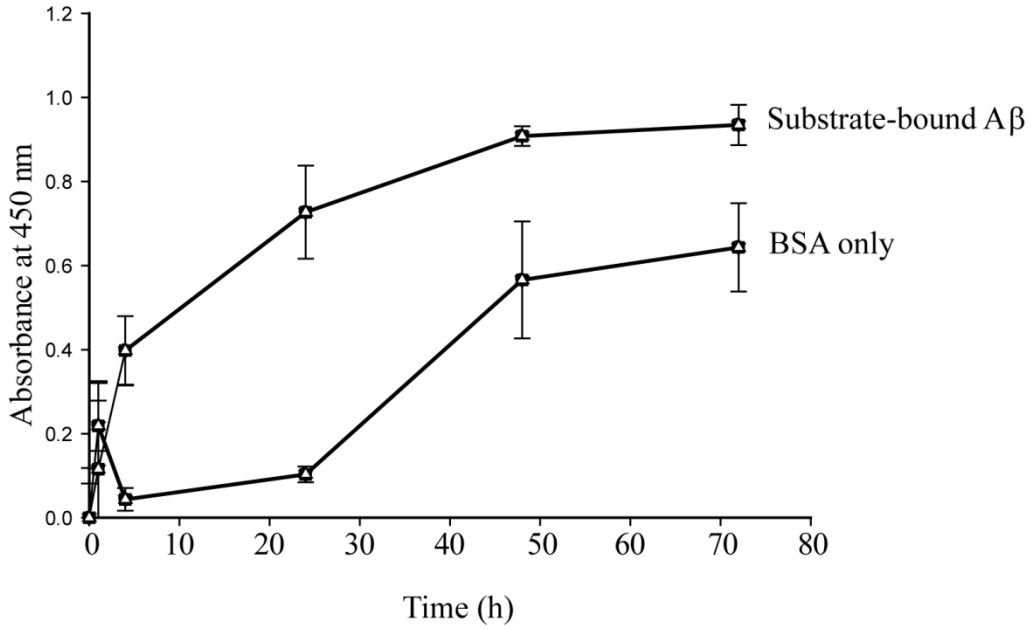

Figure 2.2. A time course study of the binding of Aβ42. A 96-well plates coated with or without Aβ42 were blocked with 200µl 1% BSA in PBS for 2h. Plate was then washed 3 X 300 µl TBS and incubated 50 nM biotin-Aβ42 in 100 µl TBS for 0, 4, 24, 48 and 72 h at room temperature with gentle agitation. The level of bound biotin-Aβ was determined by reaction with TMB and measured the absorbance at 450 nm wavelength as described in methods. The specific binding of biotin Aβ42 to Aβ in each well was calculated by subtracting to the control (without incubation with biotin Aβ42). Data are expressed as means ± SEM of n=3, $p < 0.03$, ANOVA.

2.3.2 Effect of salt concentration, metal ions and Aβ-binding proteins on Aβ-oligomerization.

To validate the assay, the effect of incubation conditions that are known to influence Aβ aggregation was studied. As salt concentration has been reported to influence Aβ aggregation (Tarus et al., 2008, Gregori et al., 2010), the effect of salt concentration on Aβ self-association was examined. The binding of biotin-Aβ42 to substrate-bound Aβ42 was measured at different NaCl concentrations (0, 10, 50, 100 and 150 nM). Increasing salt concentration increased the binding of biotin-Aβ42 to substrate-bound Aβ42 (Fig. 2.3), consistent with the view that salt concentration favours Aβ aggregation.

The presence of metal ions has also been shown to affect Aβ aggregation (Klement et al., 2007). Therefore, the effect of metal ions on the binding of biotin-Aβ to substrate-bound Aβ was studied. Consistent with previous studies, Zn^{2+} and Cu^{2+} (20 µM) greatly increased Aβ-oligomerization. Similarly, binding of biotin Aβ was significantly increased in the presence of Ca^{2+} and Mg^{2+} at physiologically relevant concentrations, 2 µM and 1 µM, respectively (Fig. 2.4). However, incubation with Ca^{2+} and Mg^{2} did not further increase binding of labelled Aβ to Aβ polymers in the presence of Zn^{2+} and Cu^{2+} (Fig. 2.4).

It has been reported that several proteins, such as ApoE3, ApoE4 (Sanan et al., 1994, Naslund et al., 1995, Bales et al., 2009) and anti-chymotrypsin (ACT) (Ma et al., 1996, Sun et al., 2002) can bind to Aβ and influence its aggregation. To validate the assay further and to confirm the previous reports that RAP can bind to Aβ and inhibit Aβ aggregation (Kanekiyo and Bu, 2009, Kerr et al., 2010), the effect of these Aβ-binding proteins on the binding of biotin-Aβ in the assay was investigated. The results showed that presence of 25 µM of Aβ-binding proteins significantly inhibited Aβ-self association (Fig. 2.5). ApoE3 and ACT

showed highest inhibition at 80%, while ApoE4 decreased Aβ aggregation by 50%. Consistent with previous studies (Kanekiyo and Bu, 2009, Kerr et al., 2010), the result showed that RAP strongly inhibited Aβ aggregation (Fig. 2.5).

2.3.3 Effect of recombinant RAP fragments on Aβ binding

To identify the Aβ-binding domain on RAP, biotin-Aβ42 was incubated on substated-bound Aβ42 in the presence of 25 μM of full-length or different recombinant RAP fragments. The level of bound biotin-Aβ42 was determined by reaction with TMB substrates and measured the absorbance at 450 nm wavelength. Consistent with previous findings, full-length RAP showed approximately 50% inhibition of Aβ aggregation. RAP fragments composed of 89-216 and 206-323 animo acid residues inhibited Aβ-self association by approximately 60% while other fragments (1-100 and 1-206) did not has any significant effect, suggesting that presence of residues 206-216 is necessary for the inhibitory effect (Figure 2.6).

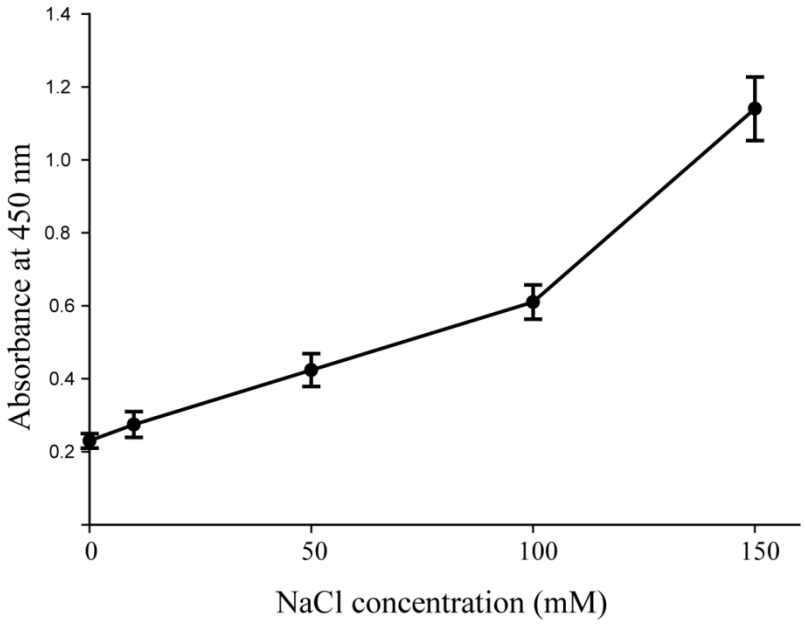

Figure 2.3. Effect of salt concentration on Aβ aggregation. Biotin-Aβ42 (50 nM) was incubated with different NaCl concentrations on an Aβ42-coated plate for 24 hr. The level of bound biotin-Aβ42 in each well was determined by reaction with TMB and measured the absorbance at 450 nm wavelength as described in methods. The specific binding of biotin Aβ42 to Aβ in each well was calculated by subtracting to the control (without incubation with biotin Aβ42). Data are expressed as means ± SEM of n=3, $p < 0.04$, ANOVA.

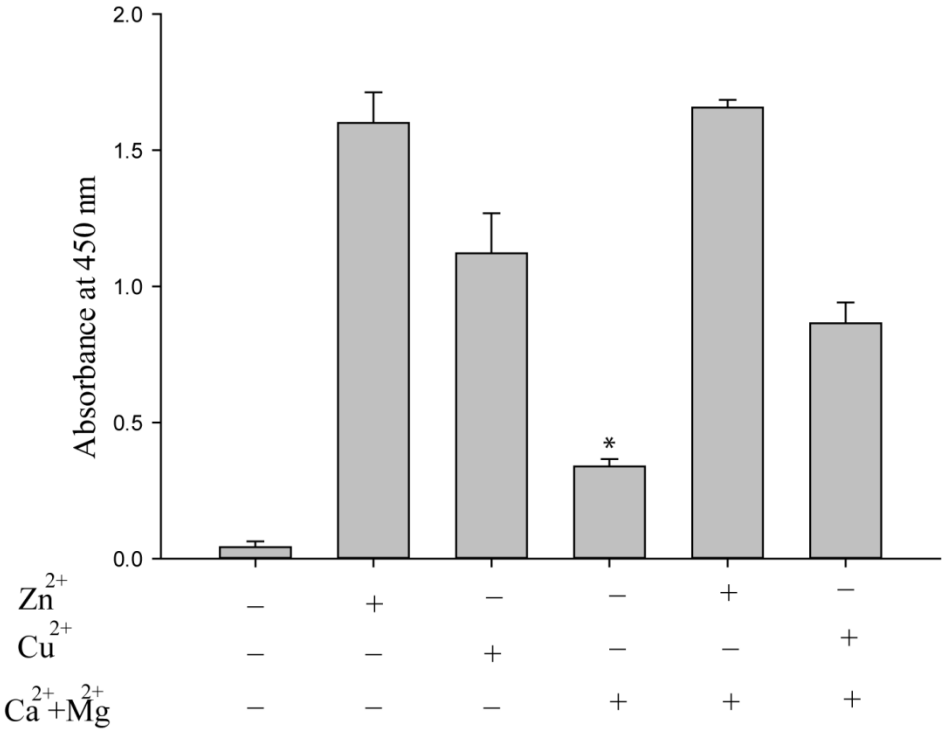

Figure 2.4. Effect of metal ions on Aβ aggregation. Biotin-Aβ42 (50nM) was incubated on an Aβ-coated plate in the presence of different metal ions (20μM $ZnCl_2$, 20 μM $CuCl_2$, 2 μM $CaCl_2$ and 2 μM $MgCl_2$) for 24 hr. Levels of bound biotin-Aβ42 in each well was determined by reaction with TMB and measured the absorbance at 450 nm wavelength as described in methods. The specific binding of biotin Aβ42 to Aβ in each well was calculated by subtracting to the control (without coating with Aβ). Data are expressed as means ± SEM of n=3, * p < 0001, Student's *t*-test.

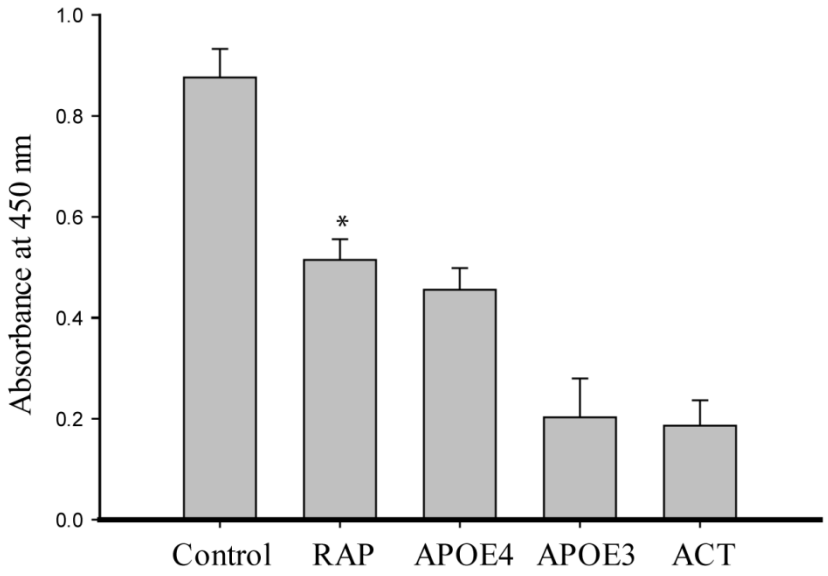

Figure 2.5. Inhibition of Aβ-self-association by putative Aβ-binding proteins. The Aβ-binding assay was carried out by incubating 50 nM biotin Aβ42 with 25μM of Aβ-binding proteins in 100μl TBS with gentle agitation for 24 hr. Level of bound biotin-Aβ42 in each well was measured by the absorbance at 450 nm wavelength and specific binding of biotin Aβ42 to Aβ in each well was calculated by subtracting to the control (without coating with Aβ42). Data are expressed as means ± SEM of n=3,* $p < 0.001$, Student's *t*-test). Abbreviations: ApoE3 & 4: apolipoprotein E 3 & 4; ACT: anti-chymotrypsin; RAP: receptor-associated protein.

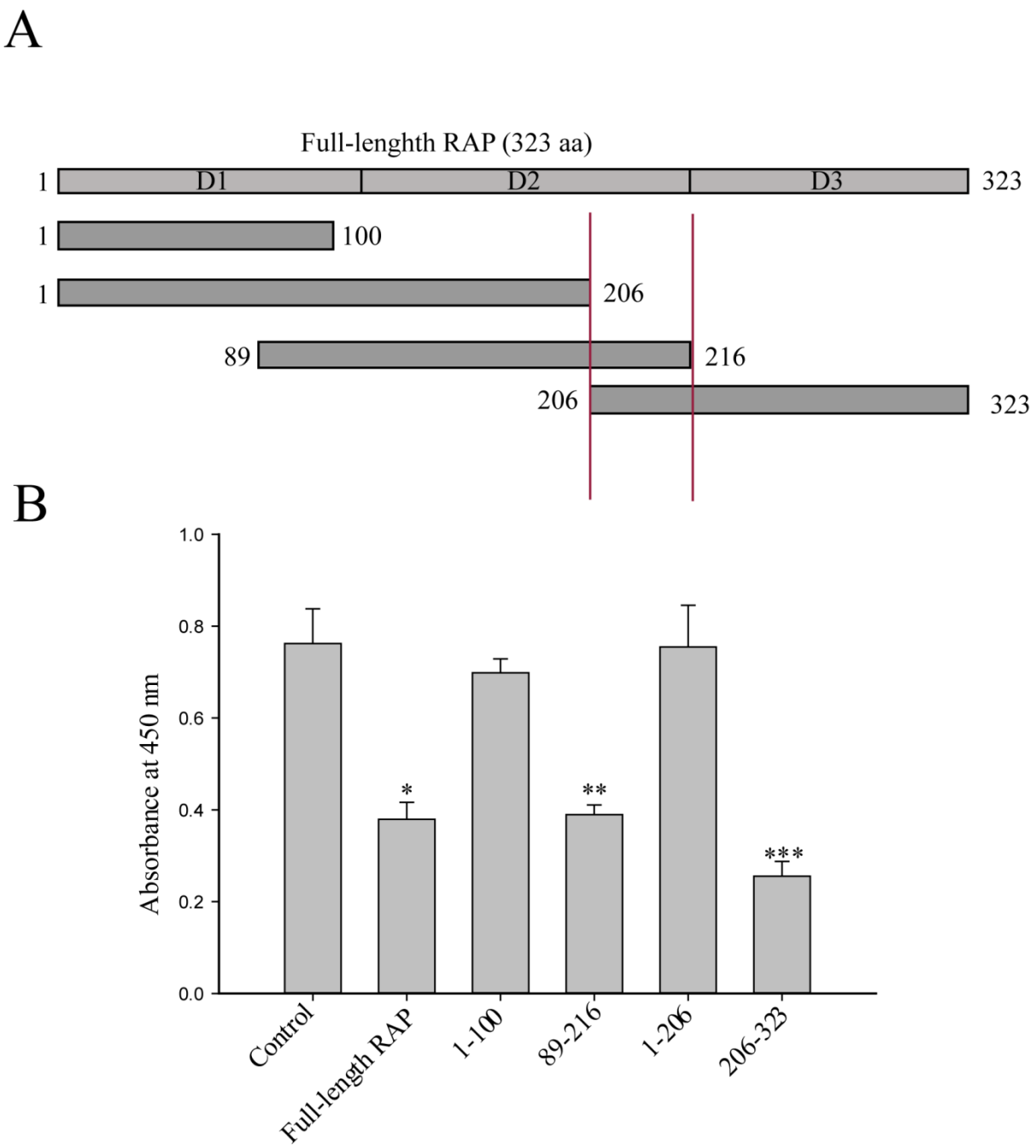

Figure 2.6. Effect of RAP fragments on Aβ self-association. (A) Representation of the different RAP fragments. (B) Biotin-Aβ42 (50 nM) was incubated with 25 μM RAP fragments in Aβ-binding assay. RAP 89-216 and RAP 206-323 inhibited Aβ-self association whereas RAP 1-100 and RAP 1-206 did not. Data are expressed as means ± SEM of n=4, * p < 0002, ** p < 0.001, *** p < 0.001, Student's *t*-test. Abbreviations: D1: domain 1; D2: domain 2, D3: domain 3.

2.4 Discussion

This study has established and validated an *in vitro* assay which allows for both quantitative and qualitative studies on Aβ aggregation. The *in vitro* assay can be potentially used as a relatively high throughput screening method for compounds that can inhibit Aβ aggregation. By testing the inhibitory effects of different RAP fragments on Aβ-self association, a putative Aβ-binding site was identified between residues 206-RVSHQGYSTE-216 in loop region between D2 and D3 of RAP. Complementary studies using co-immunoprecipitation and surface plasma resonance may help to confirm this finding.

Aβ monomers aggregate to form the fibrillar structures which are then deposited as amyloid plaques. It has been proposed that the Aβ aggregation process contains 2 steps: nucleation and elongation (Murphy, 2002, Pellarin and Caflisch, 2006). Aβ monomers are believed to self-associate into a nucleus through a rate-limiting nucleation step, which then proceeds into a soluble intermediate possibly a protofibril. Such a model is in agreement with the findings that there is a lag phase during Aβ fibrillization that can be eliminated by addition of preformed Aβ seeds (Jarrett and Lansbury, 1993, Lomakin et al., 1996). Protofibrils can progress to form fibrils either via assembly of monomeric Aβ or association of protofibrils themselves (Nichols et al., 2002). Since Aβ aggregates are the main component of amyloid plaques and generally believed to be the primary cause of AD, a potential treatment for AD could be based around the use of pharmacological agents that can bind to Aβ and inhibit Aβ aggregation. Although numerous compounds can inhibit Aβ fibrillization in-vitro and in-vivo (Frisardi et al., 2010, Bieschke et al., 2011), none of them has been approved for treatment. This may be partly due to lack of a reliable assay screening for inhibitory agents for Aβ

aggregation. None of the current methodologies can measure all forms of Aβ aggregates. For examples, turbidity assays can only measure large aggregates but do not detect intermediate molecular weight forms (Atwood et al., 1998). SDS-PAGE electrophoresis may be used to detect SDS-stable low molecular weight aggregates but cannot be used to detect oligomeric species that are not SDS-stable or high molecular weight aggregates that cannot penetrate into the polyacrylamide gels (Klug et al., 2003). Other methods such as atomic force microscopy (AFM) and thioflavin T fluorescence also have their limitations. For example, depending on the physical probe used, AFM images may not represent the true sample topography due to tip convolution effects (Dufrene, 2002). In addition, soluble Aβ aggregates that do not bind to AFM surfaces may not be observed by this technique. Moreover, AFM cannot be used to monitor Aβ aggregation in real-time. On the other hand, although thioflavin T fluorescence may be used to measure the time course of Aβ aggregation, it cannot detect Aβ species that do not bind to Thioflavin T (LeVine, 1999, Cloe et al., 2011). The simple assay developed in this study differs from other methods in that it can determine the binding of additional Aβ molecules to those already aggregated. Therefore it can potentially monitor many different types of Aβ aggregates.

Under similar conditions to the present experiments, it was demonstrated that Aβ can form fibrils on plates by using Congo red staining (Bohrmann et al., 1999). The concentration of biotin Aβ42 used in this study is relatively low and more relevant to physiological Aβ concentration (3 to 5 nM). The results of the studies reported in this chapter show that biotin-Aβ42 can bind to substrate-bound Aβ40 coated on wells of the 96-well plates (Fig.2.1). Interaction between Aβ40 and 42 is supported by a large body of literature. Aβ42 is known to more readily aggregate and be more neurotoxic than Aβ40 (Caughey and Lansbury, 2003). It

was shown that Aβ42 is deposited early in the senile plaques; and immunostaining shows that Aβ42 is often localized to the core of amyloid plaques (Younkin, 1998, Saito et al., 2011). Recent studies suggested that Aβ42 may act as a seed for the formation of amyloid plaques in vivo (Fryer and Holtzman, 2005, Gaspar et al., 2010).

The result indicates that biotin-Aβ binds weakly to BSA as significant binding occurred at longer time of incubation (Fig. 2.2). The fact that Aβ binds to albumin is not surprising. Previous studies have showed that almost 90% of Aβ in the plasma may be bound to albumin and that binding of albumin to Aβ can inhibit Aβ fibril formation (Biere et al., 1996, Bohrmann et al., 1999, Reyes Barcelo et al., 2009). At the physiological concentration of 40 mg/ml, albumin is capable of binding to 90% of the Aβ42 and Aβ40 and the binding has been demonstrated in a 1:1 ratio (Kuo et al., 2000). Using circular dichroism spectroscopy, it was demonstrated that the dissociation constant of HSA: Aβ complex (K_d) is 5 ± 1μM (Rozga et al., 2007). Due to its high affinity binding for copper, albumin can reduce the aberrant Aβ:Cu interaction (Perrone et al., 2010). However, it has been recently demonstrated that albumin binds to Aβ oligomers and competes for the addition of Aβ monomer, inhibiting fibril growth (Milojevic et al., 2009). Aβ-binding sites are located on three different domains of albumin and a single binding site per one Aβ oligomer prevents the formation of insoluble aggregates (Milojevic and Melacini, 2011). The abundant presence of albumin in the plasma may explain the reason why Aβ deposition rarely occurs in the periphery (Fukuchi et al., 1996). In this study, 24 hr incubation was chosen for further experiments because Aβ was found to bind to BSA in the wells at minimal level after this time (Fig. 2.2). The non-specific binding of biotin-Aβ42 was determined as the binding of biotin Aβ42 to wells with BSA, but without

Aβ42. The results from this study suggest that one should be cautious to use BSA as a blocking reagent to study Aβ aggregation.

It has been reported that increasing the ionic strength can increase Aβ aggregation. Using a turbidity assay, it was found that Aβ aggregation was strongly dependent upon NaCl concentration (Snyder et al., 1994). Ionic strength favours hydrophobic interactions and increases the rate the conversion of α-helix to β-sheet structures (Lin et al., 2008). High concentrations of salt are also suggested to stiffen the Aβ fibrillar structure (Zidar and Merzel, 2011). In this study, increasing the NaCl concentration from 0 to 150 mM increased Aβ-self association which is consistent with the idea that hydrophobic interactions are important for Aβ aggregation (Fig. 2.3).

There is also clear evidence that metal ions can influence Aβ aggregation. Metal ions such as Cu^{2+}, Zn^{2+}, Al^{3+} and Fe^{3+} have been shown to promote Aβ aggregation (Mantyh et al., 1993, Bush et al., 1994, Atwood et al., 1998, Exley, 2006, Yumoto et al., 2009, Miller et al., 2010, Bolognin et al., 2011, Liu et al., 2011). It has been reported that Cu^{2+} increases Aβ toxicity by inducing radical oxidative damage (Huang et al., 1999, White et al., 1999, Exley, 2006) . However, Zn^{2+} is suggested to play a protective role against Aβ neurotoxicity (Lovell et al., 1999, Cardoso et al., 2005, Garai et al., 2007). High micromolar concentrations of these metals have been found in the core and surrounding regions of the amyloid plaque. In this study, it was shown that $Cu^{2+}$and Zn^{2+} markedly promoted Aβ-self association, which further helped to validate the assay (Fig. 2.4).

There is some good evidence showing that Ca^{2+} can increase Aβ aggregation. Isaacs et al. (2006) first demonstrated that Ca^{2+} can increase the formation of early protofibrils and accelerate the conversion of protofibrils into Aβ fibrils. This effect can occur at 20-fold lower concentrations than the physiological concentration of extracellular Ca^{2+} (2 mM) (Isaacs et al., 2006). In another study, it was shown that physiological concentrations of Ca^{2+} increase the formation of Aβ oligomeric species (Itkin et al., 2011). Mg^{2+} ions at concentration of 1 mM were also shown to increase Aβ sedimentation (Klug et al., 2003). Consistent with those studies, the results show that Ca^{2+} and Mg^{2+}markedly increased Aβ-self association (Fig.2.4).

Several endogenous proteins have been found to bind to Aβ and influence its aggregation. Studies have shown that apolipoprotein E (ApoE), a major risk factor for late onset AD, can form complexes with Aβ and promote Aβ fibrilization. The Aβ-binding region is located on the C-terminal of ApoE (Strittmatter et al., 1993b, Tamamizu-Kato et al., 2008). Other studies have shown that ApoE inhibits Aβ aggregation by preventing the formation of a nucleus (Wood et al., 1996, Beffert and Poirier, 1998, Holtzman et al., 1999). Similarly, antichymotrypsin (ACT) can bind to Aβ, leading to inhibition or promotion of Aβ aggregation depending on the relative concentrations of Aβ and ACT (Fraser et al., 1993, Eriksson et al., 1995, Sun et al., 2002). An in-vivo study has found that increased ACT expression promotes amyloid deposition (Nilsson et al., 2001). In this study, all Aβ-binding proteins (RAP, ApoE4, ApoE3 and ACT) at 25 μM concentration inhibited the binding of biotin-Aβ to substrate-bound Aβ (Fig.2.5). Taken together, this in vitro assay may be valuable to screen potential molecules that can bind to and inhibit Aβ aggregation. However, further studies are clearly needed to determine if these molecules can also inhibit Aβ aggregation in AD brain.

Studies showed that RAP can form a 46 kDa complex with Aβ in vitro (Kanekiyo and Bu, 2009, Kerr et al., 2010). Therefore, it is likely that RAP interacts with Aβ in 1:1 ratio. However this does not rule out the possibility that RAP may bind to more than one Aβ molecule. Although our study did not show how Aβ binds to RAP, it is reasonable to assume that the binding may involve hydrophobic interactions. The binding of Aβ to the loop region between D2 and D3 may potentially affect the flexibility of RAP structure. RAP has been known to bind to several other proteins such as HSPG, LDL receptor family and sortilin receptors through the D3 domain (Warshawsky et al., 1993b, Orlando and Farquhar, 1994, Tauris et al., 1998). Three amino acid clusters in the D3 (R^{282}VSRSREK^{289}, R^{203}LRR^{206} and R^{314}ISRAR^{319}) were shown to be involved in binding to HSPG and LRP1 (Melman et al., 2001). By binding to Aβ though distinct binding site, RAP facilitates the cellular uptake of Aβ, possibly through a HSPG-mediated mechanism (Kanekiyo and Bu, 2009). Complementary experiments such as co-immunoprecipitation or surface plasmon resonance (SRP) may help to confirm the Aβ-binding site on RAP. Based on the Aβ-binding region found here, it is possible to generate different RAP mutants to further characterise the significant of amino acid residues that are involved in the binding.

Chapter 3: Effects of RAP on APP processing and Aβ production in cell culture

3.1 Introduction

The 39-kDa receptor-associated protein (RAP) was first identified by co-purification with the LDL receptor (Strickland et al., 1991). RAP is an ER-resident protein that can be found in all human tissues examined so far with highest expression in brain and kidney (Lorent et al., 1994). RAP is known to bind to all LDL receptor family members and to antagonize the binding of other ligands. Studies have shown that RAP acts as a chaperone for the correct folding and trafficking of the LDL receptor family (Strickland et al., 1990, Bu and Rennke, 1996, Bu et al., 1997). Structurally, RAP contains 323 amino acid residues and consists of three independent domains. Each domain contains three α-helical structures and is connected to the other domains by flexible linkers, which allow RAP to adapt different conformations (Medved et al., 1999, Lee et al., 2007a).

The LDL receptor family comprises a number of the cell-surface receptors that are involved in many physiological functions including lipoprotein and cholesterol metabolism, cell development and differentiation, and clearance of ligand proteins (Strickland et al., 1995, Herz and Bock, 2002). Accumulating evidence suggests that the LDL receptors are involved in the pathogenesis of AD (Marzolo and Bu, 2009). Among the LDL receptor family, LRP1 is proposed to play an important role in the metabolism of APP and Aβ (Andersen and Willnow, 2006, Bu et al., 2006). RAP has been found to bind to LRP1 at multiple sites

involving all three of its domains. Domain 3 reportedly has the strongest binding-affinity for LRP1 (Lee et al., 2006).

Several studies have implicated RAP in AD pathology. Polymorphism of RAP has been linked to the risk of AD (Sanchez et al., 2001). Other studies have shown that human APP transgenic mice, in which RAP has been knocked out, have increased Aβ deposition and neurodegeneration (Van Uden et al., 2002, Xu et al., 2008). However, it is unclear from these studies whether the increased Aβ deposition is due to an increase in Aβ production or a decrease in Aβ clearance, or both. Furthermore, a recent study shows that RAP is decreased in the AD brain (Provias and Jeynes, 2010).

To test the hypothesis that increased Aβ amyloid in RAP-deficient mice is due to an increase in Aβ production, the effect of RAP on APP processing and Aβ production was investigated. Two experimental strategies were employed. The first one involved over-expression of RAP in sweAPP695-Chinese hamster ovary (CHO) cells and sweAPP695-SH-SY5Y neuroblastoma cells. In these studies, the effect on APP processing and Aβ production were analysed by western blotting. As a second strategy, the effect of exogenously added RAP on APP metabolism was also examined in cultures of primary cortical neurons derived from human sweAPP695-expressing Tg2576 transgenic mice. The results suggest that RAP may decrease Aβ production by altering APP proteolytic processing pathways and that decreased level of RAP in the brain could contribute to the pathogenesis of AD.

3.2 Materials and methods

3.2.1 Materials

A human RAP cDNA inserted into the expression vector pcDNA3.1 plasmid at the *BamHI* and *EcoRII* restriction sites was kindly provided by Dr. Dudley K. Strickland (University of Maryland, USA). Anti-RAP mouse monoclonal 7F1 antibody was purchased from Santa Cruz Biotechnology (Melbourne, Australia). A polyclonal rabbit APP C-terminal antibody, a monoclonal mouse 6E10 antibody which recognizes 3-8 amino acid residues on the Aβ sequence, mouse immunoglobulin (mouse IgG), rabbit immunoglobulin (rabbit IgG) and monoclonal mouse α-actin antibody were purchased from Sigma-Aldrich (Sydney, Australia). Effectene transfection reagent was purchased from Qiagen (Melbourne, Australia). Polyclonal goat anti-rabbit and goat anti-mouse antibodies conjugated with horseradish peroxidase (HRP) were purchased from Dako (Melbourne, Australia). Dulbecco's modified Eagle's medium (DMEM) and Neurobasal medium were purchased from Sigma (Sydney, Australia).

3.2.2 Cell culture

Chinese ovary hamster (CHO) cells stably expressing Swedish APP695 mutant (sweAPP695) (K670N/M671L) in the expression vector pIRES*puro2* were obtained from Molecular Science and Biotechnology Institute, University of Melbourne. Cells were grown in RPMI medium containing 10% (v/v) fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin in an atmosphere containing 5% CO_2 at 37^{o}C. SH-SY5Y neuroblastoma cells stably expressing

sweAPP695 (K670N/M671L) in the expression vector pIRES*hyg* were kindly provided by Professor Nigel Hooper (Univeristy of Leeds, UK). These cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 100 U/ml penicillin/streptomycin at 37^{o}C. For maintaining stably transfected sweAPP695 cells, 7.5 μg/ml puromycin was used for sweAPP695-CHO cells, and 100 μg/ml hygromycin was used for sweAPP695-SH-SY5Y cells. For splitting, the culture medium was removed and the cells were washed with pre-warmed PBS. Trypsin solution was added and the cells were incubated in 37^{0}C for 5 min. Cells were resuspended in growth medium and plated on new culture flasks.

Human APP transgenic mice Tg2576 expressing the sweAPP695 (K670N/M671L) under the control of the prion promoter (Hsiao et al., 1996) were purchased from Taconic, USA . Primary cortical neurons (PCNs) were prepared from cerebral cortices of Tg2576 newborn as follows. Briefly, cerebral cortices were dissected and incubated in HBSS buffer containing 0.06% (w/v) DNase I, 0.25% (w/v) papain and 1.8 g/L glucose in 37^{o}C for 15 min. The cortices were washed twice with Neurobasal medium (Invitrogen, Mount Waverley, Australia). The cortical cells were dissociated and grown in 12-well plates (Falcon, NSW, Australia) in complete Neurobasal medium containing 2 % (v/v) B27 supplement, 1 mM glutamine and 100 U/ml penicillin/streptomycin. After 3 days in vitro (DIV), half of cell culture medium was replaced with fresh medium.

3.2.3 Preparation and analysis of RAP pcDNA3.1

To propagate plasmids, RAP pcDNA3.1 (350ng) was mixed with 50 µl of Electrocompetent E. coli TOP1 (in 10% glycerol) in a pre-chilled cuvette. Transformation was carried out by electroporation. Cells then were plated onto a plate containing 1.5% agar and grown overnight at 37^{o}C. A single colony was picked and transferred into 50 ml Terific broth and grown overnight with shaking at 37^{o}C. Cells were mixed with glycerol (1:1) and stored at -80^{0}C as a stock.

RAP-pcDNA3.1 plasmid was extracted from 50 ml TB broth E.coli cell culture using an Ultraclean Endotoxin-free Midi Plasmid Prepkit (Qiagen, Melbourne, Australia). Plasmid DNA was then precipitated with ethanol and resuspended in 60 ul TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The plasmid DNA concentration was measured by using Nanodrop spectrometer (Thermo Scientific, Wilmington, DE, USA).

To check the plasmid integrity, RAP pcDNA3.1 (800ng) was digested with EcoRI (1 unit) or BamHI (1 unit) in total volume of 10 µl at 37^{o}C for 5 min. The reaction mixtures were loaded with 2 µl of 6 x gel-loading buffer (see Appendix) into a 1% (w/v) agarose gel containing SYBR safe (1:20,000). The gel was run in TAE solution (40 mM Tris-acetate, 1 mM EDTA) at 90 mV for 40 min.

To confirm the plasmid integrity further, RAP pcDNA3.1 plasmid was sequenced. Two primers which are complementary to pcDNA3.2 plasmid sequence were used: T7 promoter

primer 5'-TAA TAC GAC TCA CTA TAG GG-3' and BGH reverse primer 5'-AAG GCA CAG TCG AGG-3'. DNA sequencing was performed using Beckman CEQ 8000 DNA sequencer.

3.2.4 Cell transfections and treatments

SweAPP695-CHO cells were grown to 80% confluence in a 12-well plate and transfected with RAP pcDNA3.1 (0.3 μg) or empty vector by following the Effectene kit protocol (Qiagen, Melbourne, Australia). Twenty-four hours after transfection, transfected cells were selected with 900 μg/ml G418 antibiotic (Sigma, Sydney, Australia). Stably transfected colonies were formed after 10 days and maintained in 900 μg/ml G418.

SweAPP695-SH-SY5Y neuroblastoma cells were grown to 80% confluence in a 12-well plate and transfected with RAP pcDNA3.1 (0.3 μg) or an empty pcDNA3.1 vector control by following the Effectene kit protocol. Cells were harvested and cultured medium was collected 24 hr after transfection.

Seven DIV primary cortical neurons from newborn Tg2576 mice were treated with 500nM RAP in complete Neurobasal medium for 72 hr. Cells were harvested and proteins were analysed by western blotting.

3.2.5 Cell lysis and determination of protein concentration

Cells in 12 well-plates were disrupted with 140 µl RIPA lysis buffer (see Appendix) with protease inhibitor (1 tablet per 10 ml RIPA buffer) (Roche, Germany) on ice for 30 min. Samples were centrifuged at 4^{o}C at 13,000 x g for 10 min and the supernatant fractions were collected. Protein concentration was quantified using a BCA protein assay kit (Thermo Scientific, Melbourne, Australia) using BSA as the standard.

3.2.6 SDS polyacrylamide gel electrophoresis and western blotting

Cell lysates containing 10 µg of proteins were combined with an equal volume of 2 x SDS sample buffer (see Appendix) and heated at 95^{o}C in a heating block for 5 min. The samples were loaded on to 8% Tris-glycine SDS-PAGE gels (see Appendix) and separated electrophoretically at 40 mA for 1.5 h. Proteins were transferred electrophoretically onto 0.22 µm nitrocellulose membranes at 300 mA for 1.5 hr at 4^{o}C. The membranes were blocked in 5% (w/v) skim milk powder in PBS containing 0.25% (v/v) Tween-20 (PBST) for 1 hr. Membranes were then incubated with primary (6E10, 1:2000) and secondary (anti-mouse HRP, 1:5000) antibodies in 5% skim milk powder in PBST overnight at 4^{o}C and for 1 hr at room temperature, respectively. The membranes were then washed extensively in PBST. For signal detection, blots were then incubated with Chemiluminescent HRP substrate (Millipore corporation, MA, USA). Chemiluminescence was captured using a Chemi-smart 5000 (Chemismart, Marne-la-Vallée, France) and quantified by using ImageJ version 1.41 software (RSB, NIH, USA).

For C99 and C83 detection, proteins were separated on 16% Tris-tricine SDS-PAGE gels (see Appendix) and incubated with 6E10 (1:1000) or APP C-terminal antibody (1:2000) overnight. Blots were stripped and stained with α-actin antibody (1:10,000) as a loading control. To determine the levels of sAPPα and sAPPβ, cell culture media (20μl) were analysed on 8% Tris-glycine SDS-PAGE gels and subjected to western blotting using a polyclonal antibody directed against sAPPβ (1:1000) and the 6E10 antibody (1:2000) for APPα.

3.2.7 Immunoprecipitation and detection of Aβ in cell culture medium

Conditioned media (800 μl) from cell cultures grown in 12-well plates were incubated with 6E10 antibody (1:1000) overnight at 4^0C with rotation and immunoprecipitated with Protein G-agarose (Roche, Australia). Samples were centrifuged and washed twice with 1 ml PBS then resuspended in 20 μl 2x sample buffer. Proteins were analysed on Tris-bicine-urea SDS-PAGE gels (see Appendix) and subjected to western blotting using the 6E10 antibody (1:1000) for Aβ detection.

3.2.8 Co-immunoprecipitation to detect RAP and APP complex

SweAPP695-CHO cells stably transfected with RAP pcDNA3.1 or empty vector were grown in T25 cell culture flasks until 90 % confluent. Cells were harvested in 1 ml RIPA buffer containing protease inhibitor. To detect RAP-APP complexes by co-immunoprecipitation, cell lysates containing 400 μg proteins were mixed with the mouse monoclonal 7F1 antibody (4 μg) or the mouse IgG (4 μg) and incubated with gentle inversion at 4^oC overnight.

Mixtures of the cell lystates and the antibodies were combined with 60 µl protein G agarose beads (Roche, Germany) and incubated with gentle inversion for 3 hr at 4^{o}C. The beads were centrifuged and washed three times with 1 ml of PBS. Pellets were combined with 20 µl SDS sample buffer, boiled for 5 min and separated electrophretically on 10 % Tris-glycine SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes and the membranes were stained with the mouse monoclonal 6E10 antibody or the rabbit polyclonal APP- C terminal antibody to detect APP. The membranes were then stripped by using stripping buffer (Thermo scientific, Sydney, Australia) and reprobed with the 7F1 antibody. In parallel experiments, cell lysates containing 400 µg proteins were incubated with the mouse monoclonal 6E10 antibody (5 µg) or the rabbit polyclonal APP- C terminal antibody (5 µg) to pull down APP. Mouse IgG and rabbit IgG was used as negative controls. Proteins were separated electrophoretically on 10 % Tris-glycine SDS-PAGE gels and subjected to western blotting using the mouse monoclonal 7F1 antibody (1:1000). The membranes were then stripped by using stripping buffer and reprobed with the mouse monoclonal 6E10 antibody (1:2000) or the rabbit polyclonal APP- C terminal antibody (1:2000).

3.2.9 Cell-surface protein labelling

SweAPP695-CHO cells stably transfected with RAP pcDNA3.1 or empty vector control were grown on a 12-well plate until 80% confluent. Cells were washed three times with 1 ml of ice-cold PBS. Cell-surface proteins were labelled with 6 mg/ml NHS-SS-biotin (Sigma, Australia) in PBS pH 8.0 at 4^{0}C. After 30 minutes, excessive NHS-SS-biotin was quenched by using 50 mM Tris pH 8.0 and cells were washed three times with 1 ml of PBS and lysed in RIPA lysis buffer containing protease inhibitor. Biotinylated proteins were

immunoprecipitated with streptavidin agarose and washed three times with 1 ml PBS. Pellets were mixed with 2 x SDS sample buffer, separated on 8% Tris-glycine SDS-PAGE gels and subjected to western blotting using 6E10 antibody (1:2000) to detect cell-surface APP.

3.2.10 Statistical analysis

All data are expressed as means of ± SEM. Statistical significance was determined by Student's t-test, and $p < 0.05$ was considered significant.

3.3 Results

3.3.1 Effect of RAP overproduction on APP processing and Aβ production in sweAPP695-CHO cells

To examine the effect of RAP over-expression on APP processing, sweAPP695-CHO cells were stably transfected with RAP pcDNA3.1 or empty vector. RAP-APP-CHO cells and APP-CHO cells were grown for 48 hr to 80% confluence in 12-well plates. Cells were harvested and the culture media were collected. The level of APP and APP proteolytic cleavage products (sAPPα, C83, sAPPβ, C99 and Aβ) was analysed by SDS-PAGE and western blotting.

To determine the level of APP, proteins in the cell lysates were separated on 8% Tris-glycine SDS-PAGE gel and subjected to western blotting using the 6E10 antibody for detection. The result showed that over-expression of RAP did not significantly affect the level of total cellular APP (Fig. 3.1). For sAPPα and sAPPβ detection, the culture media were analysed by

western blotting using the 6E10 and the sAPPβ antibody, respectively. The results showed that while the level of sAPPα was not significantly affected, the level of sAPPβ was significantly decreased by overproduction of RAP (Fig. 3.2A,B). In addition, RAP transfection led to a significant amount of RAP secretion into the media (Fig. 3.2A). Levels of APP-C terminal fragments in cell lysates were analysed by western blotting using the APP-C terminal antibody, which can detect both C99 and C83. To confirm the identity of bands as C99 fragments, western blot membranes were stripped and reprobed with the 6E10 antibody, which can only detect C99 but not C83 fragments. Data showed that RAP over-expression resulted in an increase in C83 level but decreased the level of C99 (Fig. 3.2C).

For Aβ detection, proteins in the conditioned media were immunoprecipitated and analysed by western blotting using 6E10 antibody. Levels of both Aβ40 and Aβ42 was found to be significantly decreased in the RAP transfected cells compared with the control (Fig. 3.3). Overall, RAP over-expression significantly increased the level of C83 in the cell lysates. Meanwhile, the level of sAPPβ, C99 and Aβ was significantly decreased, suggesting that over-expression of RAP resulted in a decrease in the β-secretase processing of APP.

A previous study by Ulery et al. (2000) demonstrated that treatment of APP770-overexpressing CHO cells with RAP resulted in an increase in the level of cell-surface APP. To check whether RAP over-expression has any effect on the level of APP on the cell membrane of sweAPP695-CHO cells, cell-surface proteins were biotinylated. The biotinylated proteins were then precipitated using streptavidin-sepharose and subjected to western blotting using the 6E10 antibody. The results showed that RAP over-expression significantly increased the level of cell-surface APP (Fig. 3.4), suggesting that the over-

expression of RAP may lead to reduce the internalization of cell-surface APP or/and increase recycling of APP to the cell surface.

RAP has been known to function as a chaperone for the maturation and correct trafficking of the LRP1 receptor (Bu and Marzolo, 2000, Herz, 2006). Studies have suggested that LRP1 could interact with APP and induce APP endocytosis from the cell membrane, leading to an increase in Aβ production (Ulery et al., 2000, Kinoshita et al., 2001, Bu et al., 2006) . To test whether the over-expression of RAP influenced the level of LRP1, cell lysates were analysed for LRP1 level by western blotting. The LRP1-C terminal antibody that can recognize the 85 kDa LRP1-C terminal fragment was used. Data showed that RAP over-expression had no significant effect on the level of LRP1 (Fig. 3.5).

The finding that over-expression of RAP led to a decrease in the β-secretase processing pathway of APP, raises the possibility that RAP could interact directly with APP and exert the effects. To test this possibility, co-immunoprecipitation was performed to detect the presence of RAP-APP complex in sweAPP695-CHO cells overexpressing RAP as described previously in materials and methods. However, the results failed to detect RAP-APP complex in the cell lystates (Fig. 3.6).

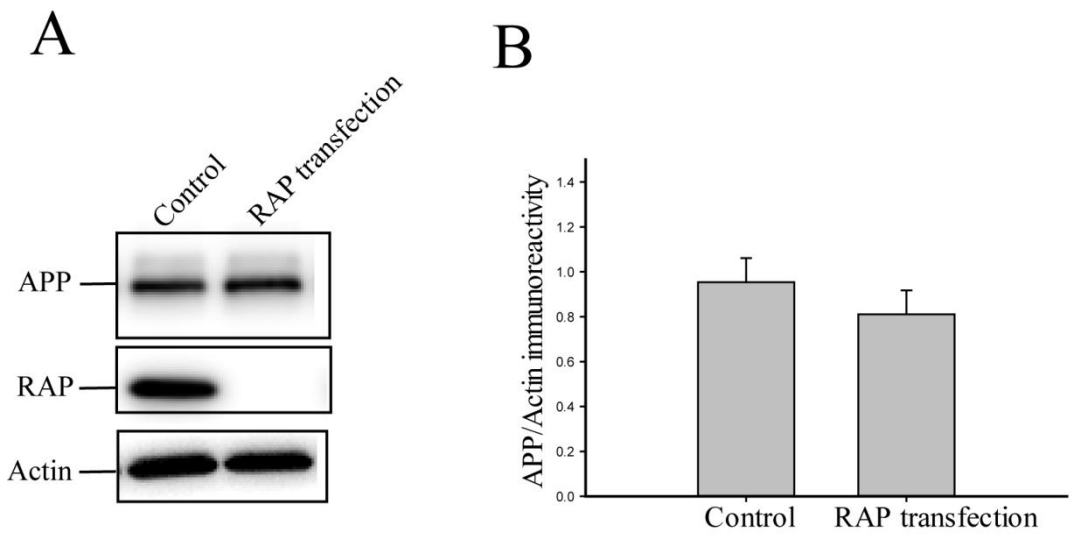

Figure 3.1. Effect of the over-expression of RAP on the total level of APP in sweAPP695-CHO cells. Cells were stably transfected with RAP or empty vector control. (**A**) Western blot analysis of total APP using the 6E10 antibody (upper panel) and RAP using the 7F1 antibody (middle panel). α-Actin immunoreactivity was shown as a loading control (lower panel). (**B**) Quantification of the total level of APP /α-actin immunoreactivity. Over-expression of RAP had no significant effect on the total level of APP in the APP695-CHO cells. Data are expressed as means ± SEM of n=3.

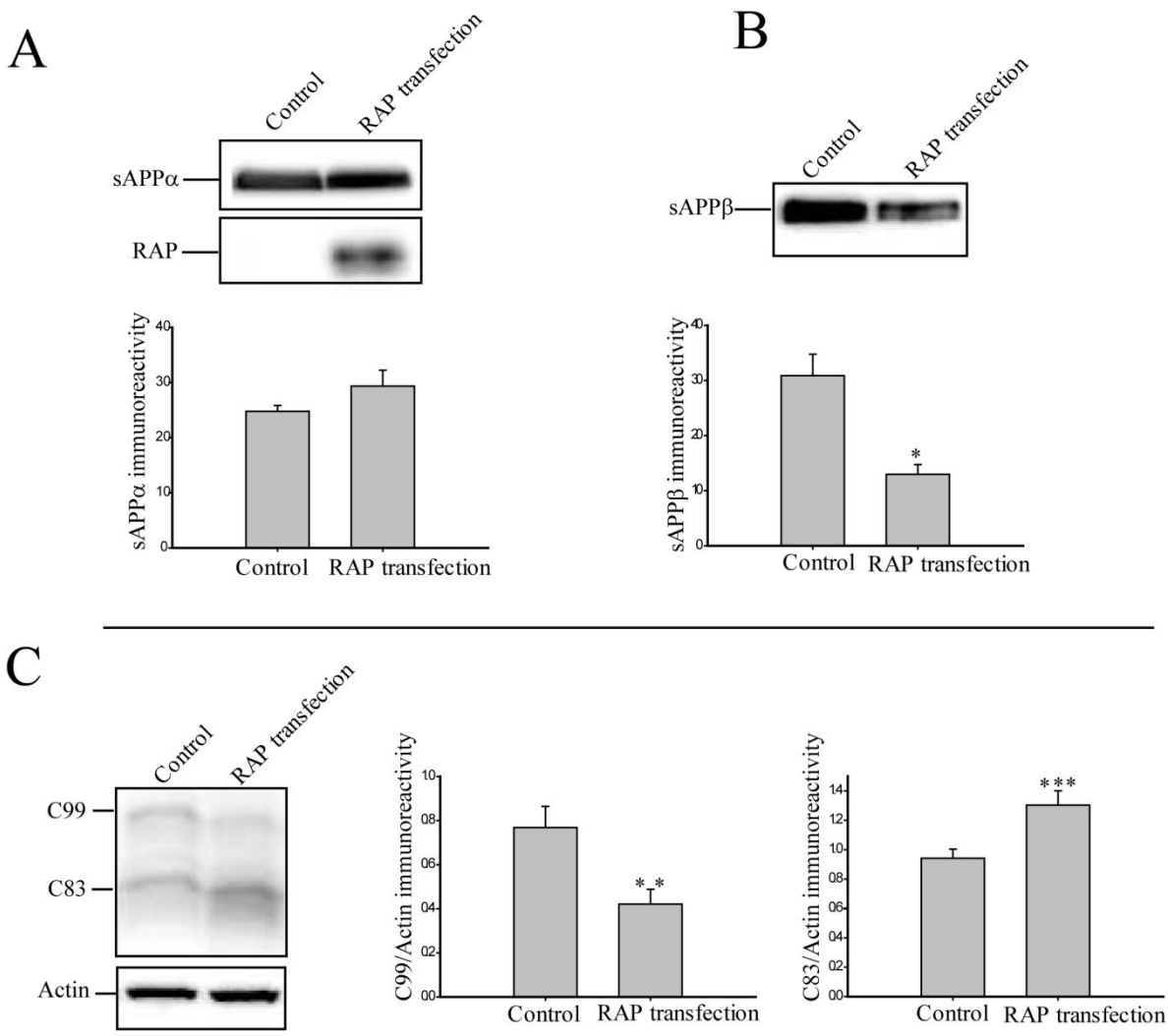

Figure 3.2 Effect of over-expression of RAP on the level of sAPPα, sAPPβ and APP C-terminal fragments in sweAPP695-CHO cells. Conditioned media from sweAPP695-CHO cells transfected with RAP or empty vector as the control were analysed by western blotting (**A**) for sAPPα using the 6E10 antibody, and RAP using the 7F1 antibody (upper panel). Quantification of sAPPα is shown (lower panel). (**B**) Western blot analysis of sAPPβ using the sAPPβ antibody (upper panel). Quantification of sAPPβ immunoreactivity (lower panel). (**C**) Cell lysates were analysed for C99 and C83 using the APP-C terminal antibody and quantification of the level C99/actin and C83/actin immunoreactivity is shown. Over-expression of RAP did not significantly affect the level of sAPPα. However, over-expression of RAP decreases sAPPβ and caused secretion of RAP into the culture medium in sweAPP695-CHO cells. Over-expression of RAP also significantly increased the level of C83 and decreased the level of C99. Data are expressed as means ± SEM of n=4, * $p < 0.006$, ** $p < 0.014$, *** $p < 0.015$.

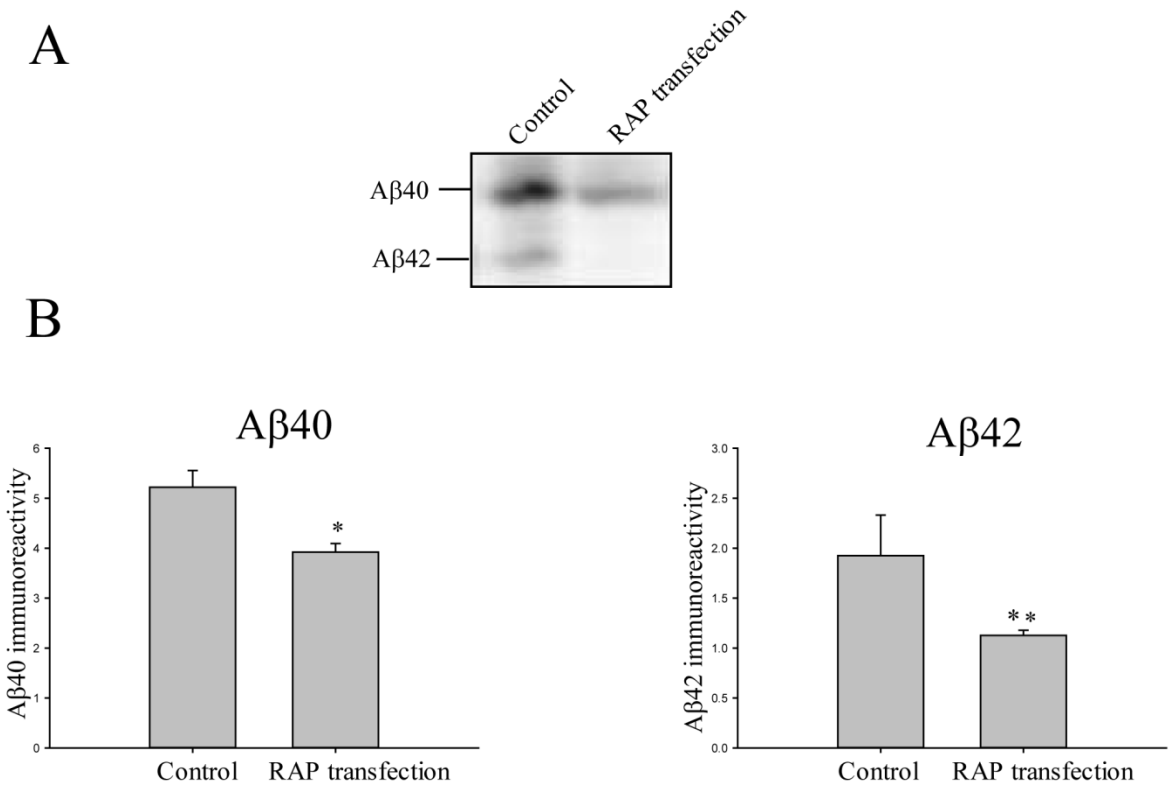

Figure 3.3. Effect of over-expression of RAP on Aβ production in sweAPP695-CHO cells. (**A**) Conditioned media from sweAPP695-CHO cells transfected with RAP or empty vector were immoprecipitated and analysed for Aβ40 and Aβ42 by western blotting using the 6E10 antibody. (**B**) Quantification of the level Aβ immunoreactivity is shown. Over-expression of RAP significantly decreased the level of both Aβ40 and Aβ2 in the medium of the sweAPP695-CHO cells, compared to the controls. Data are expressed as means ± SEM of n=3, * $p < 0.026$, ** $p < 0.01$.

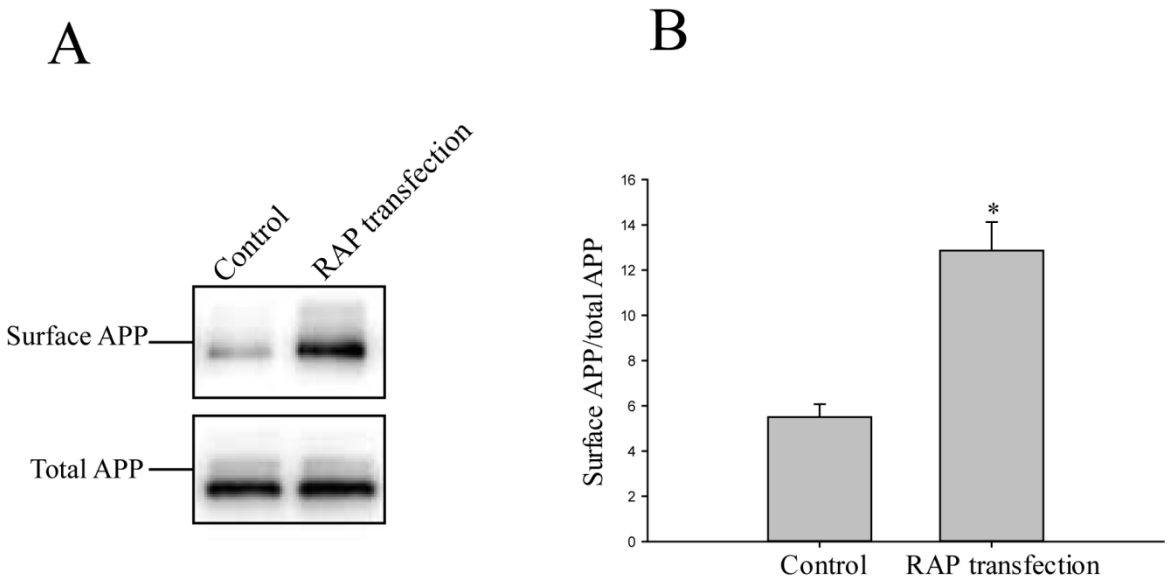

Figure 3.4. Effect of the over-expression of RAP on the level of cell-surface APP in sweAPP695-CHO cells. Cells were stably transfected with RAP pcDNA3.1 or empty vector control. (**A**) Cells were labelled with NHS-SS-Biotin and analysed for cell-surface APP using the 6E10 antibody (upper panel). The level of total APP in the cell lysates is shown (lower panel). (**B**) Quantification of the level of surface APP/total APP in the cell lysates. Over-expression of RAP significantly increased the level of cell-surface APP in the sweAPP695-CHO cells. Data are expressed as means ± SEM of n=3, * $p < 0005$.

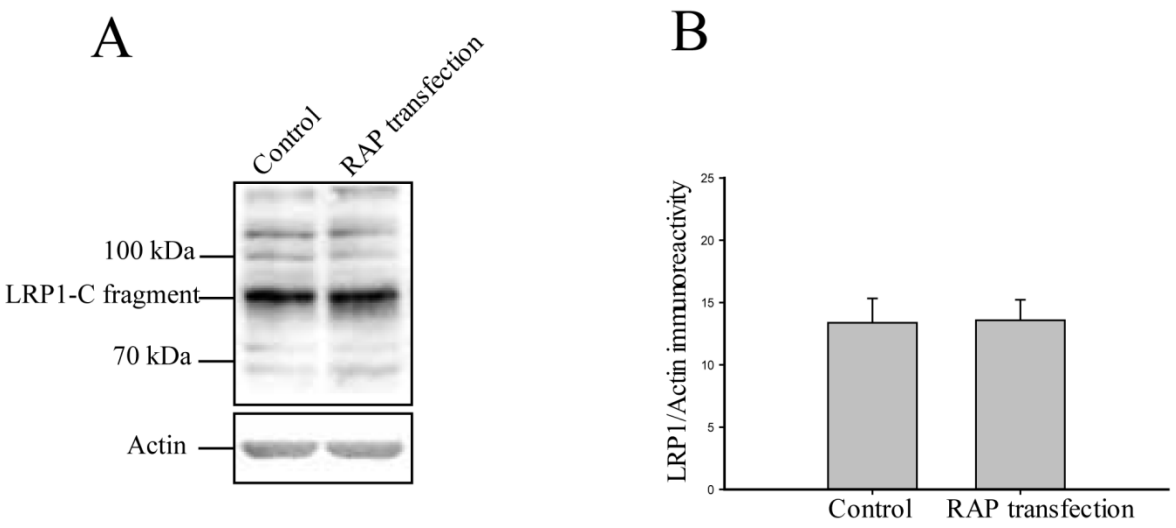

Figure 3.5. Effect of over-expression of RAP on the level of LRP1 in sweAPP695-CHO cells. Cells were stably transfected with RAP pcDNA3.1 or empty vector control. (**A**) Cell lysates were separated on 8% Tris-glycine SDS polyacrylamide gels and subjected to western blotting using polyclonal the LRP1-C terminal antibody (upper panel). Membranes were stripped and reprobed with the α-actin antibody (lower panel). (**B**) Quantification of LRP1/actin immunoreactivity was shown. Over-expression of RAP did not significantly change the level of LPR1 in sweAPP695-CHO cells. Data are expressed as means ± SEM of n=3.

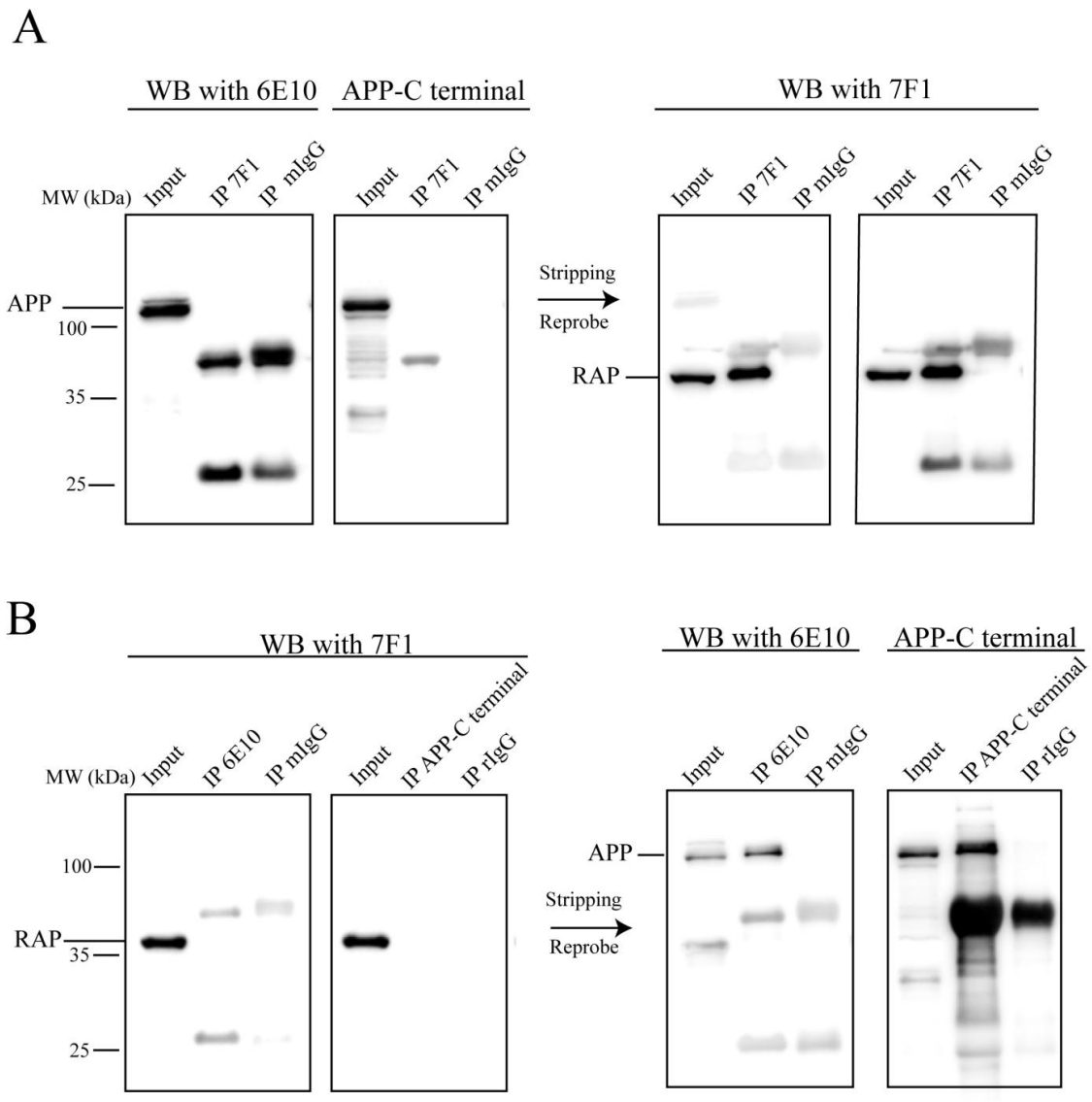

Figure 3.6. Immunoprecipitation of RAP and APP in sweAPP695-CHO cells overexpressing RAP. (**A**). Cell lysates from sweAPP695-CHO cells stably transfected with RAP pcDNA3.1 were immunoprecitated with the mouse 7F1 antibody (anti-RAP antibody) or the mouse IgG as a control. Proteins were separated electrophretically on 10 % Tris-glycine SDS-PAGE gels and subjected to western blotting using the mouse 6E10 antibody or the rabbit polyclonal APP- C terminal antibody to detect APP. The membranes were then stripped reprobed with the 7F1 antibody. (**B**) In parallel experiments, cell lysates were immunoprecipated with the mouse monoclonal 6E10 antibody or the rabbit polyclonal APP-C terminal antibody to pull down APP. Mouse IgG and rabbit IgG was used as negative controls. Proteins were separated electrophretically and subjected to western blotting using the mouse 7F1 antibody. The membranes were then stripped and reprobed with the mouse 6E10 antibody or the rabbit APP- C terminal antibody. WB: western blotting.

3.3.2 Effect of RAP overproduction on APP processing and Aβ production in sweAPP695-SH-SY5Y cells

Because CHO cells are not of neuronal origin, one might argue that they are not a good model to study AD pathogenesis. To confirm the effect of RAP overproduction on APP metabolism and Aβ production in a neuronal cell line, RAP was overexpressed in sweAPP695-SH-SY5Y neuroblastoma cells. Levels of total APP and APP proteolytic products were then analysed by western blotting.

Twenty-four hours after transfection of sweAPP695-SH-SY5Y cells with a RAP pcDNA3.1 or with an empty vector, cells were harvested and analysed on 8 % Tris-glycine SDS-PAGE for total APP and RAP. Over-expression of RAP was found to have no significant effect on total level of APP (Fig. 3.7). To further examine if APP processing was influenced by RAP over-expression, levels of APP proteolytic products, sAPPα, sAPPβ, C83 and Aβ were measured by western blotting. The results showed that while the level of sAPPα was unchanged, the level of C83 was significantly increased by over-expression of RAP (Fig. 3.8A, C). In contrast, the level of both sAPPβ and Aβ production was significantly decreased (Fig. 3.8B, D), suggesting that the over-expression of RAP might lead to a decrease β-secretase cleavage of APP in sweAPP695-SH-SY5Y cells. However, this study could not detect a quantifiable level of C99, possibly due to its low level in this cell type.

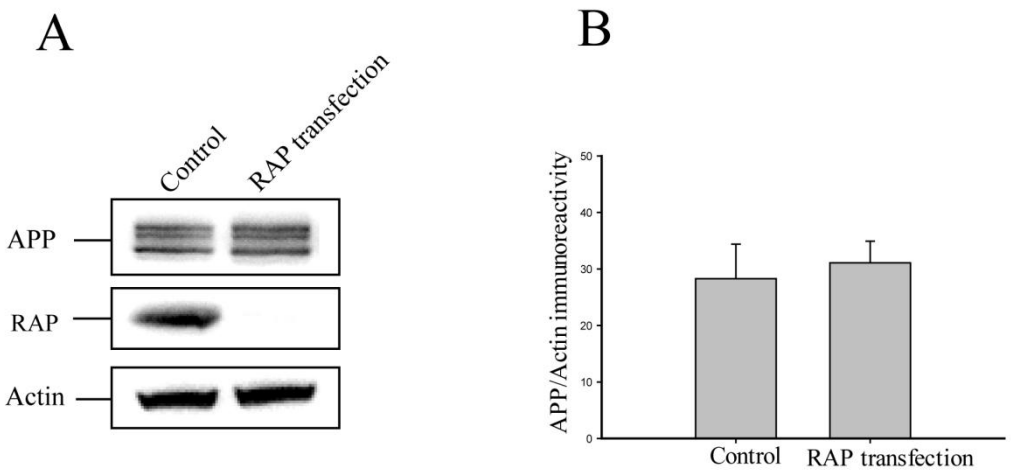

Figure 3.7. Effect of over-expression of RAP on the level of total APP in sweAPP695-SH-SY5Y cells. Cells were transfected with RAP pcDNA3.1 or control vector for 24 hr. (**A**) Cell lysates were analysed for total APP and RAP by western blotting using the 6E10 (upper panel) and the 7F1 antibody (middle panel), respectively. β-Actin immunoreactivity is shown as a loading control (lower panel). (**B**) Quantification of the level of total APP /β-actin immunoreactivity. There was no significant effect of RAP over-expression on the level of total APP. Data are expressed as means ± SEM of n=3.

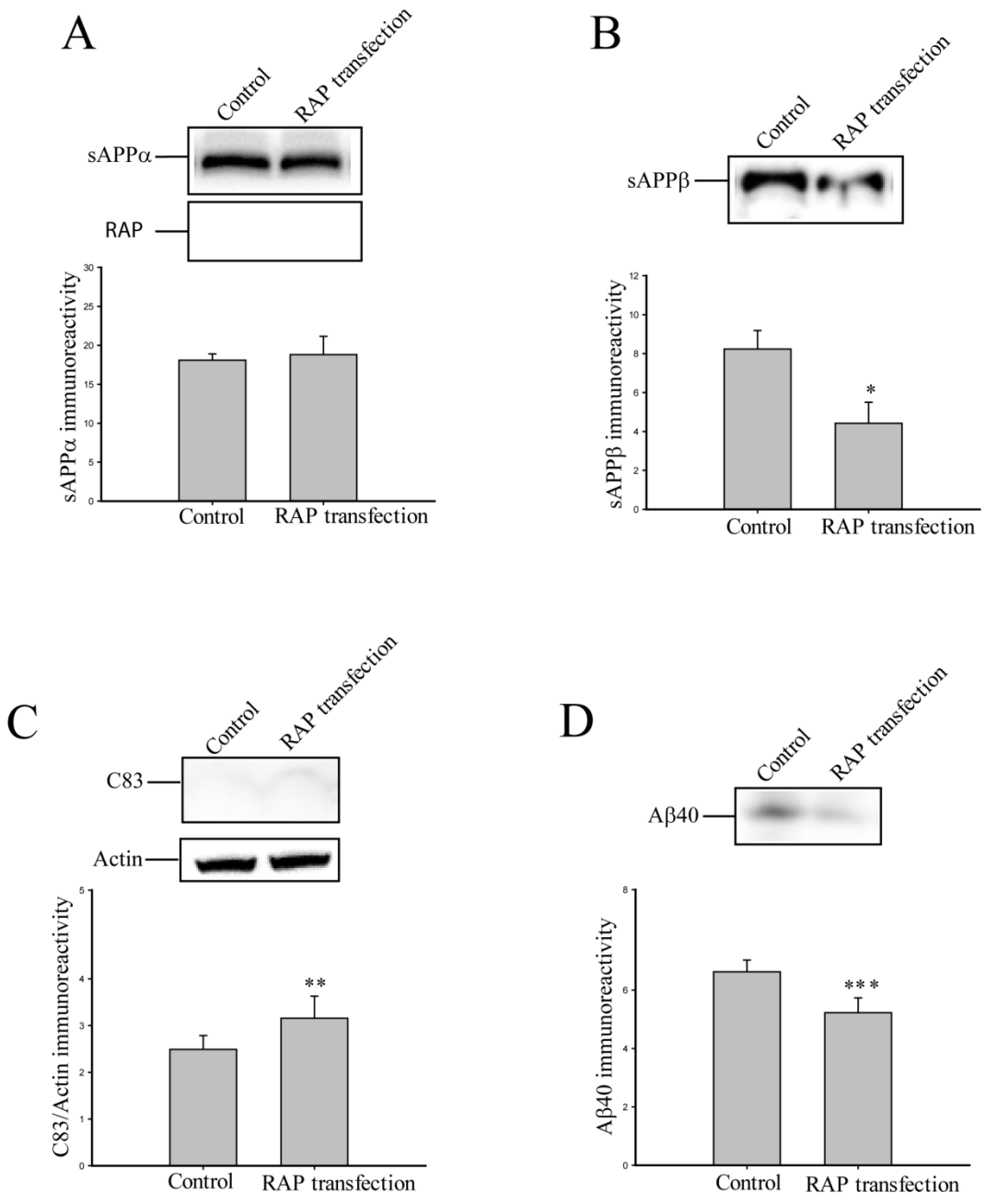

Figure 3.8. Effect of over-expression of RAP on APP processing in sweAPP695-SH-SY5Y cells. Conditioned media from sweAPP695-SH-SY5Y cells transfected with RAP or empty vector control were analysed and quantified for (**A**) sAPPα using the 6E10 antibody and RAP using the 7F1 antibody; and (**B**) sAPPβ using the sAPPβ antibody. (**C**) Cell lysates were analysed for APP-C terminal products using the APP-C terminal antibody. (**D)** Conditioned media were immunoprecipitated and analysed for Aβ using the 6E10 antibody. Over-expression of RAP significantly increased the level of C83 and decreased the level of sAPPβ and Aβ in the media of the APP695-SH-SY5Y cells compared to the mock transfected controls. Data are expressed as means ± SEM of n=3, *$p < 0.033$, **$p < 0.03$, ***$p< 0.045$.

3.3.3 Effect of RAP on APP processing and Aβ production in Tg2576 primary cortical neurons

The finding that RAP can be actively taken up across plasma membrane (Kim et al., 2004, Pan et al., 2004, Prince et al., 2004), raises the possibility that exogenous RAP may cross the cell membrane, and influence APP processing and Aβ production. To examine this possibility, PCNs at 7 DIV from Tg2576 transgenic mice overexpessing sweAPP695 were treated with RAP (500 nM) or PBS as the control for 72 hr. Cells were harvested and levels of APP proteolytic products were measured by western blotting.

The results showed that RAP treatment did not influence the total level of APP compared with the control (Fig. 3.9). There was a trend towards a decrease in the total level of C99 in the cell lysates, although this was not significant ($p = 0.061$). The total level of C83 was unaffected by the treatment of RAP (Fig. 3.10). However, RAP treatment led to significantly increase the level of sAPPα in the culture medium (Fig. 3.11A). Furthermore, RAP treatment caused a decrease in Aβ40 secretion into the culture medium (Fig.3.11B). This study failed to detect Aβ42, possibly due to the low level of Aβ42 production in the PCNs, Taken together, the data suggest that RAP treatment decreases the β-secretase cleavage of APP, lowering the production of Aβ in the PCNs.

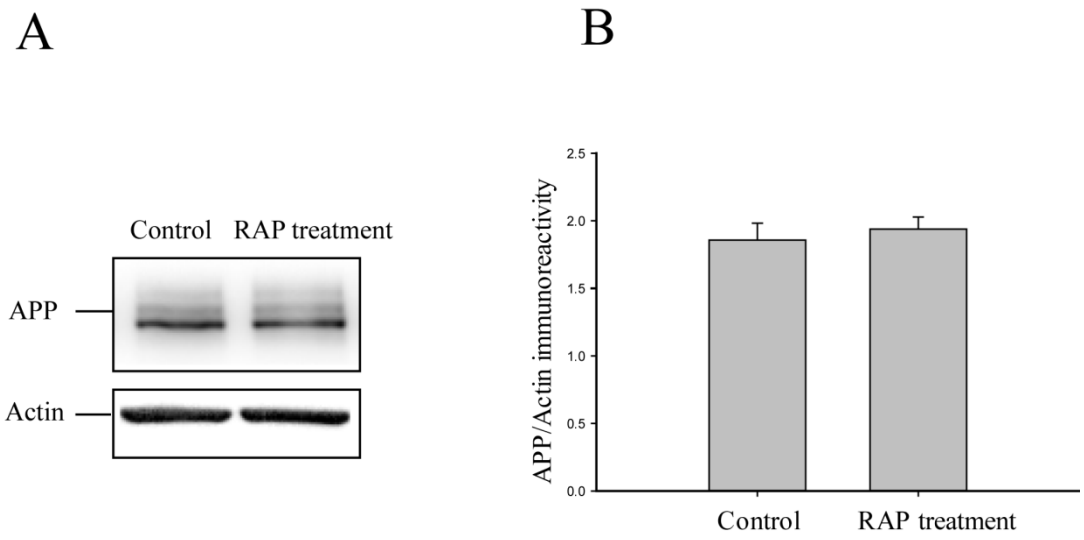

Figure 3.9. Effect of exogenously added RAP on the level of APP in the mouse cortical neurons derived from Tg2576 mice. Cells were treated with 500 nM RAP for 72 hr. Cell lysates were analysed for total APP. (**A**) Western blot analysis of APP using the 6E10 antibody (upper panel). Membranes were stripped and reprobed with the α-actin antibody as a loading control (lower panel). (**B**) Quantification of the level of total APP/β-actin immunoreactivity. The figure shows that there was no significant change on the level of APP by RAP treatment. Data are expressed as means ± SEM of n=3 replicates.

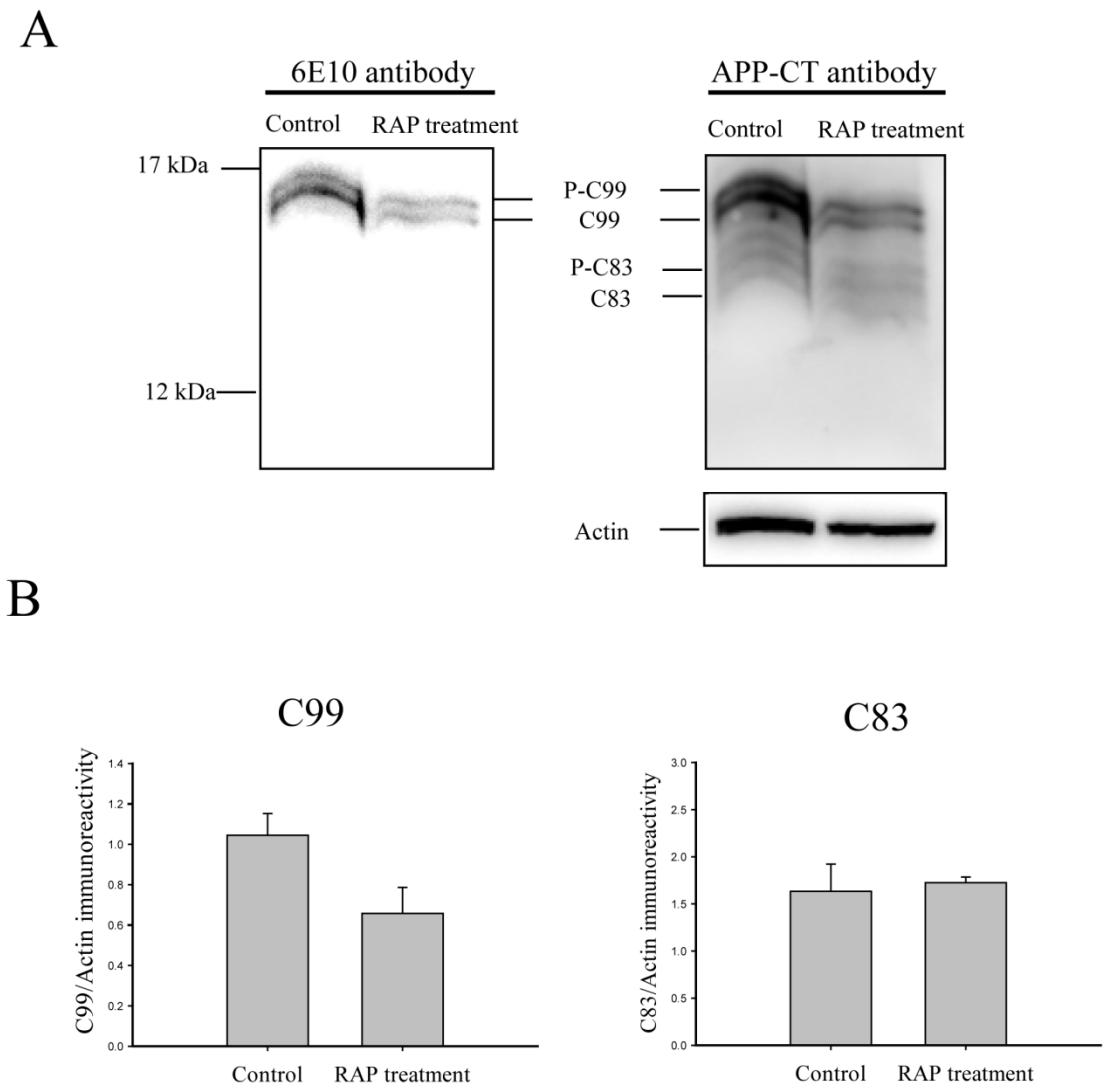

Figure 3.10. Effect of exogenously added RAP on the level of APP-C terminal fragments in primary cortical neurons derived from Tg2576 mice. Cells were treated with 500 nM RAP for 72 hr and cell lysates were analysed for APP-C terminal fragments. (**A**) Western blot analysis of APP-C terminal using different antibodies. 6E10 antibody (left panel) only recognizes C99, whereas APP-C terminal antibody (right panel) recognizes all C99, C89 and C83. Membranes were stripped and reprobed with the actin antibody. (**B**) Quantification of the ratio of C99/actin and C83/actin immunoreactivity. Levels of C99 were lower in the treatment group, although the difference was not significant ($p = 0.061$). Level of C83 was unaffected by treatment of RAP in the primary cortical neurons ($p = 0.766$). Data are expressed as means ± SEM of n=3 replicates; (P: phosphorylated).

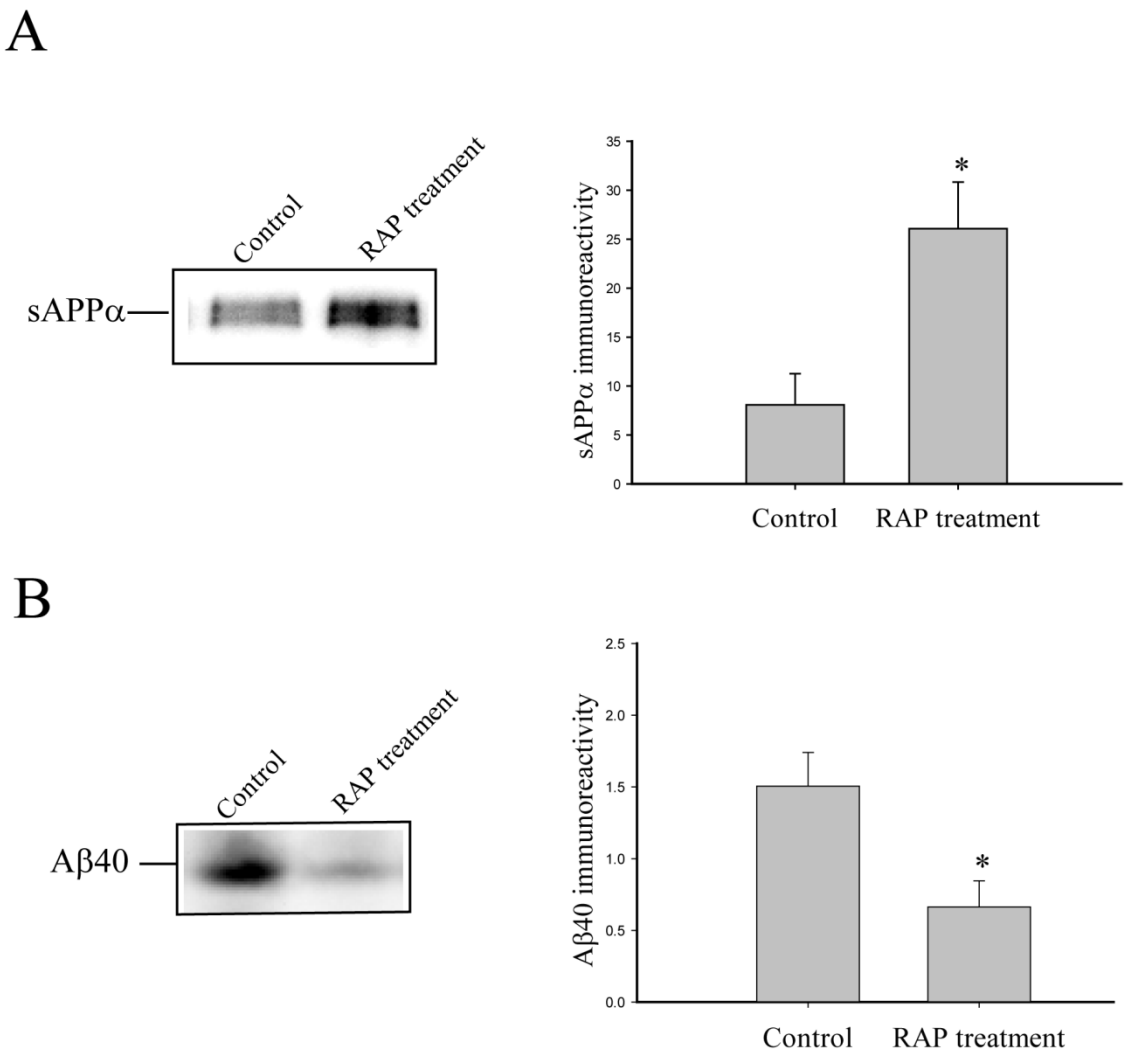

Figure 3.11. Effect of exogenously added RAP on the level of sAPPα and Aβ40 in the culture medium of primary cortical neurons derived from Tg2576 mice. Cells were treated with 500 nM RAP for 72 hr and culture medium were analysed for APPα and Aβ by Tris-bicine urea SDS-PAGE and western blotting using 6E10 antibody. (**A**) Western blot analysis of sAPPα (left panel) and quantification of sAPPα immunoreactivity (right panel). (**B**) Western blot analysis of Aβ40 (left panel) and quantification of Aβ40 immunoreactivity (right panel). Treatment of RAP significantly increased the level of sAPPα and decreased Aβ production in the primary cortical neurons. Data are expressed as means ± SEM of n=3 replicates,* $p < 0.05$.

3.4 Discussion

Emerging evidence suggests that RAP has a role in APP processing and Aβ metabolism. RAP has been shown to inhibit Aβ aggregation and increase the cellular uptake of Aβ (Kanekiyo and Bu, 2009, Kerr et al., 2010). Treatment of RAP was shown to decrease Aβ production in CHO cells overexpressing APP770 isoform (Ulery et al., 2000). In addition, transgenic mouse models of AD which are deficient in RAP showed early memory deficits and increased Aβ deposition in the brain (Van Uden et al., 1999, Xu et al., 2008). However, it is unknown from these *in vivo* studies whether increased Aβ deposition in the brains of RAP-deficient mice was due to decreased Aβ clearance or increased Aβ production. Therefore, the aim of present study was to determine if RAP has a role on APP processing and Aβ production by overexpression of RAP in cell lines and by treatment of the primary cell cultures with RAP. The results suggest that RAP modulates APP metabolism by decreasing the amyloidogenic pathway, subsequently lowering the level of Aβ production. The results from this study support the view that RAP plays an important role in APP trafficking and processing.

There is some evidence from previous studies (Bu et al., 2006, Marzolo and Bu, 2009, Wagner and Pietrzik, 2011) suggesting that RAP may indirectly regulate APP processing and Aβ production via a LDL family receptor-dependant mechanism. At least five members of the LDL receptor family, LRP1, LRP1B, megalin/LRP2, SorLA and ApoER2, which have trafficking and ligand-binding activities dependent on RAP, have been suggested to interact with APP and regulate APP trafficking and processing. APP exists in three isoforms (APP695, APP751 and

APP770). The longer isoforms (APP751 and APP770) containing KPI domains are found mainly in astrocytes and glial cells, while the non KPI-containing APP695 isoform is expressed mainly in neuronal cells and is considered to be the major source of Aβ (Rohan de Silva et al., 1997). Among the major members of the LDL receptor family, LRP1 has been most extensively studied in AD pathogenesis. It was shown that LRP1 can bind to and increase the cellular uptake of soluble KPI-containing APP isoforms but not sAPPα695 (Kounnas et al., 1995). Another study reported that LRP1 mediated the internalization of the complex between KPI-containing APP and epidermal growth factor binding protein (EGFBP) (Knauer et al., 1996). Ulery et al. (2000) showed that LRP1 can facilitate Aβ production from KPI-containing APP isoforms and that reatment of RAP increased the level of cell-surface APP and sAPPα, and decreased Aβ production from cell cultures expressing APP770 but not APP695. Introducing LRP1 into LRP1-defficient CHO cells resulted in decreased sAPPα secretion and a substantial increase in Aβ levels (Ulery et al., 2000). The results of the study suggested that an LRP1/APP770 interaction via extracellular domains on the cell surface modulate APP processing in such a way that the amyloidogenic pathway is favoured. Those results were confirmed by another study (Goto and Tanzi, 2002) in which RAP treatments decreased both the total level of Aβ and the ratio of Aβ42/total Aβ level in CHO cells expressing APP751. Studies (Kinoshita et al., 2001, Kinoshita et al., 2003, Cam et al., 2005) have provided evidence that LRP1 can interact with KPI-containing APP isoforms through ectodomains and cytoplasmic domains. The ectodomain interaction which involves KPI domain of APP and domain II or IV of LRP1 is RAP-sensitive because expression and exogenous treatment of RAP has been shown to decrease this interaction (Kinoshita et al., 2001) (Cam et al., 2005). The LRP1/APP cytoplasmic interaction via adaptor protein, Fe65, is RAP-insensitive (Kinoshita et al., 2001). This proposed model fits well with

reports that RAP can only bind to four extracellular domains but not the cytoplasmic tail of the LRP1 (Bu and Rennke, 1996, Obermoeller-McCormick et al., 2001). Therefore, the binding of RAP to extracellular domains of LRP1 is unlikely to affect the cytoplasmic interaction between APP and LRP1. Later studies (Pietrzik et al., 2002, Pietrzik et al., 2004) have shown that the C-terminal of LRP1 modulates APP processing and that interaction between the cytoplasmic domains rather than the ectodomains of APP and LRP1 is responsible for the effects on APP metabolism and Aβ production. In this study, cell cultures expressing the non KPI-containing APP695 isoform were used and the level of LRP1 did not significantly change by the over-expression of RAP. Therefore the effect of RAP over-expression on APP processing and Aβ production is unlikely to be mediated via a LRP1-dependent mechanism.

Previous studies on the effects of exogenous RAP treatment on Aβ production in cells that were deficient in LPR1 have yielded conflicting results. RAP treatment of LRP1-deficient fibroblasts expressing APP695 did not affect Aβ production within 24 hr (Kang et al., 2000). However, in another study by Goto et al. (2002) it was found that in the absence of LRP1, treatment of RAP resulted in a decrease in the ratio of Aβ42 to total Aβ in APP751-expressing CHO cells. In the present study, the level of LRP1 did not significantly change following the over-expression of RAP, consistent with previous studies (Qiu et al., 2001, Veinbergs et al., 2001, Qiu et al., 2004). Although further studies in LRP1-deficient neuronal cells are clearly needed, it seems likely that RAP may decrease Aβ production in a LRP1-independent mechanism.

Xu et al (2008) demonstrated that knock down of RAP expression led to increased Aβ deposition in the brain of APP/PS1 transgenic mice (Xu et al., 2008). Because a partial reduction of RAP did not significantly reduce the levels of LRP1 or SorLA, the authors of the study suggested that RAP may directly regulate APP processing and Aβ production. The finding that RAP can bind to Aβ as shown in previous studies (Kanekiyo and Bu, 2009, Kerr et al., 2010), suggests that RAP might also bind to APP and influence its trafficking and processing. Given the fact that a part of the Aβ sequence in the APP is embedded in the membrane, it is unclear whether RAP could bind to APP at Aβ domain. Attempts to detect RAP/APP complex by co-immunoprecipitation were not successful, despite trying many experimental conditions including using different lysis buffers (RIPA, phosphate buffer) and cross-linking by glutaraldehyde (data not shown).

The present study shows that the over-expression of RAP in sweAPP695-CHO cells and sweAPP695-SH-SY5Y cells can lead to the secretion of RAP into the medium (Fig.3.2A). Although RAP is mainly found in ER and Golgi, it is possible that secretion may occur as a result of saturation of its HNEL-ER retention signal (Bu et al., 1995). Therefore, RAP could potentially modulate APP processing both by acting intracellularly and/or on the cell surface. Furthermore, because RAP has recently been shown to bind to both Aβ40 and Aβ42 and increase their cellular uptake, it is possible that secreted RAP may increase cellular Aβ uptake and hence further decrease Aβ in the medium.

The current data from this study could not explain why the over-expression of RAP did not affect the total level of APP nor sAPPα, but significantly changed the level of C83, sAPPβ, C99 and

Aβ. Because the majority of cellular APP (about 90%) is processed via the α-secretase pathway to produce sAPPα and C83 (Filippov et al, 2012), it is tempting to speculate that the effect of RAP over-expression on the level of sAPPα might have been masked, due to extensive amount of sAPPα present in the cell medium. It might be also possible that the degradation of C83 was increased by RAP over-expression.

To examine if exogenous RAP has any effect on APP processing and Aβ metabolism, primary cortical neurons derived from Tg2576 newborn mice overexpressing sweAPP695 were treated with human recombinant RAP for 3 days. Previous studies showed that RAP is actively transported across the BBB and taken up across cell membranes through receptor-mediated mechanisms involving HSPG, LRP1 and megalin (Warshawsky et al., 1993b, Pan et al., 2004). RAP is a heparin-binding protein and three basic amino acid clusters in the domain 3 (R^{282}VSRSREK^{289}, R^{203}LRR^{206} and R^{314}ISRAR^{319}) show high affinity binding for both HSPG and LRP1 (Orlando and Farquhar, 1994). Other studies have demonstrated that exogenously added RAP is rapidly transported into the cells through endocytic trafficking. RAP could bind to megalin (Czekay et al., 1997) and LRP1 (Wilsie and Orlando, 2003, Laatsch et al., 2012) and the complexes were internalized via clathrin-coated vesicles. The RAP/megalin and RAP/LRP1 complexes were then delivered to early endosomes and late endosomes, where the β-secretase processing of APP occurs. In late endosomes, the complexes were dissociated and RAP was transported to lysosomes for degradation (Czekay et al., 1997, Laatsch et al., 2012). It was reported RAP can bind to LRP1 through all three domains with D3 domain having highest binding affinity (Migliorini et al., 2003). Using different RAP mutations, it was recently demonstrated that a single lysine of the two-lysine recognition motifs (K256 and K270) in the

D3 domain of RAP is sufficient to mediate endocytosis by LRP1 (van den Biggelaar et al., 2011). The results from the present study showed that treatment of RAP significantly increased sAPPα and decreased Aβ40 in the medium, consistent with the effects of RAP over-expression in sweAPP695-CHO and sweAPP695-SH-SY5Y cells; and in agreement with previous studies (Ulery et al., 2000, Goto and Tanzi, 2002). However, the present study failed to detect Aβ42 in the medium of the PCNs, possibly due to the low level of its production. In addition, current data cannot explain why the level of C99 and C83 was not significantly affected by RAP treatment.

Accumulating evidence suggests that wild-type APP (wtAPP) and sweAPP utilise different cellular mechanisms for Aβ production. On the one hand, the generation of Aβ from wtAPP occurs in the endocytic compartments and requires the APP internalization from the cell surface (Haass et al., 1993, Koo and Squazzo, 1994). The APP endocytosis can facilitate Aβ production by bringing APP and BACE, which is enriched in lipid rafts, into close proximity. Moreover, the amyloidogenic pathway is favoured in the acidic pH environments in endosomes (Tan and Evin, 2011). Meanwhile, retention of APP at the cell surface favours the non-amyloidogenic pathway, decreasing Aβ production. The decrease in APP internalization by mutation in endocytosis motifs or by potassium depletion results in increase retention of APP at the cell surface and favours the α-secretase pathway (Perez et al., 1999). On the other hand, increasing evidence suggests that the β-secretase processing pathway of sweAPP occurs mainly in Golgi-derived vesicles in the secretory pathway (Haass et al., 1995, Thinakaran et al., 1996, Yoon et al., 2007, Belyaev et al., 2010, Yamakawa et al., 2010, Griffiths et al., 2011). Cells expressing sweAPP produce 6-8-fold more Aβ than cells expressing wtAPP (Citron et al., 1992). Deletion of the APP internalization motif did not affect Aβ production, implying that Aβ could be generated in the secretory pathway (Citron et al., 1995, Steinhilb et al., 2002, Yamakawa et al., 2010).

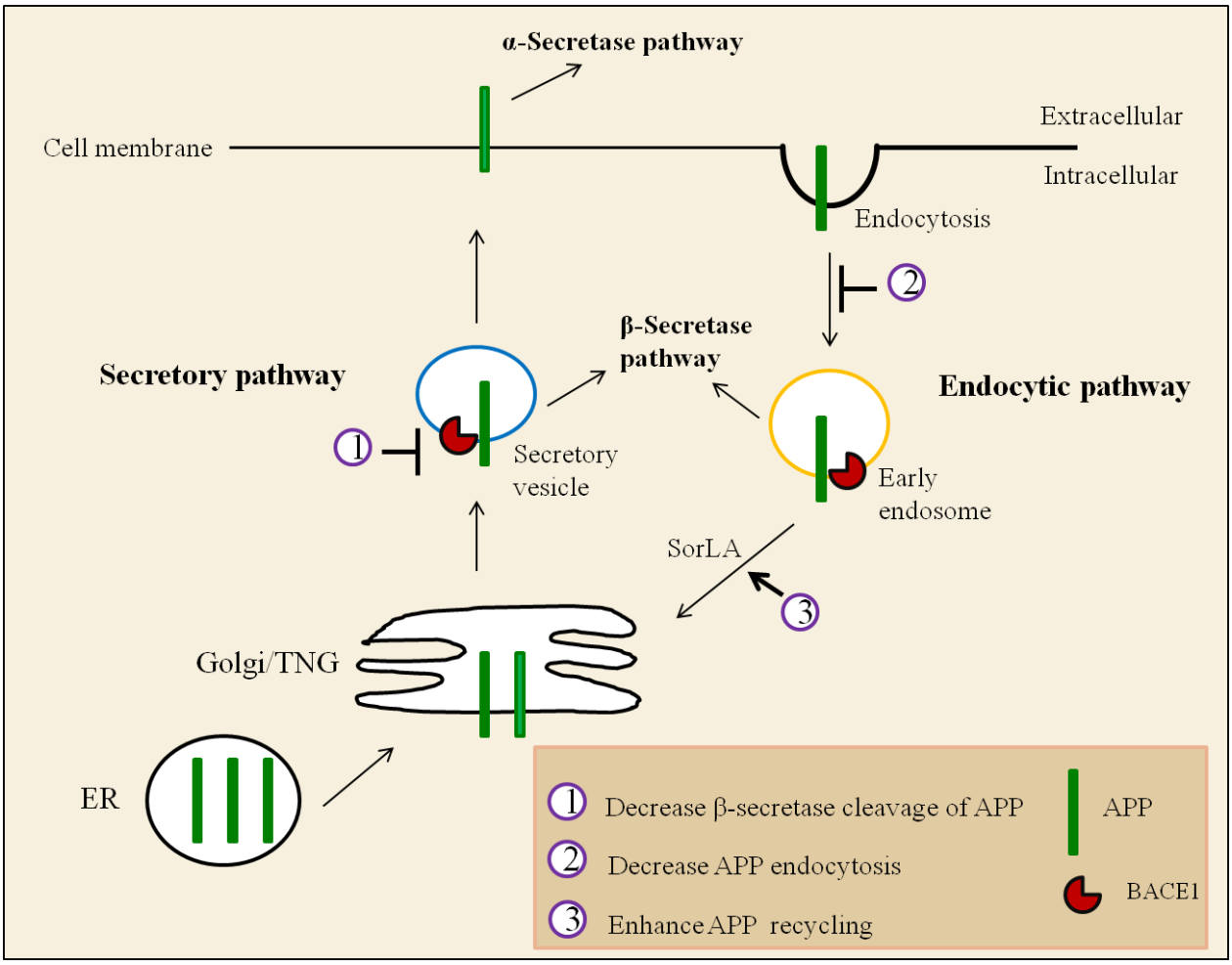

Figure 3.12. A simplified model of proposed roles of RAP in APP trafficking and processing. Following the synthesis and maturation of APP (green) in the ER and Golgi, APP is trafficked to the cell membrane in the secretory pathway. On the way to the cell membrane, APP can undergo proteolytic processing by β-secretase (BACE1, red) to produce Aβ in the secretory vesicle. At the cell surface, APP can be cleaved via the α-secretase pathway or it can be internalized into the early endosome, where APP can be processed by BACE1. SorLA may shuttle APP to the Golgi/TGN and decreases its cleavage by BACE1 in the early endosome, thus increasing APP recycling to the cell surface and lowering Aβ production. Several effects of RAP are proposed in this model, including reducing the β-secretase cleavage of APP in the secretory pathway (1), inhibiting of cell-surface APP internalization (2), and promoting APP recycling (3). Abbreviations: ER, endoplasmic reticulum; TGN, trans-Golgi network; SorLA, sortilin-related receptor with A-type repeats.

However, several other studies suggested that the β-secretase cleavage of sweAPP can occur in both secretory and endocytic compartments, with the majority of Aβ being produced through the secretory pathway (Perez et al., 1996, Steinhilb et al., 2002, Small and Gandy, 2006, Jager et al., 2009). It is noteworthy that RAP is reportedly co-localized with APP-C terminal fragments and PS1 fragments in the secretory vesicles (Annaert et al., 1999, Verdile et al., 2000). Therefore, the findings that RAP increased the level of cell-surface APP and decreased the β-secretase processing pathway of sweAPP695 could be due to: (**1**) a decrease in the β-secretase cleavage of sweAPP in the secretory pathway possibly by influencing BACE1 level/activity or BACE1:APP interaction; (**2**) an inhibition of APP endocytosis; (**3**) promotion of APP recycling via the Golgi/TGN network to the cell membrane possibly by increasing SorLA level/activity (Fig. 3.12).

Interactions between RAP and LRP, between LRP and APP, and between LRP and its ligands (α2-macroglobulin, ApoE and Aβ) may create a complex network which can modulate amyloidogenesis. Furthermore, the fact that RAP also interacts with other members of LDL receptors such as SorLA, megalin, ApoER2 and LDLR, which are all known to be involved in APP processing and Aβ metabolism, adds an additional level to the complexity to the system.

In summary, this study demonstrated that RAP can modulate APP processing and lower Aβ production in sweAPP695-CHO cells, sweAPP695-SH-SY5Y cells and sweAPP695-primary cortical neurons. However, the mechanism in which RAP mediates the effect is unclear. Previous studies found that RAP binds to Aβ and increase its cellular uptake and inhibit neurotoxicity (Kanekiyo and Bu, 2009, Kerr et al., 2010). Taken together, the data suggests that RAP can

lower Aβ production and increase Aβ clearance. Further studies need to clarify the mechanisms underlying the effects of RAP on the APP processing. Increasing level of RAP in the brain or using RAP-mimic drugs may potentially provide therapeutic benefits for AD treatments.

Chapter 4: Identification of RAP and other Aβ-binding proteins in human CSF

4.1 Introduction

The interstitial fluid of the brain is continuous with the CSF. Therefore changes in AD brain biochemistry can be reflected in changes in the CSF. The fact that Aβ does not form Aβ fibrils in biological fluids may be due to its low concentration or the presence of inhibitory factors of Aβ aggregation. Binding of specific factors to Aβ may potentially result in modulation of Aβ-aggregation in the brain. Some of these factors may be also present in the CSF. Studies have shown that the presence of CSF can promote (Ono et al., 2005, Ikeda et al., 2010) or inhibit Aβ fibril formation (Wisniewski et al., 1993), suggesting the existence of Aβ-interacting factors in the CSF. Previous studies (Kanekiyo and Bu, 2009, Kerr et al., 2010) and the results from the Chapter 2 have demonstrated that RAP can bind to Aβ, leading to an inhibition of Aβ aggregation and increased Aβ clearance. The aim of this study was to identify if RAP and other proteins could bind to Aβ in the human CSF. The low level of Aβ present in the human CSF could make Aβ:CSF protein complexes, if formed, undetectable by using traditional antibody-based coimmunoprecipitation (Yerbury et al., 2007). To overcome this issue, this study employed a pull-down approach using synthetic Aβ42 immobilized to agarose beads. Proteins were then visualized by silver staining and the identities of predicted proteins were confirmed by using specific antibodies. Although this study failed to demonstrate that Aβ could bind to RAP in the human CSF, preliminary data suggested that clusterin (CLU) is likely to be a major Aβ-binding

protein in the CSF of both AD patients and healthy controls. Additional experiments using 2D electrophoresis and mass spectrometry may help to further confirm this finding.

4.2 Materials and methods

4.2.1 Materials

Human CSF from AD and non-AD patients obtained from Dr. Lucilla Parnetti (University of Perugia, Italy). For this preliminary study, two CSF samples from patients with AD and two from healthy control that were matched for sex (female) and age (75 years old) were used for this study. Streptavidin-Sepharose and clusterin antibody (H-330) were purchased from Santa Cruz (Sydney, Australia). D-biotin was purchased from Sigma (Sydney, Australia). Siver-staining kit was purchased from Bio-rad (Melbourne, Australia).

4.2.2 Pull-down of RAP and other Aβ-binding interactors in human CSF

To precipitate RAP and other Aβ-binding proteins from human CSF, 10 μg biotin-Aβ42 inDMSO (as previously described in Chapter 2) or D-biotin as the control were incubated with 25μl streptavidin-Sepharose beads in 500 μl PBS at room temperature for 1h with rotation. Biotin-Aβ42-conjugated beads were centrifuged at 500 x g for 3 min and washed three times with 500 μl PBS. CSF (300 μl) which contained ~0.3 mg of proteins from AD patients and controls were added to the beads and the volume was made up to 500 μl with PBS buffer containing proteinase

inhibitor (1 tablet per 10 ml PBS). The mixtures were incubated with gentle rotation at 4^0C overnight. Beads were spun down at 500 x rpm, washed three times in 1 ml PBS containing 1% NP40 and pellets were collected.

4.2.3 Silver staining and western blotting

Samples bound to the Aβ-Sepharose from human CSF were mixed with 2 x SDS sample buffer and heated at 95^0C for 5 mins. Proteins were separated electrophoretically on 12% Tris-glycine SDS-PAGE gels. To visualize proteins on SDS gels, a silver stain kit (Bio-Rad laboratories) was used. After electrophoresis, gels were immersed in a fixing solution (50% methanol, 10% acetic acid and 5% fixative enhance concentrate) and gently agitated for 20 min using a mixer (Bartelt Instruments Pty. Ltd, Melbourne, Australia). Gels were washed twice with 100 ml distilled water (15 min/wash). A solution containing 10% silver complex solution, 10% reduction solution and 10% image developer reagent was prepared and mixed with an equal volume of developer accelerator reagent immediately before use. Gels were incubated in the staining solution with shaking until the development had occurred (up to 30 min) after which reaction was stopped by replacing the staining solution with 5% (v/v) acetic acid solution.

To determine the identity of protein bands by western blotting, samples were separated on 12 % Tris-glycine SDS-PAGE gels and proteins were transferred onto 0.22 µm nitrocellulose membranes. The membranes were blocked in 5% skim milk in PBST (0.25% [v/v] Tween-20 in PBS for 1 h. Incubations with a clusterin antibody (1:1000) or anti-RAP antibody (1:1000) and

secondary (anti-rabbit HRP, 1:5000) antibody in 5% skim milk PBST were performed overnight at 4^{o}C and for 1 h at room temperature, respectively. Membranes were then incubated with Chemiluminescent HRP substrate (Millipore corporation, MA, USA). Chemiluminescence was captured using a Chemi-smart 5000 (Chemismart, Marne-la-Vallée, France).

4.3 Results

To examine if RAP and other proteins could bind to Aβ in human CSF, biotin-Aβ42 immobilized to streptavidin-sepharose was used as bait to pull down RAP and Aβ-interacting proteins in the CSF samples from AD and healthy control. In this experiment, excessive amounts of biotin-Aβ42 and D-biotin should be sufficient enough to block all reactive streptavidin in the sepharose beads. Bound proteins were separated on SDS PAGE gels and subjected to silver staining. The result shows that two distinct protein bands with molecular mass of 35 and 70 kDa were pulled down with Aβ42 in the CSF of both AD and healthy control (Fig. 4.1A lane 3 and 6). These two bands were not found in absence of human CSF samples, excluding the possibility that they are aggregated forms of biotin-Aβ42 (Fig. 4.1A lane 1). Furthermore, when D-biotin was used instead of biotin-Aβ42, little or no 35 and 70 kDa bands were observed, indicating the specificity of the bindings (Fig. 4.1A lane 2 and 5).

To examine the possibility that protein bands contain RAP, anti-RAP antibody was used to analyse the CSF by western blotting. However, the result failed to detect the presence of RAP in

the CSF (data not shown). To determine the identity of the two protein bands, a database on known human CSF proteins from 2-D SDS-PAG reference maps was used (Lee, 2001). It was shown that clusterin (CLU) in the human CSF moved at a similar position with the 35 kDa band under reducing conditions. Furthermore, the large band of the 35 kDa protein in the silver-stained gel could possibly

represent different glycosylated forms of CLU (Fig. 4.1A lane 3 and 6). To test the hypothesis that the 35 kDa band protein was CLU, a specific antibody for CLU was used to analyse the CSF by western blotting. The result shows that CLU in the CSF of both AD patients and healthy control was pulled down using Aβ and separated at 35 kDa on the SDS PAGE gel (Fig. 4.2 lane 4 and 7). The fact that CLU is a major protein in the human CSF and the identical patterns of the protein bands in the silver-stained gel and western blot (Fig. 4.1 and 4.2) suggest that the 35 kDa protein band in the silver-stained gel may represents CLU. Interestingly, the 70 kDa band also shows immunoreactivity with the CLU antibody, suggesting that this band is likely to be an SDS-stable CLU dimer (Fig 4.2 lane 4 and 7). However, it cannot completely rule out the possibility that the large band at 35 kDa in the silver-stained gel may contain more than one protein.

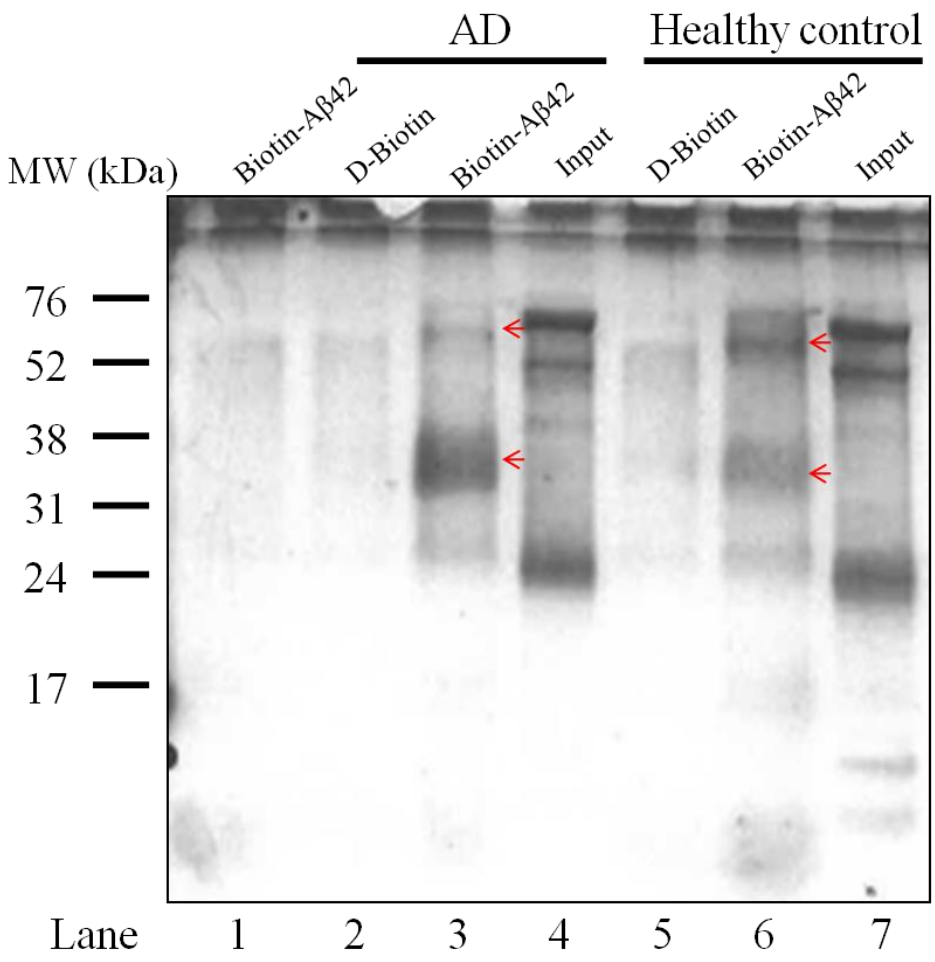

Figure 4.1. Analysis of Aβ-binding proteins in human CSF of AD and healthy control by silver staining. (**A**) Proteins that could bind to Aβ42 were pulled down by streptavidin-sepharose. After being separated on a 12 % Tris-glycine SDS-PAGE gel, proteins were subjected to silver staining. Controls for the experiments included: biotin-Aβ42 incubated without CSF (lane 1); D-biotin was used instead of biotin-Aβ42 (lane 2 and 5). The figure shows that two distinct 35 and 70 kDa protein bands (arrows) were immunoprecipitated with Aβ42 in the CSF of both AD (lane 3) and healthy control (lane 6).

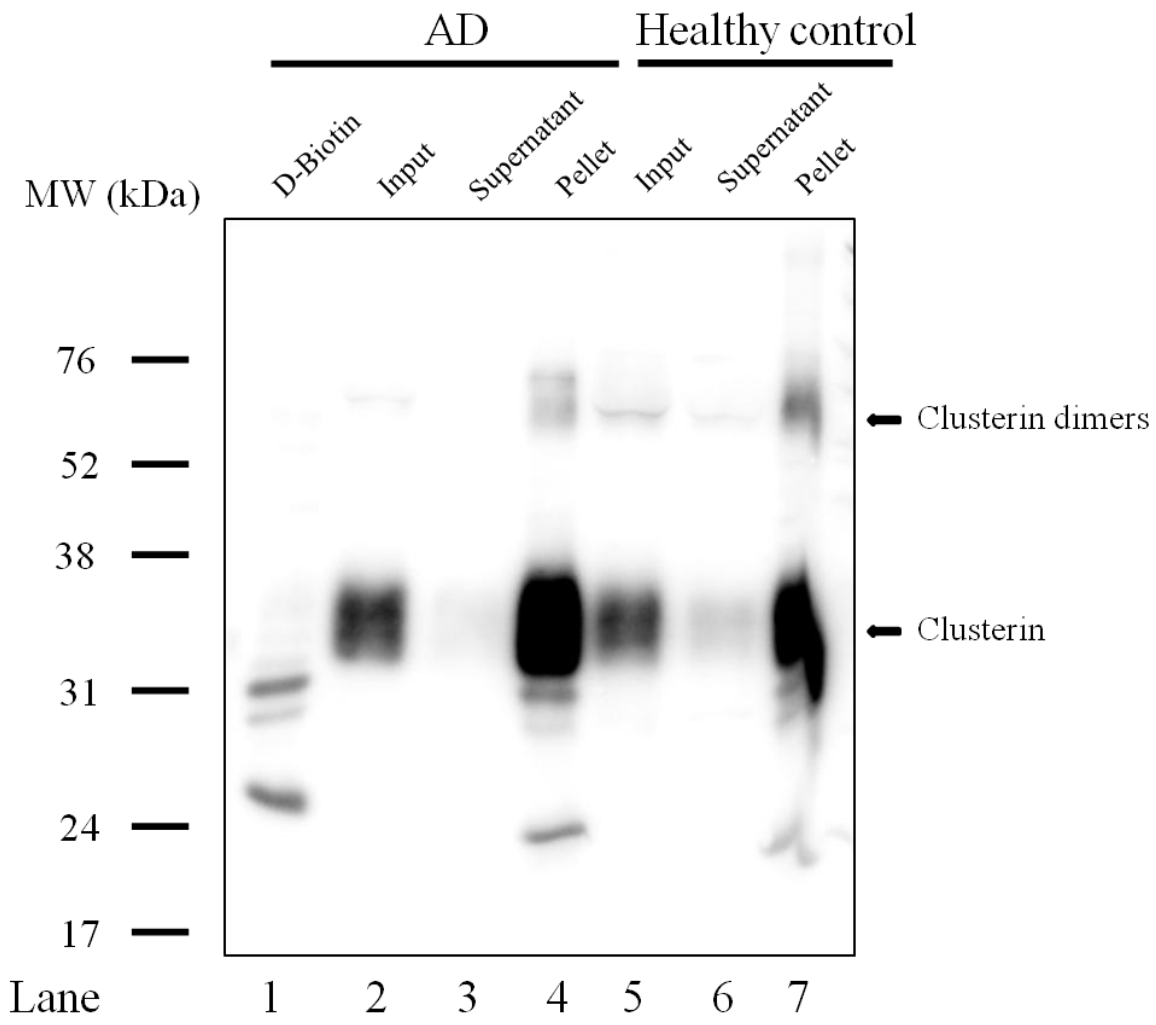

Figure 4.2. Identification of the Aβ-interacting protein in human CSF by western blotting. Aβ42-binding proteins were pulled down by streptavidin-sepharose and subjected to western blotting. Blots were stained with a clusterin antibody (1:1000) and an anti-rabbit-HRP secondary antibody. D-biotin was used instead of biotin-Aβ42 as the control (lane 1). The figure shows that two clusterin-immunoreactive protein bands (35 and 70 kDa) were concentrated after pulling down the CSF of both AD (lane 4) and healthy control (lane 7) with Aβ42. While the 35 kDa band is CLU protein, the 70 kDa protein band most likely represents SDS-stable CLU dimers.

4.4 Discussion

This study failed to show that Aβ can bind to RAP in the human CSF, however preliminary data suggested that clusterin is likely to be a major Aβ-binding protein in the human CSF from both AD patients and healthy controls. Moreover, both clusterin monomers and dimers were found after pulling down using Aβ. This finding could be explained by the fact that CLU normally exists in 70 kDa heterodimers in the CSF (Rosenberg and Silkensen, 1995, Nilselid et al., 2006) and was pulled down with Aβ42 immobilized to sepharose beads. Under SDS-reducing conditions, the 70 kDa CLU heterodimers dissociated and released 35 kDa CLU momoners. Further experiments using 2D electrophoresis and mass spectromertry will help to confirm this finding.

Under normal conditions, RAP is known to primarily reside in the endoplasmic reticulum and Golgi apparatus, due to the presence of HNEL-ER retention signal {Bu, 1997 #40;Lee, 2006 #73}. However, RAP can be secreted into the extracellular space when the retention signal is disrupted (see Chapter 1) or by the overexpression of RAP (see Chapter 3). Therefore, it could be possible that under pathological conditions such as in AD, RAP might be secreted extracellularly into the CSF. Yet the result of this study could not detect the presence of RAP in the human CSF by using western blotting.

Previous studies showed that CLU binds to Aβ *in vitro* with a high affinity (K_d = 2nM) and the binding is reversible. The stoichiometry for the interaction is 1:1 ratio and CLU was shown to

have 5 times higher affinity for fresh Aβ than aggregated forms (Matsubara et al., 1995, Narayan et al., 2011). It was also demonstrated that a CLU/Aβ complex exists in human CSF under physiological conditions (Ghiso et al., 1993, Calero et al., 2000). However, it still remains unclear which region of CLU sequence is involved in the binding with Aβ and how they actually interact. Using biophysical methods, a very recent study has shown that CLU binds and sequesters Aβ40 oligomers formed from both the disaggregation of Aβ40 fibrils and the aggregation of Aβ40 monomers, leading to an inhibition of the further dissociation or growth of these Aβ40 oligomers (Narayan et al., 2011). However, it still remains to determine whether and how CLU and Aβ oligomers interact *in vivo*. The effect of the binding of clusterin on the neurotoxicity of Aβ40 oligomers is also unclear. By forming long-lived complexes with Aβ oligomers (Narayan et al., 2011), CLU may prevent them from interacting with other molecules and promote their degradations *in vivo* (Nuutinen et al., 2007, Wyatt et al., 2011) .

Apart from CLU, the result from the silver-stained gel did not show other known Aβ-binding proteins such as ApoE isoforms, transthyretin and ACT, suggesting that CLU is the main Aβ-binding protein in the CSF. Previous data showed that the presence of other Aβ-binding proteins at physiological concentrations such as ApoE, ACT and transthyretin did not inhibit the formation of Aβ/CLU complexes (Matsubara et al., 1995), indicating that the formation of Aβ/CLU complexes is favoured in physiological conditions. Given the fact that CLU is present at much higher concentration (20 to 60 nM) (O'Bryan et al., 1990, Nilselid et al., 2006) than Aβ (1 to 10 nM) (Mehta et al., 2000, Yerbury et al., 2007) in the CSF, it could act as an Aβ-protein carrier and sequester Aβ from the brain. However, it remains to be determined if there is any change in the level of CLU and if there is any difference in the interaction between CLU and Aβ

in the CSF of AD patients and healthy controls. Further studies are also needed to examine differences in CLU variants associated with AD risks.

Chapter 5: Conclusions and future directions

The Aβ-binding site is most likely to be located between amino acid residues 206 and 216 in the loop region between the D2 and D3 domains of RAP (Fig. 2.6). This study provided a new insight into RAP: Aβ interaction and may offer a potential to develop RAP-mimicking drugs to inhibit Aβ deposition and increase cellular Aβ uptake for AD. Future studies could further confirm the Aβ-binding site on RAP by using complementary experimental approaches, such as co-immunoprecipiation and surface plasmon resonance (SPR) to detect Aβ:RAP fragment complexes. The nature and mechanism of the Aβ: RAP interaction should be also investigated, for example, by studying the interaction of Aβ with different RAP mutants.

Both RAP over-expression in cell lines and treatment of primary cortical neurons with RAP decreases the β-secretase processing pathway of APP, thus lowering Aβ production. RAP over-expression increases the level of cell-surface APP (Fig. 3.4) and leads to secretion of RAP into the extracellular space (Fig. 3.2 and 3.8). However, the underlying mechanism for the effects of RAP in the present study is unclear. Because RAP increases the α-secretase cleavage and decreases the β-secretase cleavage of APP, further experiments should examine if there is any change in APP subcellular location, APP endocytosis, BACE1 level/ activity, α-secretase level (ADAM10 and ADAM17) and SorLA level/activity.

To further determine whether RAP decreases Aβ production via a LRP1-independent mechanism, RAP could be overexpressed in cells lacking LRP1 and the effects on APP processing and Aβ production could be examined. Moreover, because RAP is a chaperone for many members of the LDL receptor family, further work also needs to determine if the level of the LDL receptor family such as LRP1B, megalin, ApoER2 and LDLR could be affected by RAP over-expression/treatment. It would be also of interest to examine if there is any difference of the effects of RAP on APP processing and Aβ production in cells expressing sweAPP versus wtAPP and to determine if RAP acts through the secretory or endocytic pathway.

Studies in AD transgenic mouse models with RAP knockout have shown an increased Aβ deposition in the brain. However, it is unclear if the mechanism is due to increased Aβ production or decreased Aβ clearance. Therefore, the effect of RAP knock down on APP processing and Aβ production also needs to be studied further.

It remains unclear from this study how exogenously added RAP can enter the cells, where it goes to after internalization and what domain of RAP is responsible for the effects on APP processing. Further studies using fluorescent-labelled RAP and different RAP fragments will need to address these issues. Future experiments should also examine if the injection of RAP or RAP-mimic peptides could reduce Aβ deposition in *in vivo* models.

It was not possible to detect the presence of RAP in the human CSF, consistent with the view that RAP mainly resides inside the cell and is not secreted into the extracellular space. Preliminary data suggests that CLU is the major Aβ-binding protein in the human CSF of both AD patients and healthy controls (Fig. 4.1 and 4.2). Further experiments using 2D gel electrophoresis and mass spectrometry need to confirm this result. It is still unclear how CLU interacts with Aβ40 and Aβ42, and which amino acid sequences are involved in the interaction. The effect of CLU:Aβ interaction on Aβ neurotoxcity also needs further study. Furthermore, it would be of interest to examine if there is any change in the level of CLU. Because CLU has been suggested to promote Aβ clearance, it would be interesting to see if there is any difference in the interaction between CLU and Aβ in the CSF of AD patients and healthy controls and if the CLU-mediated clearance of Aβ is impaired in AD brain. The effects of different CLU variants on AD risks also need to be investigated.

Appendix: Commonly used solutions

Luria-Bertani broth (LB):

Per litre:

10 g Tryptone

5 g Yeast extract

10 g NaCl

Adjust pH to 7.5 with 1 M NaOH and autoclave

Terrific broth (TB):

Per litre:

12 g Trytone

24 g Yeast extract

4 ml Glycerol

Autoclave and add 100 ml sterile potassium phosphate (2.31 g KH_2PO_4; 12.54 g K_2HPO_4)

6xGel-loading buffer:

30% (v/v) glycerol

0.25% (w/v) bromophenol

4xSDS sample buffer

400mM Tris-HCl, pH=6.8

8% (w/v) SDS (sodium dodecyl sulphate)

0.4% (w/v) bromophenol

40% (v/v) glycerol

400 mM β-mercaptoethanol

Tris Buffered Saline (TBS)

8 g NaCl

0.2 g KCl

3 g Tris base

Dissolve in 0.8 L H_2O and adjust to pH 7.4 with HCl. Make up to 1L with H_2O

Phosphate Buffered Saline (PBS)

137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO4

2 mM KH_2PO4

Radioimmunoprecipitation (RIPA) lysis buffer

50 mM Tris

150 mM NaCl

05% [w/v] sodium deoxycholate

1% [v/v]) NP40

Tris-tricine SDS gel

- Separating gel (16.5% T/ 6% C) (10ml)

  3.33 ml Separation gel monomer

  3.33 ml 3x Gel buffer

  1 ml Glycerol

  2.3 ml H_2O

  33µl 10% APS

  3 µl TEMED

- Stacking gel (4 %T/ 3%C) (5 ml)

  400 µl Stacking gel monomer

  1.25 ml 3x Gel buffer

  3.22 ml H_2O

  25 µl 10% APS

  5 µl TEMED

- Separating gel monomer (49% T/ 6 %C) (200ml)

  93 g acrylamide

  6 g N,N'-methylene-bisacrylamide

  Heat at 50^{o}C for 1 hr and filter through 0.22 µm filter

- Stacking gel monomer (49% T/ 3 %C) (200ml)

  93 g acrylamide

  3 g N,N'-methylene-bisacrylamide

Heat at 50^{o}C for 1 hr and filter through 0.22 µm filter

- 3x Gel buffer (500ml)

  181.71 g Tris base

  15 ml 10% SDS

  Adjust to pH 8.45 with HCl

- Anode buffer (1L)

  24.23 Tris base

  Adjust to pH 8.9 with HCl

- Cathode buffer (1L)

  12.22 Tris base

  17.92 Tricine

  10 ml 10% SDS

  pH 8.25

Tris-bicine urea SDS gel

- Separation gel (10 ml)

  2.5 ml Separation gel buffer

  1.67 ml Acrylamide monomer

  1 ml H2O

  4.8 g Urea powder

  100 µl 10 %SDS

  40 µl 10% APS

  5 µl TEMED

  Top up to 10 ml with H_2O

- Stacking gel (5 ml)

  1 ml Stacking gel buffer

  200 µl Acrylamide monomer

  20 µl 10 %SDS

  770 µl H_2O

  8 µl 10 % APS

  2 µl TEMED

- Comb gel (2 ml)

  1.5 ml Comb gel buffer

  375 µl Acrylamide monomer

  30 µl 10 % SDS

  1.064 ml H_2O

  24 µl 10% APS

  3 µl TEMED

- Acrylamide monomer (60 %T/ 5% C) (100 ml)

  57 g Acrylamide (57%)

  3 g Bis-acrylamide (3%)

- Separation gel buffer (100 ml)

  1.6 M Tris (19.38 g)

  0.4 M H_2SO_4 (2.17 ml)

- Stacking gel buffer (100 mL)

  0.8 M Bis Tris (16.74 g)

  0.2 M H_2SO_4 (1.09 ml)

- Comb gel buffer (100 ml)

  0.72 M BisTris (15.07 g)

  0.32 M Bicine (5.22 g)

- Cathode buffer (1L)

  0.2 M Bicine (32.64 g)

  0.1 M NaOH (4 g)

  0.25 % SDS (25 ml 10% SDS)

- Anode buffer (1L)

  0.2 M Tris (24.22 g)

  0.05 M H_2SO_4 (2.8 ml)

Transfer buffer (1x)

- 100 ml Methanol
- 14.4 g Glycine
- 3.03 g Tris-base
- Dissolve in 1 L of H_2O

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