Defining the role of p75 neurotrophin receptor ($p75^{NTR}$) in the development of Alzheimer's disease

**A THESIS SUBMITTED IN TOTAL FULFILMENT
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THESIS SUMMARY

The dysregulation of neurotrophins and their receptors plays a crucial role in the pathological process of sporadic Alzheimer's disease (AD). Here, we investigated the potential functions of $p75^{NTR}$ in the development of AD. We have found that $p75^{NTR}$ interacts with APP and Aβ, as a $p75^{NTR}$ ligand, promotes the interaction. To address the significance of this $p75^{NTR}$/APP interaction in AD, we discovered that $p75^{NTR}$ transfection increased amyloidogenic processing of APP in CHO^{APP695}. Aβ enhances APP amyloidogenic processing in mouse cortical neurons of AD/$p75^{+/+}$, but not in AD/$p75^{-/-}$ neurons via upregulation of APP and BACE1 expression. $A\beta_{42}$ increases the internalization of APP and the internalization of BACE1 through $p75^{NTR}$. In addition, Aβ and proNGF increased the APP/BACE1 interaction. The $A\beta_{42}$/$p75^{NTR}$ association regulates the phosphorylation of APP-Thr668 and phosphorylation of Tau in mouse cortical neurons.

It was shown that Sortilin interacts with BACE1, mediates retrograde trafficking of BACE1 and promotes Aβ generation. We have elucidated that BACE1, the rate-limiting enzyme processing APP, interacts with $p75^{NTR}$, as a co-receptor for Sortilin, and regulates its proteolytic processing. Our results present that BACE1 interacts with p75ECD. Aβ and proNGF significantly enhanced the BACE1/$p75^{NTR}$ interaction. The ratio of p75ECD/p75FL in $BACE^{+/+}$ mouse brain was significantly higher than in $BACE^{-/-}$ mouse brain. p75ECD is increased in cell lysates, but reduced in culture medium, of HEK-293T cells co-transfected with BACE1/$p75^{NTR}$ plasmids. To address the physiological function of p75ECD in AD, we found that p75ECD significantly rescued Aβ and proNTs-induced impairment of neurite outgrowth in cortical neurons.

The neurotrophin receptor $p75^{NTR}$ mediates both neurotrophic and neurodegenerative signals and its ectodomain shedding from the cell surface are physiologically regulated. We have conducted an *in vivo* study to investigate the effects of p75ECD-Fc recombinant protein on cognitive function and neuropathology features of AD in an AD mouse model. Our data showed that i.p delivery of p75ECD-Fc was not effective on cognitive function in APPswe/PS1DE9 (AD) mouse. p75ECD-Fc improved the process of learning, but not memory impairment in tau pathology-related tyrosine phosphorylation (PR5) mouse model. p75ECD-Fc significantly decreased the size and number of Aβ plaques in AD mouse brain through inhibition of BACE1 expression. p75ECD-Fc significantly reduced GFAP levels in AD mouse. Moreover, p75ECD-Fc was not effective in restoring the level of synaptic proteins, including the vesicle-associated membrane protein (VAMP2) and synaptosomal-associated protein 25 (SNAP-25) in AD mouse brain. p75ECD-Fc did not change ChAT levels, but it significantly reduced Tau phosphorylation and inhibited BACE1 expression in PR5 mouse brain.

We further investigated the expression and regulation of Sortilin, as a $p75^{NTR}$ co-receptor, in AD. Our data showed that Sortilin expression is significantly increased in human AD brains and in brains of 6-month old APPswe/PS1dE9 transgenic mice in comparison with relevant control groups. $A\beta_{42}$ enhanced the protein and mRNA expression level of Sortilin in SH-SY5Y cells. In addition, proBDNF also significantly increased the mRNA and protein expression of Sortilin. We found the inhibition of $p75^{NTR}$ and ROCK, but not JNK, suppressed constitutive and $A\beta_{42}$-induced expression of Sortilin.

Taken together, the full length of $p75^{NTR}$ mediates APP processing and contributes to AD pathogenesis via Aβ-induced upregulation of BACE1, APP and Sortilin, whereas

the p75ECD fragment is a novel neurotrophic molecule and protects the brain from toxicity induced by Aβ and proNTs.

DECLARATION

'I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the test.'

Khalil Saadipour

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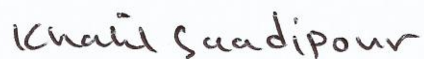

PUBLICATIONS AND SEMINARS ARISING FROM THIS THESIS

Publications:

1. Wang YJ, Zeng F, **Saadipour K**, Lu JJ, Zhou XF. $p75^{NTR}$- A molecule with multiple functions in amyloid-beta metabolism and neurotoxicity (2014); J. Neurotoxicity Research (*Handbook*).

2. **Saadipour K**, Yang M, Lim Y, Georgiou K, Sun Y, Keating DJ, Liu J, Wang YR, Gai WP, Zhong JH, Wang YJ, Zhou XF. Amyloid beta $_{1\text{-}42}$ ($A\beta_{42}$) up-regulates the expression of Sortilin via the $p75^{NTR}$/RhoA signalling pathway (2013); J. Neurochemistry 127(2):152-62. doi: 10.1111/jnc.12383.
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3. Yang M, Virassamy B, Lekha Vijayaraj S, Lim Y, **Saadipour K**, Wang YJ, Han YC, Zhong JH, Carlos R. Morales CR, Zhou XF. The Intracellular Domain of Sortilin Interacts with Amyloid Precursor Protein and Regulates Its Lysosomal and Lipid Raft Trafficking (2013); PLoS One **8**(5): e63049. doi: 10.1371/journal.pone.0063049.

4. Yao X[¥], Jia S[¥], **Saadipour K**[¥], Wang S, Zeng F, Wang Q, Wang Y. Zhong J, Zhou H, Zhou XF and Wang YJ. $p75^{NTR}$ ectodomain is a physiological neuroprotective molecule against amyloid-beta toxicity in the brain of Alzheimer's disease (Under review). ***¥ First equal co-authors***

5. **Saadipour K**, Lim Y, Zhou XF. A simplified method for the brain meninges removal of neonatal mouse for cortical neuron culture (Under review).

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Conference abstracts:

1. **Khalil Saadipour**, Yoon Lim, Jia Liu, Damien J. Keating, YeRan Wang, Jin-hua Zhong, Yan-Jiang Wang and Xin-Fu Zhou. Aβ induces BACE1 upregulation and enhances APP processing through cross-talk with $p75^{NTR}$. Alzheimer's association International Conference (AAIC), 12th-17th of July 2014, Copenhagen, Denmark. Poster presentation.

2. **Khalil Saadipour**, Yoon Lim, Jia Liu, YeRan Wang, Damien J. Keating, Yan-Jiang Wang and Xin-Fu Zhou. BACE1 regulates the proteolytic processing of $p75^{NTR}$ via interacting with its extracellular domain. Australasian Neuroscience Society 34th Annual Meeting, Jan 2014, Adelaide, Australia. Oral presentation.

3. **Khalil Saadipour**, Miao Yang, Kristen Georgiou, Yoon Lim, Shen Liu, Ying Sun, Wei-Ping Gai, Damien Keating and Xin-Fu Zhou. Amyloid $beta_{1-42}$ up-regulates expression of Sortilin mRNA and protein in SH-SY5Y human neuroblastoma cells. Australian Neuroscience Society 33rd Annual Meeting, Feb 2013, Melbourne, Australia. Poster presentation.

4. **Khalil Saadipour**, Miao Yang, Yoon Lim, Kevin Smith, Shen Liu, Ying Sun, Yan-Jiang Wang and Xin-Fu Zhou. Amyloid beta mediates APP processing through $p75^{NTR}$ in Alzheimer's disease. Australian Society for Medical Research (ASMR), 6th June 2012, Adelaide, Australia. Poster presentation.

AWARDS

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ABBREVIATIONS

AA	Amino acid
Aβ	Amyloid beta/ Beta amyloid
AD	Alzheimer's disease
ADAM	A disintegrin and metalloproteinase
AICD	APP intracellular domain
ANOVA	Analysis of variance
AP	Anteroposterior (axis)
APLP	Amyloid precursor-like protein
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
BACE1	Beta-site amyloid precursor protein cleaving enzyme 1
BBB	Blood–brain barrier
BCA	Bicinchoninic acid (kit)
BDNF	Brain-derived neurotrophic factor
BF	Basal forebrain
BFCN	Basal forebrain cholinergic neurons
bp	base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cdk5	Cyclin-dependent protein kinase 5
CFP	Cyan fluorescent protein
CGNs	Sensory and cerebellar granule neurons
ChAT	Choline acetyltransferase
CHO^{APP695}	Chinese hamster ovary cells expressing APP695 protein
CNS	Central nervous system

CO2	Carbon dioxide
Co-IP	Co-immunoprecipitation
CREB	cAMP responsive element binding (signalling)
CSF	Cerebrospinal fluid
CTF	C-terminal fragment
Cy3	Cyanine-3 fluorescence dye
DAB	3,3'-Diaminobenzidine
DAPI	4' 6-Diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonuclease I
DRG	Dorsal root ganglion
DS	Down syndrome
DV	Dorsoventral (axis)
Dyrk1A	Dual specificity tyrosine-phosphorylation-regulated kinase 1A
ECD	Extracellular domain
EDTA	Ethylene diamine tetraacetic acid
EEA1	Early endosome antigen 1
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAD	Familial Alzheimer’s disease
FBS	Fetal bovine serum
Fc	related to IgG “Fc” chain
FRET	Förster resonance energy transfer

FRET AB	FRET Acceptor bleaching
Gab1	GRB2-associated-binding protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM130	cis-Golgi matrix protein
GO	Glucose oxidase
GRP78	Glucose-regulated protein
GSK-3β	Glycogen synthase kinase 3β
GTP	Guanosine triphosphate
HA-tag	Hemagglutinin-tag
HAB	Head activator binding protein
HEK-293T	Human embryonic kidney-293T cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
HRP	Horseradish peroxidase
ICD	Intracellular domain
ICC	Immunocytochemistry
IGF-1R	Insulin-like growth factor 1 receptor
IHC	Immunohistochemistry
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IS	Interstitial (fluid/space)
JNK	c-Jun N-terminal kinases
kb	kilobase
kDa	kilodalton
KPI	Kunitz protease inhibitor
LAMP1	Lysosomal-associated membrane protein 1

LM	Lateromedial
LRP	Lipoprotein receptor-related protein
LTD	Long-term depression
LTP	Long term potentiation
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinase
MAP-2	Microtubule-associated protein-2 (antibody)
MAPs	Microtubule-associated proteins
MARK	Microtubule-affinity-regulating kinase
MBGIs	Myelin-based growth inhibitors
mRNA	Messenger RNA
MTT	Methyl Thiazoly Blue Tetrazolium Bromide (assay)
MW	Molecular weight
MWM	Morris water maze
N	Normal
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NBM	Nucleus Basalis of Meynert
NC	Negative control
NEB	New England Biolabs
NEP	Neprilysin
NFTs	Neurofibrillary tangles
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NgR	Nogo receptor
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4

NTs	Neurotrophins
OB	Olfactory bulb
OD	Optical density
OmGP	Oligodendrocyte myelin glycoprotein
OS	Oxidative stress
p3	Peptide 3
p53	Tumour protein p53
p75ECD-Fc	Extracellular domain of $p75^{NTR}$
p75KO	p75knockout or $p75^{-/-}$ (mouse)
$p75^{NTR}$	p75 neurotrophin receptor
p75WT	p75wild type or $p75^{+/+}$ or 129sv (mouse)
PBS	Phosphate-buffered saline
PBS-CM	Phosphate-buffered saline with calcium chloride and magnesium chloride
PBST	Phosphate-buffered saline with Tween-20
PC	Positive control
PC12 cells	Rat adrenal pheochromocytoma cells
PCR	Polymerase chain reaction
PDL	Poly-D-Lysine
PF (4%)	Paraformaldehyde solution
PHFs	Paired helical filaments
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLC-γ1	Phospholipase C-γ1
PMSF	Phenyl methane sulfonyl fluoride
PNS	Peripheral nervous system

PR5	Tau pathology-related tyrosine phosphorylation (mouse)
proBDNF	Precursor form of brain-derived neurotrophic factor
proNGF	Precursor form of Nerve Growth Factor
proNTs	Precursor form of neurotrophins
PS1 or 2	Presenilin-1 or 2 (enzyme)
RAGE	Receptor for advanced glycation end products
RhoA	Ras homolog gene family, member A
RIPA	Radioimmunoprecipitation assay (buffer)
ROCK	Rho-associated protein kinase
ROI	Region of interests
RPM	Revolutions per minute
RT-PCR	Real-time quantitative PCR
SAPK1b	Stress activated protein kinase 1b
sAPPα	non-Amyloidogenic soluble form of APP
sAPPβ	Amyloidogenic soluble form of APP
Scr.	Scramble
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of mean
SNAP-25	Synaptosomal-associated protein 25
SORCS	Sortilin-related Vps10p domain containing receptor 1
SorLA	Sorting protein-related receptor with A-type repeats
SPSS	Statistical Package for the Social Sciences
SVZ	Sub-ventricular zone
TACE	Tumour necrosis factor-alpha converting enzyme
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline with Tween 20

2x Tg	Double transgenic (mouse)
TGN	Trans-Golgi network
Tm	Melting temperature
TMD	Transmembrane domain
TNF-alpha	Tumour necrosis factor-alpha
Trk	Tyrosine protein kinase/ Tropomyosin-related kinase (receptor)
V	Voltage
VAMP2	Vesicle-associated membrane protein 2
Vps10p	Vacuolar protein sorting 10 protein
vs	versus
WT	Wild type
YFP	Yellow fluorescent protein

CHAPTER 1: INTRODUCTION

1.1 Alzheimer's disease (AD)

Dementia is a clinical term, describing the deterioration of neurons, the associated cognitive dysfunction and reduced capacity for independent living. Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most common form of dementia characterized by impairment of cognitive function (Katzman 1986, Selkoe 2002, Blennow, de Leon et al. 2006). More than 24 million worldwide people had dementia in 2001, and it is estimated the number of people who are suffering from AD to double every two decades up to 81 million in 2040 (Ferri, Prince et al. 2005). Based on onset age prior to or after 60 years old, AD is grouped into early and late-onset, respectively. The prevalence of late-onset AD increases during aging without reaching a plateau (Morris 1999). The main pathologic hallmarks of AD include accumulation of extracellular amyloid beta (Aβ) peptide containing plaques, and intracellular neurofibrillary tangles of abnormally phosphorylated Tau, astrocytic gliosis, reactive microglia, inflammation, and neuronal and synaptic loss (Geula 1998, Lee, Goedert et al. 2001, McGeer and McGeer 2001, Schubert, Ogata et al. 2001, Selkoe 2001). Histological studies show these hallmarks of AD in different parts of the brain such as the parietal and temporal cortex, the hippocampus, the entorhinal cortex, and the amygdala. These result in malfunction of psychomotor performance and cognitive function in AD patients. For instance, deterioration of cholinergic neurons in the basal forebrain is one of the earliest and most important pathological manifestations in this disease (Bowen, Smith et al. 1976, Davies and Maloney 1976, Whitehouse, Price et al. 1982, Whitehouse, Struble et al. 1982, Coyle, Price et al. 1983, Pearson, Sofroniew et al. 1983). Episodic amnesia and

verbal disorders are also common problems seen in AD (Hodges, Salmon et al. 1990, Henry, Crawford et al. 2004).

There are some evidences showing that development of AD is accelerated by Down syndrome (DS). The amyloid precursor protein (APP) gene, encompassing ~ 400 kb of DNA, is located in the mid-portion of the long arm of human chromosome 21 and as such people with DS have an extra copy of chromosome 21 and are more prone to develop AD (Kang, Lemaire et al. 1987, Lamb, Sisodia et al. 1993). In addition, there is a significant association between inflammatory mechanisms in the ethology of cardiovascular disease and AD (Finch 2005). For example, inflammatory factors and abnormalities in cholesterol metabolism in DS are proposed to contribute to the development of AD (Finch 2005).

1.2 Amyloid precursor protein (APP) and its processing and function in AD

APP is a single membrane-spanning protein with a large extracellular amino-terminal domain (ECD) and a small intracellular cytoplasmic domain (ICD). The ECD has a cysteine-rich subdomain close to the extreme amino terminus which is followed by three subdomains, one of which may have neuroprotective functions (De Strooper, Annaert et al. 1999). Sequential structural studies on APP indicate that APP has 653 shared amino acids (AA) belonging to a type-I transmembrane protein family known as the amyloid precursor-like protein (APLP) (Wasco, Bupp et al. 1992). In mammals the APP family consists of APP, APLP1 and APLP2 (Koo, Sisodia et al. 1990, Vetrivel, Cheng et al. 2004). Three major isoforms of APP including APP695, APP751, and APP770 are identified according to their AA numbers. The APP751 and APP770 have a 56 amino acid Kunitz protease inhibitor (KPI) domain within

their extracellular regions and they are expressed in most tissues, but APP695 does not have KPI within ECD and is mainly expressed in neurons. Based on this, the APP695 isoform consequently contributes to Aβ generation and the development of AD (Riddell, Christie et al. 2001, Small and Gandy 2006). However, some evidence indicates that the protein and mRNA levels of APP751 and APP770 isoforms are increased in AD and overexpression of these isoforms subsequently results in over production of Aβ in the brain of AD patients (Ehehalt, Keller et al. 2003). Many studies have been done on APP since it was first identified, but its exact biological function is still not fully understood. APP has multiple functions in the CNS such as neurite outgrowth and synaptogenesis, neuronal protein trafficking along the axons, transmembrane signal transduction, cell adhesion, and calcium metabolism. (Turner, O'Connor et al. 2003, Zheng and Koo 2006). Several fragments are produced when full length APP is processed by different proteolytic enzymes during intracellular trafficking through amyloidogenic and non-amyloidogenic pathways (Figure 1-1). Each segment has different functions, so the net biological effect of APP perhaps depends on the sum of functions of all APP metabolites in neurons (Zhang, Thompson et al. 2011). Shared similarities in structural and proteolytic processing of APP and other transmembrane receptors such as Notch imply that APP may have similar functions as Notch receptors (Zhang, Thompson et al. 2011). APP has several agonists such as Aβ (Lorenzo, Yuan et al. 2000), F-spondin (Ho and Sudhof 2004), and netrin-1 (Lourenco, Galvan et al. 2009) and each one triggers different intracellular signalling pathway when they bind APP.

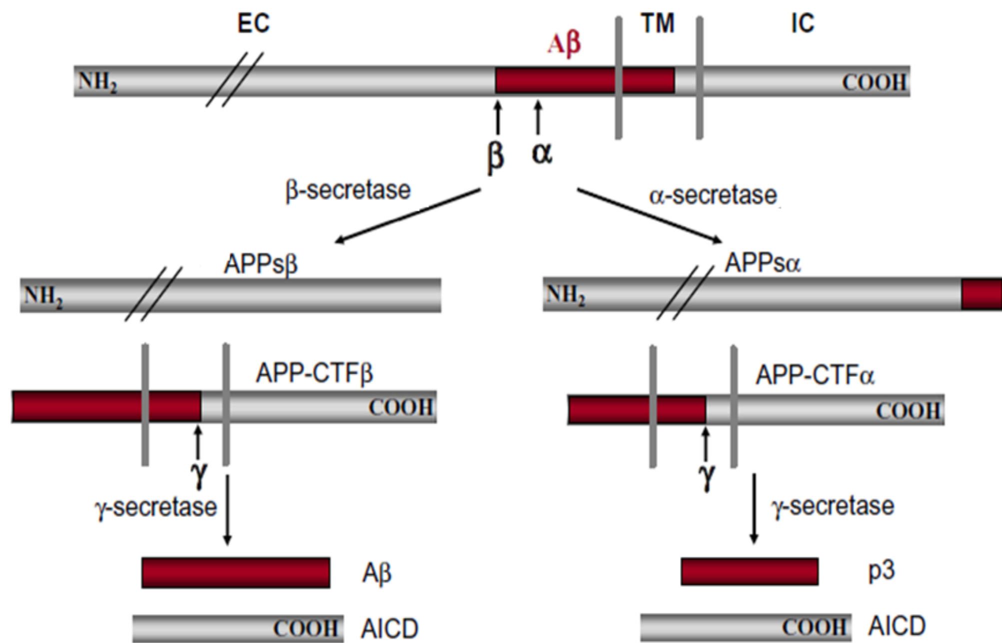


Figure 1-1: The enzymatic processing pathways of amyloid precursor protein (APP) in neurons

EC: Extracellular; TM: Transmembrane; IC: Intracellular domain of APP; APPsβ: APP secreted β; APPsα: APP secreted α; CTFβ: β-secretase generates C-terminal fragment; CTFα: α-secretase generates C-terminal fragment; Aβ: Amyloid beta; p3: peptide 3; AICD:APP Intracellular domain (Zheng and Koo 2006).

APP is synthesized in the endoplasmic reticulum (ER) and subsequently transported through the Golgi apparatus to the trans-Golgi-network (TGN) in neurons in order to complete the process of glycosylation and maturation (Weidemann, Konig et al. 1989, Hartmann, Bieger et al. 1997, Xu, Sweeney et al. 1997, Greenfield, Tsai et al. 1999). $A\beta_{x\text{-}42}$ is mainly generated in the ER. TGN also contributes to $A\beta_{40}$ production, as a fragment of APP and subsequently $A\beta_{40}$ is packaged into post-TGN secretory vesicles. $A\beta_{42}$ ($A\beta_{1\text{–}42}$ plus $A\beta_{x\text{-}42}$) also is made in the TGN and packaged into secretory vesicles. Two types of Amyloid peptides, detergent and non-detergent extractable soluble fragments are produced in TGN (Greenfield, Tsai et al. 1999). On the other hand, when APP is moved from TGN-derived secretory vesicles to the cell surface, the non-amyloidogenic processing of APP, which is constitutively cleaved by α- secretase in the Aβ sequence at 17 amino acids NH2- terminal to the single transmembrane domain of APP, occurs in lipid rafts. The α-secretase is a zinc metalloproteinase and membrane-bound endoprotease which cleaves APP at the Lys16-Leu17 bond of Aβ and releases a large soluble ectodomain of APP known as sAPPα (Sisodia 1992, Roberts, Ripellino et al. 1994). The processing of APP in the non-amyloidogenic pathway also results in an 83-amino acid C-terminal fragment (CTFα) which is retained in the cytoplasmic membrane and lipid raft (Sisodia 1992, Kojro and Fahrenholz 2005).

In contrast to Aβ, which has neurotoxic and neurodegenerative effects on neurons, sAPPα appears to have critical neuroprotective effects such as neuronal plasticity, neurite outgrowth, synaptogenesis, and cell survival. sAPPα increases long term potentiation (LTP) and modulates neuronal excitability (Ishida, Furukawa et al. 1997, Taylor, Ireland et al. 2008). sAPPα rescues neurological abnormalities caused

by APP deficiency such as cognitive dysfunction, deficits in synaptic maturation and neuronal maintenance in the brain of APP knockout mice (Ring, Weyer et al. 2007). In addition, sAPPα modulates cell adhesion and regulates proliferation of non-neuronal cells (Furukawa, Sopher et al. 1996, Mattson 1997). It was reported that sAPPα can act as a regulator of neuro-proliferation in the brain and sub-ventricular zone (SVZ) progenitor cells in the adult CNS (Ohsawa, Takamura et al. 1999, Caille, Allinquant et al. 2004).

The amyloidogenic processing pathway of APP which is initiated by BACE1 (β-site amyloid precursor protein cleaving enzyme 1), results in generation of soluble fragment of APPβ (sAPPβ) and subsequently Aβ and CTFβ production. In this process BACE1 plays a critical role in Aβ production. BACE1 is a type I transmembrane protein with 501 AA, was identified by 5 groups during 1999-2000 (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000). BACE1 has two aspartic protease active site motifs, DTGS (residues 93–96) and DSGT (residues 289–292), and mutation of either aspartic acid inactivates the proteolytic function of BACE1 and inhibits the processing of APP and Aβ generation (Hussain, Powell et al. 1999, Bennett, Denis et al. 2000). BACE1, like other β-secretases, has a luminal active site which provides a suitable topological orientation for APP processing in the intracellular compartment. BACE1, as well as other β-secretases, has six luminal cysteine residues that form three intramolecular disulfide bonds and several N-linked glycosylation sites (Haniu, Denis et al. 2000). This enzyme cleaves APP at the amino terminus, between residues 671 and 672 of APP, and generates the N-terminal end of Aβ (Sinha, Anderson et al. 1999). Several studies have shown that overexpression of

BACE increases Aβ production, so it is suggested that BACE1 is responsible for the generation Aβ (Vassar, Bennett et al. 1999). While BACE1 contributes to Aβ generation in AD pathogenesis, it is also necessary for normal brain function as BACE1 deficiency in mice results in impairments of hippocampal neuronal plasticity and regulation of myelination and cognitive deficits (Laird, Cai et al. 2005, Willem, Garratt et al. 2006).

In the non-amyloidogic and amyloidogic APP processing pathways, after production of CTFα and CTFβ, the residue of APP is cleaved by other enzymes which belong to the γ-secretase family. This results in p3 and Aβ production in the non-amyloidogic and amyloidogic pathways, respectively (Figure 1-1). In contrast to Aβ and sAPPα, p3 does not have any known physiological or pathological function in AD. γ-secretase plays a critical role in production of $A\beta_{40}$ and $A\beta_{42}$ as well as the intracellular domain of APP (AICD) which are often considered as therapeutic targets in AD (Zhao, Tan et al. 2007). AICD plays an important role to regulate the interaction of APP with various cytosolic factors which control intracellular trafficking of APP and this process is controlled by the phosphorylation site of AICD (Tamayev, Zhou et al. 2009). Over expression of AICD promotes cell death in different cells. For example, AICD31, AICD57, or AICD59 isoforms induce apoptosis after transfection in HeLa, H4, N2a or PC12 cells (Lu, Rabizadeh et al. 2000, Kinoshita, Whelan et al. 2002). AICD phosphorylation on Thr668 modulates AICD/FE65 interaction to induce apoptosis in cells (Chang, Kim et al. 2006). AICD mediates Tau hyper phosphorylation via glycogen synthase kinase-3beta (GSK3β) which and then leads to neuronal microtubule destabilization and cell-cell adhesion and eventually cell death (Kim, Kim et al. 2003, Muller, Concannon et al. 2007). In

addition, it is reported that AICD induces cell death signalling through activation of caspase-3 and p53 tumour suppressor protein genes (Checler, Sunyach et al. 2007).

1.3 Aβ and its function in the brain

In 1984 Aβ, ~ 4 kD a peptide with a β-pleated sheet structure (Figure 1-2), was characterized and implied to contribute to AD pathogenesis (Glenner and Wong 1984). Overproduction of Aβ in the brain results in neurosynaptic malfunction, formation and accumulation of neurofibrillary tangles and eventually cell death in the AD brain (Selkoe 1998). The loss of neuronal synapses appears before other symptoms and accompanies cognitive impairment (Shankar and Walsh 2009). $A\beta_{39}$, $A\beta_{40}$ and $A\beta_{42}$ peptides, which differ only in their carboxyl terminus are responsible for the pathogenesis of AD.

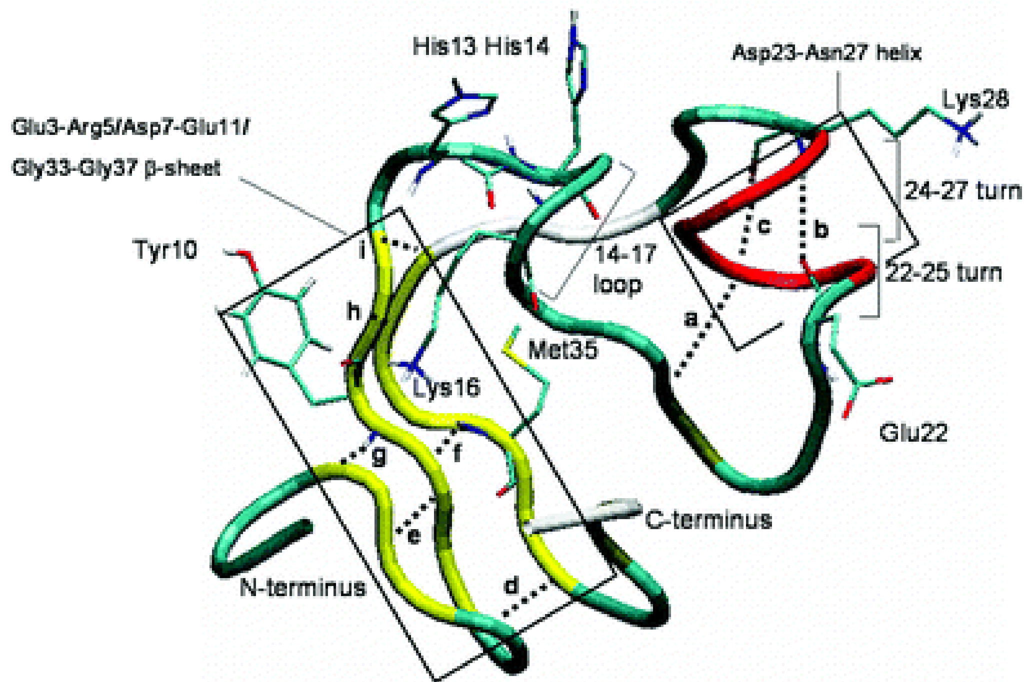


Figure 1-2: The structure of $A\beta_{42}$ monomer in AD (Rauk 2008).

$A\beta_{40}$ and $A\beta_{42}$ are more toxic than other forms. $A\beta_{42}$ in comparison with $A\beta_{40}$ is more hydrophobic and has greater potential to form oligomers, fibrils and amyloidogenic plaques (Burdick, Soreghan et al. 1992, Jarrett, Berger et al. 1993, Iwatsubo, Odaka et al. 1994). Furthermore, some studies show that the ratio of $A\beta_{42}/A\beta_{40}$ increases in familial AD (FAD) (Borchelt, Thinakaran et al. 1996, Scheuner, Eckman et al. 1996, Walsh and Selkoe 2007). After APP processing by β- and γ-secratase, Aβ, which is produced within some cell organelles such as the ER, TGN and endosome/lysosome, is secreted out of cells. This extracellular Aβ can be internalized by cells. The accumulation of Aβ in neurons in different regions of the brain such as the hippocampus and entorhinal cortex, areas involved in cognitive function, induces cell degeneration and AD (Gouras, Tsai et al. 2000). Studies on DS brain also demonstrate some neuropathologic changes similar to AD (Finch 2005). The intracellular accumulation and deposition of Aβ inside and outside neurons and astrocytes consequently results in the formation of diffuse plaques in DS patients but not in aged matched non-DS patients. Furthermore, development of neuritic plaques and neurofibrillary tangles has been observed in the brain of DS patients (Gyure, Durham et al. 2001). A steady level of Aβ as a result of the balance between Aβ production and clearance is critical to have a healthy brain. Disruption of this balance can result in accumulation and deposition of Aβ in the brain and eventually AD (Figure 1-3) (Zlokovic 2004, Wang, Zhou et al. 2006).

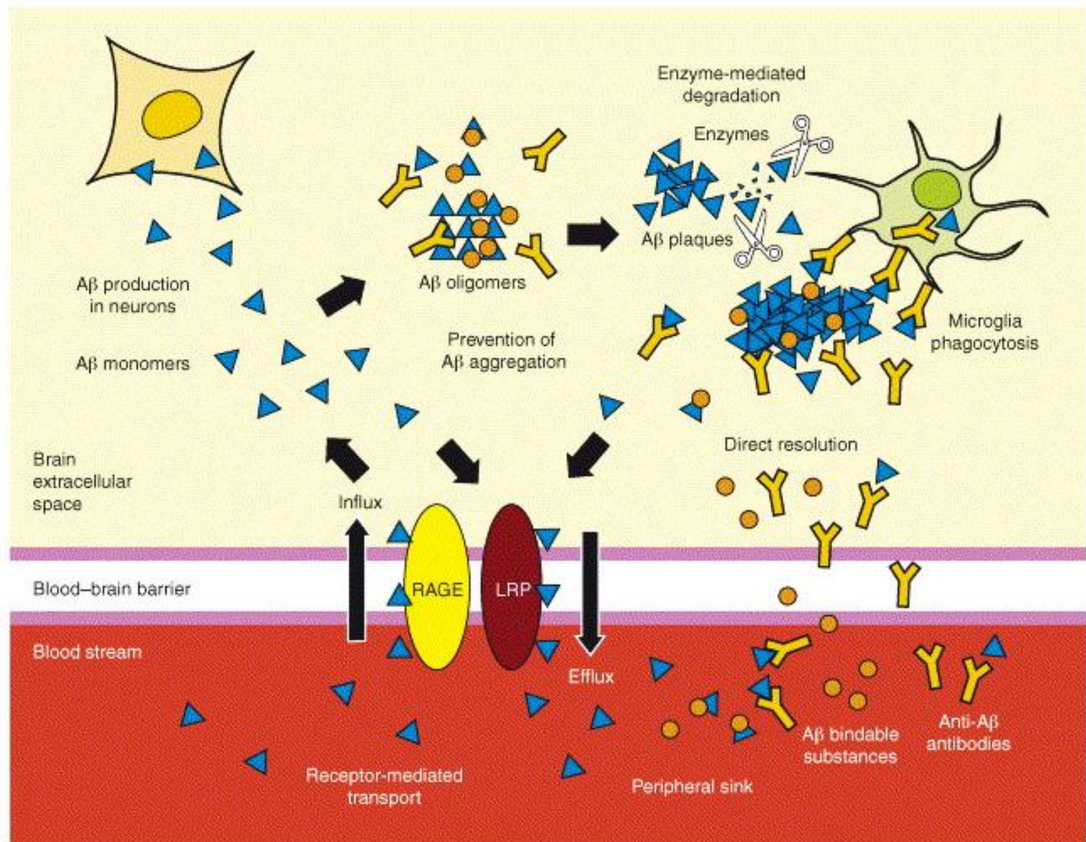


Figure 1-3: Mechanisms of Aβ homeostasis in the brain.

A steady-state level of Aβ in brain is a consequence of the balance between production and clearance. Many receptors such as receptor for advanced glycation end products (RAGE) and lipoprotein receptor-related protein (LRP) mediates Aβ transportation across the BBB. Neprilysin (NEP) and insulin-degrading enzyme (IDE) degraded Aβ in intra- and extra cellular space. Peripheral anti-Aβ antibodies and Aβ-bindable substances are able to enter the brain at low levels, where they prevent Aβ aggregation and resolve Aβ fibrils. By binding to peripheral Aβ they also exert as a peripheral sink to promote the efflux of Aβ from the brain and disrupt the Aβ equilibrium between the brain and the blood, resulting in the clearance of Aβ from the brain (Wang, Zhou et al. 2006).

The choroid plexus, which forms a barrier between blood and brain cerebrospinal fluid (CSF), regulates Aβ concentration in the CSF. It is reported that 10 to 15% of soluble Aβ are slowly cleared via interstitial (IS) bulk flow into the bloodstream (Barde, Edgar et al. 1982). Aβ transportation across the BBB is mediated by receptors such as lipoprotein receptor-related protein (LRP) (Lambert, Wavrant-De Vrieze et al. 1998, Kang, Pietrzik et al. 2000, Shibata, Yamada et al. 2000, Van Uden, Mallory et al. 2002), P-glycoprotein (Lam, Liu et al. 2001, Vogelgesang, Cascorbi et al. 2002), receptor for advanced glycation end products (RAGE)(Yan, Zhu et al. 2000, Deane, Du Yan et al. 2003) and gp33p/megalin (Zlokovic, Martel et al. 1996). A percentage of Aβ is eliminated by enzymes which belong to the zinc metalloendopeptidase family. For example, Neprilysin (Liepinsh, Ilag et al. 1997, Iwata, Tsubuki et al. 2001, Yeo, Connie Hung et al. 2004, Caccamo, Oddo et al. 2005), Insulin-degrading enzyme (Coulson, Reid et al. 2000, Farris, Mansourian et al. 2003, Morelli, Llovera et al. 2004), endothelin-converting enzyme (Eckman, Reed et al. 2001, Feng, Kim et al. 2010), and Angiotensin-converting enzyme (He and Garcia 2004) all contribute to the degradation of Aβ in the brain.

1.4 The role of ApoE on Aβ aggregation and clearance in AD

Homo sapiens apolipoprotein E (ApoE) is a glycoprotein consisting of 299 AA with variable levels of post-translational sialylation through O-linked glycosylation at the threonine 194 residue (Wernette-Hammond, Lauer et al. 1989). ApoE is highly expressed in human liver and brain, but it is mainly expressed in non-neuronal cells such as astrocytes and glia cells in the brain (Pitas, Boyles et al. 1987, Grehan, Tse et al. 2001). ApoE acts as a ligand in receptor-mediated endocytosis of lipoprotein particles (Fagan, Holtzman et al. 1999). The ε4 allele of ApoE is a major genetic risk

factor for AD. It is reported that ApoE highly interacts with Aβ (Naslund, Thyberg et al. 1995) and increases Aβ toxicity via inducing a pathological β-sheet conformational change in Aβ (Wisniewski and Frangione 1992). Studies on mice brain showed that the ApoE-Aβ interaction disrupts Aβ clearance through the BBB. Moreover, it is reported that the disruptive effect ApoE4 on Aβ clearance is more than that of either ApoE3 or ApoE2 (Deane, Sagare et al. 2008).

1.5 The role of protein phosphorylation in AD

Phosphorylation of proteins modifies protein behaviours such as enzymatic activity, subcellular localization, ligand binding and protein-protein interaction. Protein phosphorylation is thought to alter the progression of AD. Therefore, the regulation of protein phosphorylation by protein kinases/phosphatases has been considered a therapeutic target for the treatment of AD (Chung 2009).

1.5.1 The role of APP phosphorylation in AD

The APP short cytoplasmic C-terminal domain is very important in the trafficking and physiological function of APP. Interaction between the C-terminal domain of APP and other proteins such as X11/Mint, Fe65 (Russo, Venezia et al. 2005) and $p75^{NTR}$ (Fombonne, Rabizadeh et al. 2009) affects APP processing either by stabilizing APP or by regulating Aβ production. The C-terminal domain of APP has several phosphorylation sites which are phosphorylated by different protein kinases. For instance, phosphorylation of APP by PKC at residues Thr654/Ser655 initiates non-amyloidogenic processing of APP and may protect the brain from AD (Gandy, Caporaso et al. 1993).

The intracellular domain of APP contains eight potential phosphorylation sites and the phosphorylation of seven regions including Y653, S655, T668, S675, Y682, T686, and Y687 (APP695 isoform numbering) are increased in AD (Lee, Kao et al. 2003). The phosphorylation of APP at Thr668 is common in AD and mediated by neuronal cyclin-dependent protein kinase 5 (cdk5) (Iijima, Ando et al. 2000), p34cdc2 protein kinase (Suzuki, Cheung et al. 1994), glycogen synthase kinase 3β (GSK-3β) (Aplin, Gibb et al. 1996), or c-jun N-terminal kinases (Standen, Brownlees et al. 2001, Taru, Iijima et al. 2002, Taru and Suzuki 2004, Kimberly, Zheng et al. 2005).

Interestingly, it has been reported that phosphorylation of APP at Thr668 enhances APP and BACE1 convergence, thus promoting APP processing and Aβ generation. Inhibition of Thr668 phosphorylation abolishes BACE1-mediated cleavage of APP and Aβ production through a reduction of the APP/BACE1 association (Lee, Kao et al. 2003).

1.5.2 Hyper-phosphorylation of Tau in AD

Tau is a neuronal protein and a member of microtubule-associated proteins (MAPs) family that promotes the assembly and stabilization of neuronal microtubules (Cleveland, Hwo et al. 1977). The human Tau gene is over 100 kb and located on the long arm of chromosome 17 at band position 17q21 and contains 16 exons. Exon 1 in the Tau gene is part of the promoter and it is transcribed but not translated. Some Tau exons including 1, 4, 5, 7, 9, 11, 12, and 13 are constitutive exons; exons 2, 3, and 10 are alternatively spliced in the adult brain. Exon 2 can appear alone, but exon 3 is usually in association with exon 2 (Sergeant, Delacourte et al. 2005). A single Tau isoform is expressed in fetal human brain while six isoforms are identified in

adult brain, with the fetal isoform corresponding to the shortest of the adult isoforms. More phosphorylation sites are present in fetal Tau protein than adult (Goedert, Jakes et al. 1993). In AD, an abnormal hyper-phosphorylation of Tau especially at Ser202 and Thr205 (Rankin, Sun et al. 2005), results in the formation of paired helical filaments (PHFs), also called neurofibrillary tangles (NFTs) (Selkoe 1991, Avila, Lucas et al. 2004). NFTs are considered another major causative characteristic of AD of Aβ amyloidosis (Hardy 2009). Over 39 phosphorylation sites have been recognized in PHFs-Tau, including multiple proline-directed serine/threonine residues (Kopke, Tung et al. 1993, Giese 2009). Several kinases and phosphatases such as GSK3β, Cdk5, Dyrk1A, PKA and microtubule-affinity-regulating kinase (MARK) contribute to phosphorylation of Tau protein. Therefore, any imbalance of these enzyme functions can result in Tau hyper-phosphorylation and formation of NFTs in AD brains (Johnson 2006, Ryoo, Jeong et al. 2007). However, phosphorylation of different sites of Tau causes different biological functions and pathological malfunctions of this protein. For instance, phosphorylation of Tau at Ser 262 decreases Tau binding to microtubules (Biernat, Gustke et al. 1993) and promotes $A\beta_{42}$-induced Tau toxicity in AD pathogenesis (Iijima, Gatt et al. 2010).

1.6 Neurotrophins (NTs) and their functions in AD

The neurotrophins are dimeric proteins that promote neuronal survival in vertebrates (Lewin and Barde 1996). All neurotrophins possess six shared cysteine residues that contribute to the formation of disulfide bonds. Neurotrophins are mainly expressed in neurons in the CNS and PNS and are first synthesized as a precursor form. The biological function of neurotrophins is regulated by specific proteases which convert precursor forms to mature forms in the cell (Thoenen 1995, Seidah, Benjannet et al.

1996, Lee, Kermani et al. 2001). For example, brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), are expressed in sensory and sympathetic nerves and modulate neuronal development, synapse formation and plasticity (Dechant and Barde 2002, Huang and Reichardt 2003, Lu, Pang et al. 2005). The physiological function of neurotrophins is not restricted to healthy neurons as they often participate in the recovery of neurons that are affected in other neurological and psychiatric pathologies including ischemia, epilepsy, depression and eating disorders (Dechant and Neumann 2002). Nerve growth factor (NGF) was the first identified neurotrophic factor and promotes survival of sympathetic and sensory neurons (Levi-Montalcini 1987, Shooter 2001). NGF improves the function of sympathetic neurons, nociceptors and temperature sensory neurons in the PNS. Moreover, NGF enhances the survival and neuronal activity of cholinergic neurons in the basal forebrain in the CNS (Chen, Nishimura et al. 1997). BDNF, a supportive factor of survival and neurite outgrowth, was the second neurotrophin discovered. It was originally extracted from pig brain (Barde, Edgar et al. 1982). So far NGF, BDNF, NT3, and neurotrophin-4 (NT4) have been identified in mammals. The majority of mature neurotrophins regulate neuronal survival, development and function via activation of tyrosine kinase (Trk) receptors (Figure 1-4 and Figure 1-5) (Reichardt 2006). Two different types of neurotrophin receptors interact with their ligands (Frade and Barde 1998). The first class of this receptor is $p75^{NTR}$, which binds with high affinity to proneurotrophins (proNGF, proBDNF, proNT-3, and proNT-4). The mature forms of neurotrophins, which are obtained by activation through proteolysis, also interact with $p75^{NTR}$, but they have more specific interactions with three Trk receptors. These receptors, TrkA, TrkB, and TrkC, are categorized as another class of mammalian neurotrophin receptor which can be activated by one or more of four mature

neurotrophins: NGF, BDNF, NT3 and NT4 (Rodriguez-Tebar, Dechant et al. 1990, Frade and Barde 1998, Chao 2003, Huang and Reichardt 2003). For instance, NGF binds specifically to TrkA; BDNF and NT4 interact with TrkB; while NT3 activates TrkC. In some cellular contexts, NT3 is also able to activate TrkA and TrkB with less efficiency (Figure 1-4) (Reichardt 2006). Trk receptors regulate neuronal development and function, including cell proliferation, cell survival, axon and dendrite growth, membrane trafficking, synaptic formation and function as well as glial differentiation and interactions with neurons through activation of different signalling pathways (Figure 1-5) (Reichardt 2006). For example, BDNF plays an important role in hippocampal plasticity and hippocampal-dependent memory. An interrupted transport of BDNF in neurons as a consequence of the polymorphism (V66M) in the BDNF gene results in impairment of hippocampal function and episodic memory (Egan, Kojima et al. 2003).

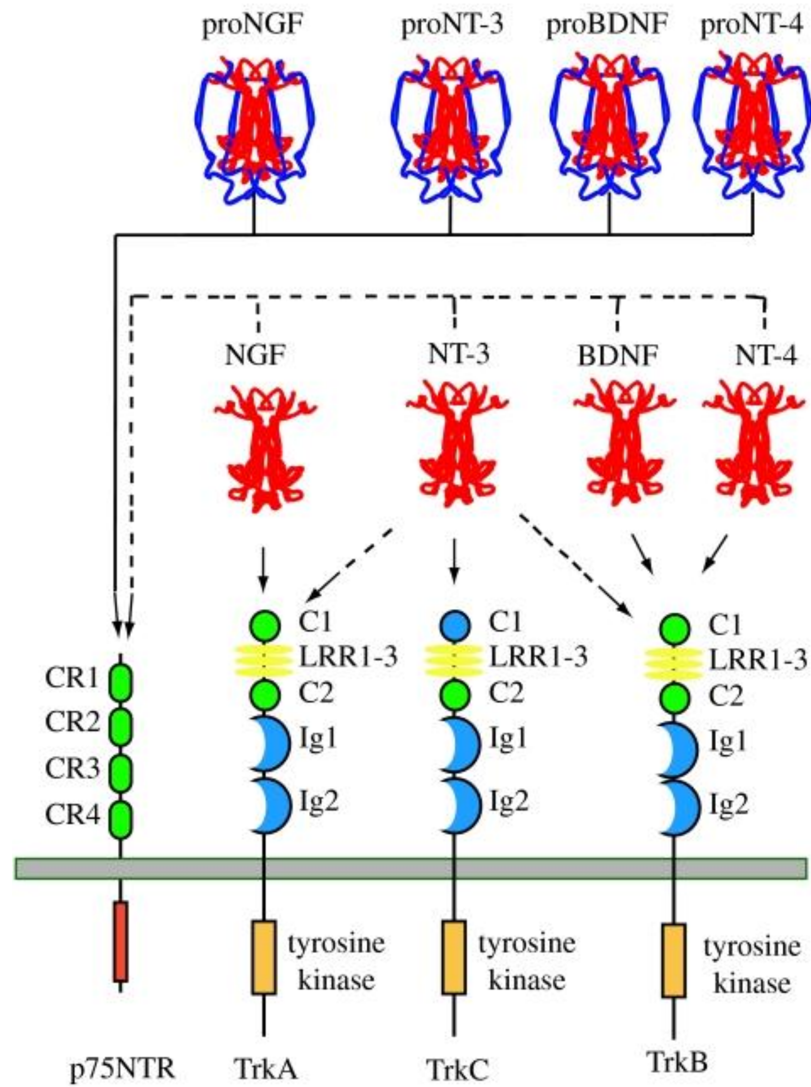
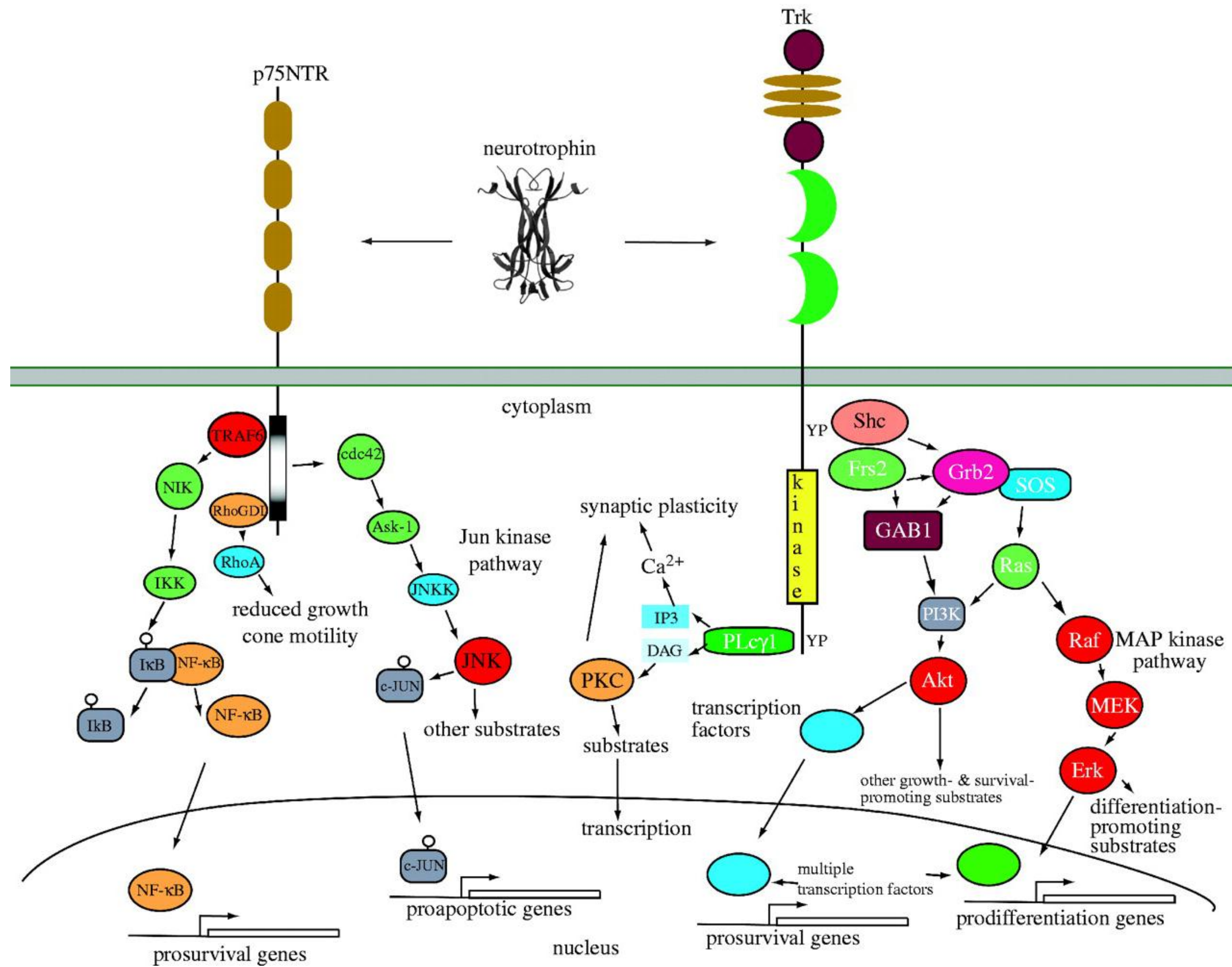


Figure 1-4: The binding of neurotrophin receptors with their ligands

All pro-neurotrophins bind $p75^{NTR}$, but not the Trk receptors. Although mature neurotrophins like proneurotrophins bind $p75^{NTR}$, they interact more potently with the three Trk receptors. For example, NGF binds TrkA; BDNF and NT4 interact with TrkB and NT3 activates TrkC. NT3 is capable to activate TrkA and TrkB with less efficiency. The interaction between ligands and neurotrophin receptors is also modulated by $p75^{NTR}$ (Reichardt 2006).



Trk
p75NTR
neurotrophin
cytoplasm
TRAF6
NIK
RhoGDI
RhoA
reduced growth cone motility
IKK
IκB
NF-κB
IκB
NF-κB
cdc42
Ask-1
Jun kinase pathway
JNKK
JNK
c-JUN
other substrates
synaptic plasticity
Ca2+
IP3
DAG
PLcγ1
PKC
substrates
transcription
YP
kinase
YP
Shc
Frs2
Grb2
SOS
GAB1
Ras
PI3K
Akt
Raf
MAP kinase pathway
MEK
Erk
transcription factors
other growth- & survival-promoting substrates
differentiation-promoting substrates
NF-κB
prosurvival genes
c-JUN
proapoptotic genes
nucleus
multiple transcription factors
prosurvival genes
prodifferentiation genes

Figure 1-5: The signalling pathways activated by neurotrophins

This depicts the different signalling pathways activated by individual neurotrophin ligands when they bind Trk and $p75^{NTR}$ receptors. The $p75^{NTR}$ activates three major signalling pathways. The activation of NF-κB by $p75^{NTR}$ results in transcription of multiple genes, and promoter of neuronal survival. The ligand-$p75^{NTR}$ interaction activates the Jun kinase (JNK) pathway and controls activation of several genes which promote neuronal apoptosis. Rho is another neuronal pathway downstream of agonist-$p75^{NTR}$ binding which regulates growth cone motility. $p75^{NTR}$ promotes apoptosis in co-operation with Sortilin. Three major signalling pathways are also activated by individual Trk receptor. Activation of Ras leads to activation of the MAP kinase-signalling cascade, which promotes neuronal differentiation and neurite outgrowth. Trk activates PI3 kinase through Ras or Gab1 promotes survival of neurons and growth other cells. Activation of PLC-γ1 results in activation of Ca^{2+}-and protein kinase C-regulated pathways that promote synaptic plasticity. Moreover, each of these signalling pathways regulates gene transcription (Reichardt 2006).

1.6.1 p75^{NTR} and its biological function

p75^{NTR} is a 75 kDa type-1 transmembrane receptor and belongs to the tumour necrosis factor (TNF) receptor family. The p75^{NTR} gene includes 10 exons and 9 introns and the protein was identified in 1973 as a receptor for NGF (Herrup and Shooter 1973). p75^{NTR} is expressed in a wide range of species and tissues and is responsible for apoptosis, survival and growth depending on the binding of different ligands and interactions with different receptors (Kaplan and Miller 2004). A high level of p75^{NTR} expression at early stages of life in the CNS and PNS indicates that p75^{NTR} contributes to growth and development of neurons. For example, p75^{NTR} regulates the development of dorsal root ganglion (DRG) (Ernfors, Hallbook et al. 1988) and sympathetic ganglia (Buck, Martinez et al. 1987). The expression level of p75^{NTR} is changed in pathological conditions. An upregulation of p75^{NTR} expression and downregulation of Trk receptors in the basal forebrain (BF) during aging were reported (Ginsberg, Che et al. 2006). Furthermore, p75^{NTR} is over-expressed after neuronal injury (Barrett and Bartlett 1994). In addition to neurons, p75^{NTR} is over-expressed in variety of glial tissues such as Schwann cells and oligodendrocytes during development and after injury (Cragnolini and Friedman 2008). p75^{NTR} interacts with pro and mature neurotrophins (Chao and Bothwell 2002, Barker 2004). The interaction between p75^{NTR} and Trk receptors with neurotrophins activates several signal transduction pathways, which subsequently promote the survival, differentiation and development of neuronal cells (Figure 1-5) (Reichardt 2006). In contrast to this, p75^{NTR} mediates apoptosis in neonatal sympathetic neurons (Bamji, Majdan et al. 1998), motor neurons (Sedel, Bechade et al. 1999), sensory neurons (Barrett and Bartlett 1994), oligodendrocytes (Casaccia-Bonnefil, Aibel et al. 1996), and embryonic proprioceptive neurons especially when Trk activation is limited (Davey and Davies 1998). Furthermore, p75^{NTR} triggers cell death in cholinergic

neurons independent of TrkA during development and after injury (Van der Zee, Ross et al. 1996). NGF stimulates neurite outgrowth of rat embryonic hippocampal and chick cilliary neurons which express $p75^{NTR}$ but not TrkA (Brann, Scott et al. 1999). Tumour necrosis factor alpha-converting enzyme (TACE)/ADAM17 is a proteolytic enzyme which cuts $p75^{NTR}$ at the juxta-membrane region and generates the extracellular domain of $p75^{NTR}$ (p75ECD) (Weskamp, Schlondorff et al. 2004). p75ECD includes 28 amino acids as a single peptide which is followed by four cysteine rich domains each of which contain 40 amino acid residues and each of them contains an N-glycosylated site (Figure 1-6)(Schor 2005).

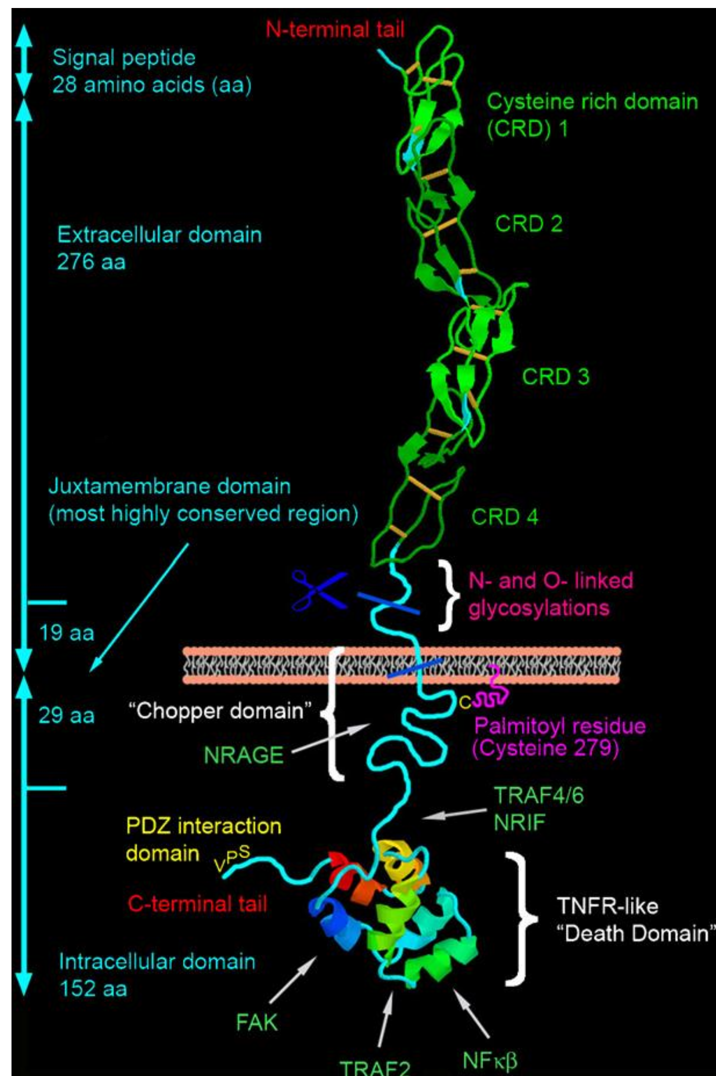


Figure 1-6: The structure of p75^{NTR}

Neurotrophins bind to p75^{NTR} via the cysteine-rich domains. Dark blue scissor lines indicate sites of regulated intramembrane proteolysis (RIP) cleavage. The Chopper domain, a flexible linker region, has been implicated in death signalling when directed to lipid-raft membranes by palmitoylation. The intracellular domain of p75^{NTR} interacts with a number of death-signalling binding partners (Frade 2000, Roux and Barker 2002, Coulson, Reid et al. 2004), can be phosphorylated (Grob, Ross et al. 1985), and its C-terminal tail may bind to a PDZ domain containing proteins known for protein trafficking and receptor complex association (Skeldal, Matusica et al. 2011).

The intracellular domain of p75^{NTR} (p75ICD) does not possess any catalytic function, but it consists of a death domain of 80 AA similar to the structure of other tumour necrosis factor receptor family. This site interacts with several signalling effectors (Liepinsh, Ilag et al. 1997, Roux and Barker 2002). The chopper domain is a cytoplasmic juxta-membrane region of the p75^{NTR} with 29 amino acids, and triggers neural cell death. It has been shown that dissociation of the chopper domain from the ectodomain causes an interaction of the other signalling proteins and the chopper domain results in an enhancement of its biological activity (Coulson, Reid et al. 2000). There is evidence to suggest that a homodimer form of p75^{NTR} binds neurotrophins in a symmetrical 2:2 receptor-ligand complex (Gong, Cao et al. 2008). A study on co-crystal structure of p75^{NTR} showed that the ectodomain of p75^{NTR} interacts with NT3 and proNGF (Kanning, Hudson et al. 2003, Feng, Kim et al. 2010). However, there is evidence showing p75^{NTR} is also able to bind neurotrophins in an asymmetric 2:1 neurotrophin/p75^{NTR} complexes form (He and Garcia 2004).

1.6.2 Expression of p75^{NTR} in the brain of AD

Cholinergic neurons in the BF, especially in the nucleus basalis of Meynert (NBM) which express p75^{NTR}, TrkA, TrkB and TrkC, are influenced by AD (Salehi, Ocampo et al. 2000). The expression of the neurotrophin receptors is changed by the progression of AD. However, there is some controversial evidence in regards to the expression levels of p75^{NTR} and neurotrophins receptors in AD patients and aging. It was reported that the neuronal expression of p75^{NTR} increased in AD (Mufson and Kordower 1992), in contrast to this, some reports demonstrate a downregulation of p75^{NTR} expression in AD brain (Arendt, Schindler et al. 1997, Salehi, Ocampo et al. 2000). Meanwhile, some report no significant differences in the expression levels of p75^{NTR} during mild cognitive impairment and AD (Goedert, Fine et al. 1989, Treanor, Dawbarn et al. 1991, Ginsberg, Che et al. 2006). Previously our laboratory showed a correlation between p75^{NTR} expression and aging in both wild type and APPSwe/PS1dE9 mice. Wang et al reported that the expression level of p75^{NTR} in APPSwe/PS1dE9 mice brains was higher than their wild type littermates (Wang, Wang et al. 2011). This is consistent with a recent study which suggests that accumulating Aβ promotes p75^{NTR} expression *in vitro* and *in vivo* (Chakravarthy, Gaudet et al. 2010).

1.6.3 p75^{NTR} and neuronal death

Neuronal death is the most common pathological characteristic of AD. Aβ as a ligand for p75^{NTR} mediates neuronal death and is consistently linked to the pathology of AD. There is some evidence indicating that p75^{NTR} plays a critical role in the early and characteristic loss of cholinergic neurons in response to Aβ in the septo-hippocampal region (Sotthibundhu, Sykes et al. 2008). The neurotoxicity function of p75^{NTR} is associated with its interaction with the different forms of Aβ. For example,

some evidences indicate that the interaction between the fibrillary form of Aβ with $p75^{NTR}$ promotes neuronal death, whereas soluble oligomeric Aβ/$p75^{NTR}$ binding has a protective function in neurons (Costantini, Della-Bianca et al. 2005). It is speculated that the protective effect of $p75^{NTR}$ may be mediated by phosphatidylinositide 3-kinase (PI3K) activity due to the function of the juxta-membrane sequence of the cytoplasmic region of $p75^{NTR}$ (Costantini, Della-Bianca et al. 2005). ProNGF induces neuronal death through an interaction with $p75^{NTR}$. ProNGF eliminates injured cells through activation of the apoptotic machinery of $p75^{NTR}$ (Beattie, Harrington et al. 2002). The level of proNGF is increased in glial cells and neurons of cortex and hippocampus in AD patients in correlation with the severity of the disease and cognitive malfunction (Fahnestock, Michalski et al. 2001, Peng, Wuu et al. 2004). ProNGF extracted from AD patients more potently induces neuronal apoptosis via $p75^{NTR}$ than proNGF extracted from healthy brains (Pedraza, Podlesniy et al. 2005). In addition, a cleavage-resistant mutated proNGF can promote neurite outgrowth and cell survival in PC12 cells which express high levels of TrkA. TrkA and ERK1/2 phosphorylation trigger apoptosis in neurons. An increased ratio of proNGF:NGF and $p75^{NTR}$:TrkA results in a shift from cell survival to apoptosis and eventually progression of AD (Clewes, Fahey et al. 2008).

1.6.4 $p75^{NTR}$ and neurite degeneration

Neuronal degeneration is a key event in AD and a main contributor of the associated cognitive disorders. Aβ plays a critical role in neurite degeneration in AD patients, but the mechanism of this phenomenon still is not fully known (Petratos, Li et al. 2008). The binding of Aβ to $p75^{NTR}$ mediates neuronal degeneration *in vitro* and *in vivo* through activation of c-Jun pathways (Morishima, Gotoh et al. 2001, Hashimoto, Kaneko et al. 2004, Yaar, Zhai et al. 2007). The neuronal degeneration

and loss of basal forebrain cholinergic neurons (BFCN) in chronic AD models and Aβ-induced toxicity are significantly abolished by deletion of the extracellular region of p75^{NTR} which suggests that p75^{NTR}/Aβ interaction promotes neuronal apoptosis (Knowles, Rajadas et al. 2009). On the other hand, a study indicates that sympathetic innervation is severely impaired in a p75^{NTR}-deficient AD mouse model. This suggests that p75^{NTR} not only interacts with APP, Aβ and induces neuronal toxicity, but promotes neuronal survival through activation of a TrkA-NGF signalling pathway especially in the sympathetic nervous system (Bengoechea, Chen et al. 2009). Based on these reports, p75^{NTR} critically modulates Aβ-induced neurite degeneration and it may become an important target to block Aβ neurotoxicity. A previous study from our laboratory demonstrated that degenerative fibers and neurites in the neocortex as well as the hippocampus express p75^{NTR} in APPSwe/PS1dE9 mice, suggesting that the p75^{NTR} may play a role in neurite degeneration (Wang, Wang et al. 2010). NGF has also been suggested to contribute to the pathogenesis of AD, but the role of its precursor, pro-NGF, in AD remains unknown. ProNGF has been shown to induce apoptosis in neuronal cell cultures through binding to p75^{NTR}. Moreover, it was reported that proNGF levels are increased in AD patient’s cerebral cortex and blocking the biological activity of pro-NGF inhibits neuronal loss (Friedman 2000, Podlesniy, Kichev et al. 2006, Volosin, Trotter et al. 2008). Previous our laboratory researchers reported that pro-NGF co-localized with Aβ plaques in AD mice brain. They illustrated that pro-NGF induces neuritic degeneration in different neuronal cell lines, as well as primary neuron cultures most likely via interaction with p75^{NTR} (Hashimoto, Kaneko et al. 2004, Wang, Valadares et al. 2010)

1.6.5 The association of p75^{NTR} with Tau phosphorylation and cognitive disorders in AD

Amino acids 29–35 of Aβ interact with p75^{NTR} and promote neurotoxicity (Yaar, Zhai et al. 2002, Coulson 2006). Aβ binds to the p75^{NTR}, but not to TrkA, and induces neuronal apoptosis (Yaar, Zhai et al. 1997, Maloney, Minamide et al. 2005) through activation of the intracellular death domain mediated-signalling pathway (Maloney, Minamide et al. 2005) and through GO, JNK, NADPH and caspase3-related mechanisms (Hashimoto, Kaneko et al. 2004). Non-peptide small molecule p75^{NTR} ligands inhibit Aβ-induced neurodegeneration and synapse impairment. Furthermore, these ligands are able to inhibit the activation of some molecules which are involved in the pathophysiology of AD including calpain/cdk5, GSK3b, c-Jun, and Tau phosphorylation, and prevent Aβ-induced inactivation of AKT and CREB. These findings support an extensive association between p75^{NTR} signalling and Aβ pathogenic mechanisms (Yang, Knowles et al. 2008). The degeneration of cholinergic neurons in the BF results in impairment of cognitive function in AD patients. Partial or complete p75^{NTR} gene knockout increases the population of cholinergic neurons in the medial septal nucleus to 13% and 28% respectively in AD mice. Subsequently an increase in the number of these neurons results in improvement of learning and memory (Naumann, Casademunt et al. 2002). Based on this, it is proposed that the cytoplasmic domain of p75^{NTR} may be responsible for the cell death effect, and complete deletion of the full length p75^{NTR} can improve cholinergic neuronal survival. There is indirect evidence to suggest that p75^{NTR} participates in formation of neurofibrillary tangles (NFTs), another hallmark of AD. The ratio of neurons which express p75^{NTR} in CA1 and CA2 subfields of hippocampus, where the neuronal death is more severe in AD, is significantly higher than other regions. Furthermore, a large proportion of p75^{NTR} in this area is co-

localized with Alz-50, the antibody which is used to detect hyperphosphorylated Tau protein in the same brain regions of AD patients (Hu, Zhang et al. 2002). Although this evidence indirectly indicates p75^{NTR} contributes to Tau phosphorylation, the exact mechanism of this is still not clear and needs to be investigated.

1.6.6 The cooperation between p75^{NTR} and other receptors

The binding of p75^{NTR} with proneurotrophins is mediated by an interaction with another co-receptor named Sortilin. It was shown that the cooperation between p75^{NTR} and Sortilin leads to apoptosis and growth inhibition in neurons (Nykjaer, Lee et al. 2004). On the other hand, mature neurotrophins enhance survival and growth of neurons through binding with p75^{NTR} and Trk receptors (Barker 2004). p75^{NTR} also works in conjunction with Trk receptors. The association between p75^{NTR} and Trk receptors regulates ligand specificity of Trk receptors and modulates the binding affinity of TrkA to NGF (Huang and Reichardt 2003). Although other neurotrophins can interact with TrkB receptors, only BDNF provides a functional response upon co-expression of TrkB with p75^{NTR}. Similarly, both NGF and NT-3 bind to TrkA, but p75^{NTR} regulates TrkA-NGF signalling. In contrast, co-expression of p75^{NTR} and TrkC decreases ligand specificity of TrkC to NT-3. p75^{NTR} interacts with a dozens of signalling molecules and mediates apoptosis, Schwann cell migration, myelination, axonal growth, and regeneration. p75^{NTR} interacts with Nogo receptor (NgR) and regulates NgR-mediated signalling in neuronal myelination (Figure 1-7) (Wang, Kim et al. 2002). CNS-derived myelin-based growth inhibitors (MBGIs) which include Nogo, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OmGP) are mediated by p75^{NTR} through the activation of RhoA signalling pathway (Yamashita, Tucker et al. 1999). Deletion of p75^{NTR} gene attenuates the activity of Rho and MBGI in mice sensory and cerebellar granule

neurons (CGNs) and subsequently rescues neurite growth impairments (Yamashita, Higuchi et al. 2002).

A recent study demonstrated that $p75^{NTR}$ directly interacts with APP and mediates the APP processing and downstream signal transduction (Fombonne, Rabizadeh et al. 2009). It was claimed that $p75^{NTR}$/APP association reduces non-amyloidogenic processing of APP and sAPPα production. Therefore the APP processing balance shifts to amyloidogenic pathway and increases Aβ generation. Moreover, in this article the authors reported that NGF and Aβ peptide, as $p75^{NTR}$ ligands, block the $p75^{NTR}$/APP interaction therefore reduces Aβ production through diverting APP processing toward non-amyloidogenic pathway (Fombonne, Rabizadeh et al. 2009).

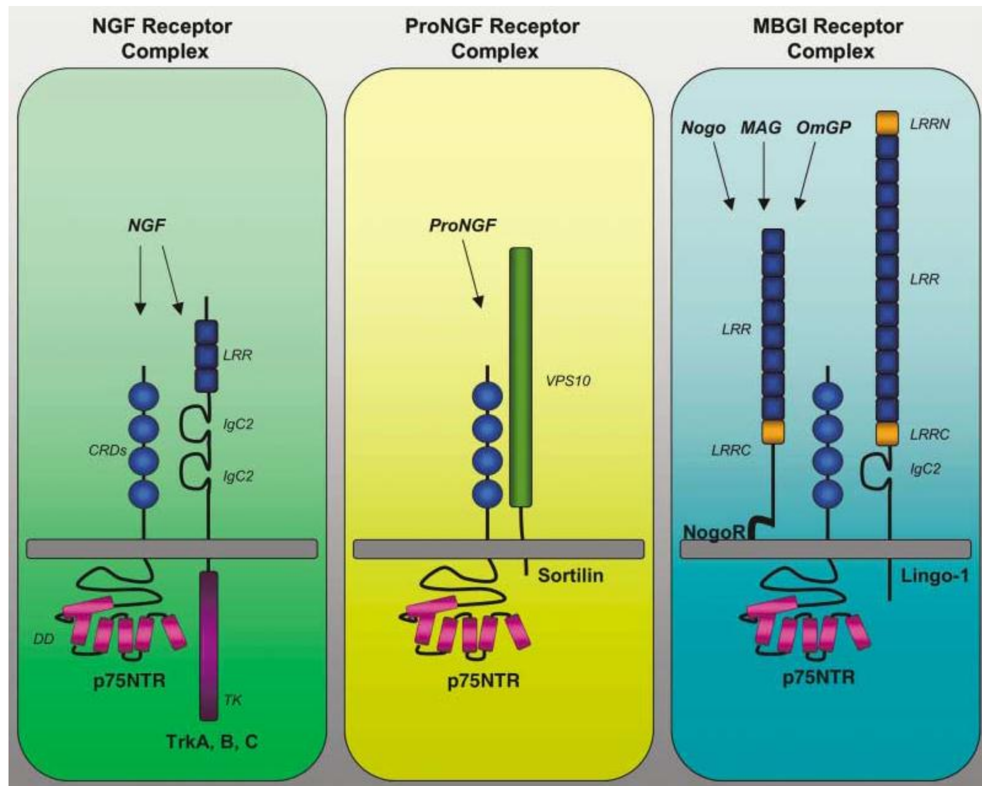


Figure 1-7: The interaction of p75^{NTR} with other receptors

p75^{NTR} signalling modulates the interaction between p75^{NTR} and TrkA and increases NGF-TrkA binding capability and discriminates between preferred and non-preferred neurotrophin ligands. A second signalling complex consisting of p75^{NTR} and Sortilin mediates pro-apoptotic signals in response to proNGF binding. A complex NogoR/Lingo-1/p75^{NTR} interaction promotes growth inhibitory signals from Nogo, MAG and OmGP (Yamashita, Tucker et al. 1999, Barker 2004).

1.7 Sortilin and its association with APP processing in AD

Sortilin is a 95-kDa type-I transmembrane glycoprotein and was first identified in human brain (Petersen, Nielsen et al. 1997). The Sortilin gene is located on chromosome 1 and encodes an extracellular domain that had not been recognized in any other vertebrate protein before (Petersen, Nielsen et al. 1997). The Sortilin receptor consists of 700 AA and belongs to the vacuolar protein sorting 10 protein (Vps10p) family. The Vps10p family has five members including Sortilin, SorCS1, SorCS2, SorCS3, and SorLA. A Vps10p is a common shared domain at the N-terminal of all members, which is either the only module of luminal/extracellular moiety or is combined with additional domains. The main function of Vps10p receptors is to contribute to protein transport and signal transduction (Hermey 2009). The Vps10p domain encompasses the entire extracellular portion of Sortilin and forms a unique 10- bladed beta-propeller structure (Figure 1-8) (Quistgaard, Madsen et al. 2009). Sortilin is also able to bind with neurotensin (Mazella, Zsurger et al. 1998). The function of Sortilin depends on the tissue type expressing Sortilin. For example, Sortilin is intensively expressed in neurons of the CNS and the PNS and modulates protein transport and signal transduction (Petersen, Nielsen et al. 1997, Nykjaer, Lee et al. 2004). In metabolic tissues including the liver, it regulates release of lipoprotein particles into the circulation (Morris, Ross et al. 1998, Arnett, Ryals et al. 2007, Kjolby, Andersen et al. 2010). The Sortilin-ICD contributes to protein sorting and internalization (Nielsen, Madsen et al. 2001, Willnow, Petersen et al. 2008). For instance, three motifs, F/YXXXXF/Y, YSVL and HDDSDEDLLE (dileucine motif), have been identified in Sortilin-ICD (Petersen, Nielsen et al. 1997) and they may mediate protein sorting, trafficking and internalization through binding to adaptor

proteins, such as AP-1, AP-2 and GGA (Nielsen, Gustafsen et al. 2007). However, Sortilin has a different gene structure compared with SorLA (Figure 1-8) (Hampe, Rezgaoui et al. 2001), and the above motifs do not all exist in SorLA ICD (Mazella 2001), suggesting that they may target different adaptor proteins. Hence, Sortilin may have diverse functions in neurons though it shares some similarity with SorLA and other family members. It was showed that the expression of SorLA is downregulated in AD. SorLA contributes to APP trafficking and processing by retaining intracellular APP in Golgi regions and subsequently prevents Aβ generation in the brain (Dodson, Gearing et al. 2006). In contrast, Sortilin is increased in AD brains, where it may up-regulate Aβ production via interaction with BACE1 (Finan, Okada et al. 2011, Saadipour, Yang et al. 2013).

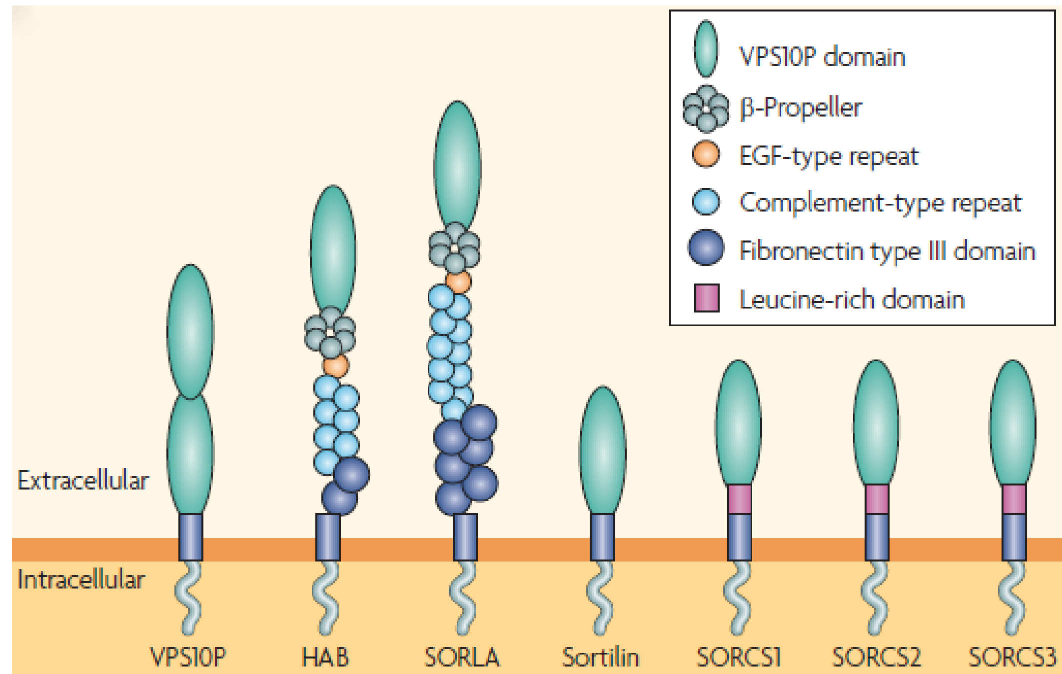


Figure 1-8: The structure of Vps10p family members including Sortilin, SorLA, SorCS-1, -2, and -3

The extracellular domains of the receptors includes one or two Vps10p domains which may carry additional modules such as leucine-rich domains, complement-type repeats, β-propeller, EGF-type repeats and fibronectin-type III domains, is involved in protein–protein interactions; HAB: head activator binding protein (Nykjaer and Willnow 2012).

Recently, it has been reported that Sortilin interacts with APP and promotes non-amyloidogenic processing of APP, unlike SorLA, which inhibits the generation of all soluble APP products (Gustafsen, Glerup et al. 2013). Our laboratory also showed that the FLVHRY motif in Sortilin-ICD plays an essential role in APP intracellular trafficking and participates in distribution of APP in lysosomes and lipid raft, and may promote lysosome-dependent degradation of APP (Yang, Virassamy et al. 2013).

1.8 Hypothesis and aim

Aβ accumulates in the brain which causes a cascade of pathological responses including loss of synaptic connections, neuronal death, inflammation, oxidative stress (OS), hyper-phosphorylation of Tau, and neurofibrilliary tangles and loss of cognitive functions. Aβ is able to bind to $p75^{NTR}$ and mediate neuronal death, such as cholinergic neurons in BF, hippocampus and different regions of the brain in AD. This results in impairment of cognitive function and psychomotor disorders (Sotthibundhu, Sykes et al. 2008). The main apoptotic consequence of $p75^{NTR}$/proNTs and $p75^{NTR}$/Aβ is mediated by interactions between $p75^{NTR}$ and the Sortilin receptor, thus the $p75^{NTR}$/Sortilin association is critical in neuronal death especially cholinergic neurons and progression of AD. It is also reported that the level of $p75^{NTR}$ is elevated during aging and AD (Mufson and Kordower 1992) and Aβ promotes the expression of $p75^{NTR}$ (Chakravarthy, Gaudet et al. 2010).

Multiple interactive mechanisms are involved in, and accelerate development of, AD. Overproduction and aggregation of Aβ, reduction of Aβ clearance, oxidative stress, and inflammation as well as genetic components all contribute to AD pathogenesis in the

brain. A number of treatments have been developed to target Aβ removal or inhibition of Aβ generation. For example, Aβ plaque deposition in the brain of APP transgenic mice was reduced after immunization with $A\beta_{42}$ (Schenk, Barbour et al. 1999). The inhibition of β- and γ-sectretase was another strategy to prevent Aβ production in AD (Ghosh, Gemma et al. 2008, Imbimbo 2008). However, there has been a complete failure to develop any AD therapy targeted of Aβ. For instance, Wischik et al demonstrates more than 25 AD clinical trials aimed at Aβ have failed (Wischik, Harrington et al. 2014). Also, Ittner LM et al showed that Tau can drive Aβ toxicity but without Tau hyperphosphorylation, Aβ has no toxicity. Thus the Aβ theory of AD may be wrong (Ittner, Ke et al. 2010).

This project has focused on investigating the role of $p75^{NTR}$ in the development of AD and proposes a new strategy to target Aβ generation through blocking the Aβ/ $p75^{NTR}$ interaction. So in this thesis, based on reports by others researchers and previous studies in our laboratory, we hypothesize;

1. A complex $p75^{NTR}$/APP/Aβ interaction mediates a feed-forward loop promoting APP processing and Aβ generation in AD

2. BACE1 regulates the proteolytic processing of $p75^{NTR}$ and mitigates neurodegenerative signals in the brain through generation of p75ECD

3. p75ECD-Fc recombinant protein attenuates behavioural deficits and neuropathology features in AD mouse models

4. $A\beta_{42}$ up-regulates the expression of Sortilin via the $p75^{NTR}$/RhoA signalling pathway

CHAPTER 2: A COMPLEX P75^{NTR}/APP/AB INTERACTION MEDIATES A FEED-FORWARD LOOP PROMOTING APP PROCESSING AND AMYLOID BETA GENERATION IN ALZHEIMER'S DISEASE

2.1 Abstract

Background: AD is pathologically characterized by deposition of Aβ peptide in the brain. The dysregulation of neurotrophins and their receptors plays a pivotal role in the development of sporadic AD. p75^{NTR} is up-regulated in AD brain, but its role still is not fully understood in the development of AD. In this study, we have investigated the potential roles of p75^{NTR} in Aβ production in AD pathology. **Methods:** FRET acceptor bleaching and co-immunoprecipitation (Co-IP) assays were employed to evaluate the interaction of APP/p75^{NTR} and APP/BACE1. To elucidate the effects of p75^{NTR} on APP processing, CHO^{APP695} cells were transfected with either p75^{NTR} or BACE1 plasmids and cell lysate was subjected to Western blot to quantify the processed fragments of APP. Cortical neurons from AD/p75$^{+/+}$ and AD/p75$^{-/-}$ mice were cultured and treated with 0.1 and 0.3 µM Aβ_{42} for 24 hours and cell lysate was harvested for BACE1 and sAPPβ quantification by Western blot. In addition, cortical neurons from p75$^{+/+}$ and p75$^{-/-}$ mice were treated with either 0.1 µM Aβ_{42} or Aβ_{25-35} for 24 hours and following this BACE1 protein level was measured by Western blot. Sucrose subcellular fractionation assay was conducted in 9-month old p75$^{+/+}$ and p75$^{-/-}$ mice brains in order to evaluate distribution of APP in subcellular compartments. A cell surface biotinylation assay was applied to assess the effects of 0.1 µM Aβ_{42} on APP, BACE1 and p75^{NTR} protein internalization. In order to evaluate the effects of Aβ/p75^{NTR} interaction on APP-Thr668 and Tau phosphorylation, mouse cortical neurons from AD/p75$^{+/+}$ and AD/p75$^{-/-}$ were treated with 1 µM Aβ_{42} for 24 hours and subsequently cell lysate then subjected to Western blot analysis. **Results:** We found that p75^{NTR} co-localized with APP in cortical neurons (74%) and their interaction was confirmed in HEK-293T cells ($p<0.05$). Aβ

enhanced the FRET signals of p75^{NTR}/APP interaction in a dose and time-dependent manner ($p<0.05$, $p<0.01$ and $p<0.001$). Co-IP data also confirmed the p75^{NTR}/APP interaction. Blocking the biological activity of Aβ_{42} using p75ECD-Fc recombinant protein reduced the FRET signals of APP/p75^{NTR} interaction ($p<0.05$). Next, we tested whether p75^{NTR}/Aβ interaction plays a significant role in APP processing and Aβ production. First we found that the transfection of p75^{NTR} construct in CHO^{APP695} significantly increased amyloidogenic processing of APP and sAPPβ production ($p<0.01$). Consistent with this, we discovered that the baseline of sAPPβ generation in AD/p75$^{+/+}$ is significantly higher than AD/p75$^{-/-}$ mouse cortical neurons ($p<0.01$) indicating p75^{NTR} regulates amyloidogenic processing of APP. In addition, Aβ_{42} increased APP and BACE1 expression as well as APP processing in a dose-dependent manner in AD/p75$^{+/+}$, but not in AD/p75$^{-/-}$ mouse cortical neurons ($p<0.05$, and $p<0.01$). Our cell surface biotinylation assay indicated that Aβ_{42} mediates APP and BACE1 endocytosis through a p75^{NTR}-dependent mechanism ($p<0.01$). Moreover, our data showed that Aβ and proNGF increased APP/BACE1 interaction ($p<0.01$). Our results demonstrated that Aβ_{42} induces APP-Thr668 and Tau phosphorylation through p75^{NTR} in mouse cortical neurons ($p<0.05$). **Conclusion:** Taken together, our study demonstrated that the Aβ/p75^{NTR} interaction promotes p75^{NTR}/APP association, induces APP and BACE1 upregulation, increases APP and BACE1 internalization and their interaction, induces APP-Thr668 and Tau phosphorylation, and therefore contributes to the pathogenesis of AD.

Key words: p75^{NTR}, APP, Aβ, BACE1, and Alzheimer's disease

2.2 Introduction

AD is characterized by accumulation of amyloidogenic plaques and neurofibrillary tangles in the brain as well as loss of cholinergic neurons and cognitive functions. Aβ plays a central role in the pathogenesis of AD (Katzman 1986, Selkoe 2002, Blennow, de Leon et al. 2006).The dysregulation of neurotrophins and their receptors in AD is a key pathological process in sporadic AD (Pedraza, Podlesniy et al. 2005, Peng, Wuu et al. 2005, Garzon and Fahnestock 2007, Cattaneo and Calissano 2012). p75^{NTR} is the common neurotrophin receptor which has been implicated to contribute to the development of AD in different ways. For instance, a high level of p75^{NTR} expression in the BFCN increases neuronal apoptosis, and therefore contributes to cognitive dysfunction during the progression of AD (Whitehouse, Price et al. 1981, Mufson, Bothwell et al. 1989, Woolf, Gould et al. 1989). *In vivo* and *in vitro* studies showed that binding Aβ to p75^{NTR} promotes neuronal death in human and mouse models (Kuner, Schubenel et al. 1998, Yaar, Zhai et al. 2002, Costantini, Rossi et al. 2005, Sotthibundhu, Sykes et al. 2008), however, there is evidence to show that Aβ/p75^{NTR} binding mediates neuronal survival (Zhang et al., 2003).

A complex p75^{NTR}/Sortilin/Aβ interaction that is mediated by proneurotrophins, results in neuronal death (Yaar, Zhai et al. 1997, Nykjaer, Lee et al. 2004, Pedraza, Podlesniy et al. 2005). The p75^{NTR} mediates apoptosis through interaction with proNGF, which is increased in the brain of AD patients (Longo and Massa 2005). Furthermore, p75^{NTR} signalling plays critical roles in nerve injury and degeneration of the nervous system (Dechant and Barde 2002, Ibanez and Simi 2012). It was reported that Aβ stimulates the expression of the p75^{NTR} receptor in SH-SY5Y human neuroblastoma cells and AD

transgenic mouse brain (Chakravarthy, Gaudet et al. 2010). Furthermore, our groups previous study also showed that the expression of p75^{NTR} in AD transgenic mouse brain is higher than wild type, suggesting Aβ is most likely a crucial factor causing p75^{NTR} upregulation in AD (Wang, Wang et al. 2011). Although there is accumulating evidence indicating p75^{NTR} interacts with Aβ and the interaction plays a key role in neuronal death and development of AD, its exact mechanism has not been fully understood. Our laboratory showed that p75^{NTR} has two opposing effects on Aβ metabolism. On one hand, consistent with other studies, p75^{NTR} signalling increases the amyloidogenic processing of APP and generates more Aβ in neurons (Costantini, Weindruch et al. 2005, Wang, Wang et al. 2011). On the other hand, p75ECD plays a critical role in the clearance of Aβ and suppresses Aβ aggregation and deposition in the brain (Wang, Wang et al. 2011). So in the present study, we are investigating how p75^{NTR} promotes amyloidogenesis. We hypothesize that a complex p75^{NTR}/APP/Aβ interaction contributes to amyloidogenic processing of APP and Aβ generation and eventually promoting a feed-forward loop driving in AD progression.

2.3 Materials and methods

Animal maintenance

The APPSwe/PS1dE9 (AD) transgenic mice bearing a chimeric mouse/human (Mo/Hu) APP695 with mutations linked to FAD (KM 593/594 NL) and human PS1 carrying the exon-9-deleted variant associated with FAD (PS1dE9) on C57BL as well as p75$^{-/-}$ (p75^{NTR}/ExonIII$^{-/-}$ or p75knockout) mice on 129sv background were obtained from Jackson Laboratory (Jankowsky, Slunt et al. 2001). In order to investigate the effects of p75^{NTR} on APP processing, p75knockout (p75KO) mice were crossed with AD mice and AD/p75$^{-/-}$ and AD/p75$^{+/+}$ strains from the crossing were used in our study. In order to confirm mice strains, genotyping was performed using primers and PCR according to supplier's instructions (Table 2-1).

Table 2-1: PCR primer pair sequences used for AD Tg and p75KO mice genotyping in this study

Mice	Primers	Sequences
AD Tg	oIMR 1597	5'- gactgaccactcgaccaggttctg-3'
AD Tg	oIMR 1598	5'- cttgtaagttggattctcatatcc-3'
AD Tg	oIMR 0944	5'- cctctttgtgactatgtggactgatgtcgg-3'
AD Tg	oIMR 1588	5'- gtggataaccctcccccagcctagacc-3'
p75KO	oIMR 0710	5'- tgttacgttctctgacgtggtgag-3'
p75KO	oIMR 0711	5'- tcagcccagggtgtgcactc-3'
p75KO	oIMR 0013	5'- cttgggtggagaggctattc-3'
p75KO	oIMR 0014	5'- aggtgagatgacaggagatc-3"

Mice were maintained on *ad libitum* food and water with a 12-hour light/dark cycle. All animal husbandry procedures performed were approved by the University of South Australia (Animal Ethics Committee approval: 16/12) in accordance with NHMRC guidelines.

Antibodies, reagents and plasmids

In this chapter following antibodies, reagents and plasmids were used; Rabbit monoclonal anti-BACE1 (D10E5, cell signalling, US), mouse monoclonal anti-human/mouse BACE1 Ectodomain from R&D (Cat.No.MAB931, US). Rabbit anti-p75ECD (9650) antibody which was used for blocking the biological activity of p75^{NTR}, and rabbit polyclonal anti-p75ICD (9992) were generously provided by Prof. Moses V. Chao (Department of Cell Biology, Skirball Institute, and New York, US). Rabbit anti-p75^{NTR} from Prof. Louis F. Reichardt (Departments of Physiology and Biochemistry and Biophysics, UCSF, US), mouse polyclonal anti-p75ECD (5B1D2) antibody was kindly provided by Prof. Yan-Jiang Wang (Department of Neurology and Centre for Clinical Neuroscience, Daping Hospital, Third Military Medical University, Chongqing, China). Mouse monoclonal anti-Aβ_{42} (6E10) and rabbit anti-sAPPβ (Covance, USA), rabbit polyclonal anti-APP C-terminal (Sigma-Aldrich, US), rabbit polyclonal anti-APP C-terminus (678-695aa) (6687) (Steiner, Kostka et al. 2000) was kindly provided by Prof. C. Haass (Ludwig-Maximilians University Munich, Munich, Germany). Sheep polyclonal anti-total Tau antibody (Antibody Technology Australia, Australia), mouse anti-Phospho-PHF-Tau pSer202+Thr205 monoclonal antibody (AT8) (Cat.No. MN1020, CiteAb, UK), rabbit anti-phosphorylated APP-Thr668 (Cell Signalling, US), rabbit anti-ER (GRP78) (abcam, US), rabbit anti-Lamp1 (abcam, US), rabbit anti-

integrin α (abcam, US), mouse anti-GM130 (BD, US), rabbit anti-EEA1 (Cell signalling, US), rabbit anti-Flotilin-1 (Sigma-Aldrich, US), human, goat and rabbit IgG (Sigma-Aldrich, US), mouse monoclonal anti-β actin (Sigma-Aldrich, US), sheep anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Osenses, Australia), polyclonal goat anti-GFP (Rockland Immunochemicals, Inc.Antibodies & Assays, Gilbertsville, PA, US) and all horseradish peroxidase (HRP)-linked and fluorescence secondary antibodies were provided from Sigma-Aldrich and Jackson Laboratories, respectively. synthetic $A\beta_{25\text{-}35}$ peptide (Cat.No.SCP0002, Sigma, US), synthetic $A\beta_{42}$ (Cat.No. 62080) and scrambled $A\beta_{42}$ (Cat.No. 62046) were purchased from American Peptide (Sunnyvale, CA, US). Mouse NGF and human proNGF recombinant proteins were purchased from Invitrogen (Australia) and ProSpec (US), respectively. Protein G sepharose (Sigma-Aldrich, US), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Cat.No.105228, Sigma-Aldrich, US), poly-D-lysine (PDL) (Cat.No.P7280, Sigma-Aldrich, US). Gel extraction Kit (Cat. No. 28704, Qiagen, Australia), DNA Purification miniprep Kit (Cat.No. A1460, Promega, Australia). Human full length BACE1-CFP was kindly provided by Dr. Patrick Keller (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). Human APP695-YFP was generously provided by Dr. Christoph Kaether (Leibniz Institute for Age Research, Fritz Lipmann Institute, Jena, Germany), pcDNA3.1 human full length p75^{NTR} was from Dr. Rainer Niedenthal, (Institute for Physiological Chemistry and Biochemistry, Hannover Medical School, Hannover, Germany). The construct of p75-YFP-CFP is generously provided by Dr. Elizabeth Coulson (The Queensland Brain Institute, The University of Queensland, and Australia). The construct of p75ICD-HA was kindly gifted by Prof. Toshihide

Yamashita (Department of Molecular Neuroscience, The University of Osaka, Japan). Human p75ECD-Myc plasmid was made by PCR, amplifying Hindi III-Xho1 fragments using forward and reverse primer 5’- GTGC AAGCTT GCCACC ATG GGG GCA GGT GCC ACC GGC -3’, 5’- GTGC CTCGAG CGG GCT GGG AGC TGC CCA TCA C-3’ in pcDNA3.1(+)- myc/his vector (From Dr. Morales, McGill University). pECFP-N1 and pEYFP-N1constructs were purchased from Clontech.

p75ECD-Fc and Fc recombinant protein preparation

Homo sapiens p75ECD-Fc and Fc constructs were individually produced using primers (Table 2-2) and PCR and then the fragments were fused together by overlapping PCR.

Table 2-2: PCR primer pair sequences used for human p75ECD-Fc and Fc cloning

Gene	Primers	Sequences
p75ECD-Fc	Forward	5’- gcgctctagaccaccatgggggcaggtgccaccgg -3’
p75ECD-Fc	Reverse	5’- ctctgtcgacttatcatttacccggagacaggg-3’
Fc	Forward	5’- aaccatgggcgacaaaactcacacatg -3’
Fc	Reverse	5’- aagaattctcatttacccggagacag -3’

Once p75ECD-Fc and Fc were generated, they were cloned into pET-28a vector (Figure 2-1 A) using Nco-I and EcoR-I restriction enzymes and then transformed into DH10B E.coli competent cells and subsequently transformed into BL21 E.coli competent cells. In order to express p75ECD-Fc and Fc recombinant peptides, BL21 E.coli stock of p75ECD-Fc and Fc was inoculated in a large volume of Luria Broth (LB) culture medium and incubated at 37°C until OD value reached to 0.4-0.6 and then 1mM IPTG was used to induce expression of p75ECD and Fc in E.coli for 3 hours at 37°C. E.coli

was spun down at 5,000 RPM at 4°C for 15 min and then pellet was re-suspended in hypotonic lysis buffer (10 mM tris, 2mM EDTA, 25 mM NaCl, 0.1% Triton X-100, 1-2mg/ml Lysozyme, pH=8.0) and after sonication supernatant was extracted by high speed centrifuging (15,000 RPM) for 30 min at 4°C. In order to harvest p75ECD-Fc and Fc, the supernatant was passed through protein-G sepharose column and then p75ECD-Fc and Fc were eluted from protein-G sepharose by applying 100 mM glycine pH 2.5. Subsequently the proteins in glycine solution were dialyzed in PBS at 4°C and purity of the peptides was checked by Coomassie blue staining (Figure 2-1B).

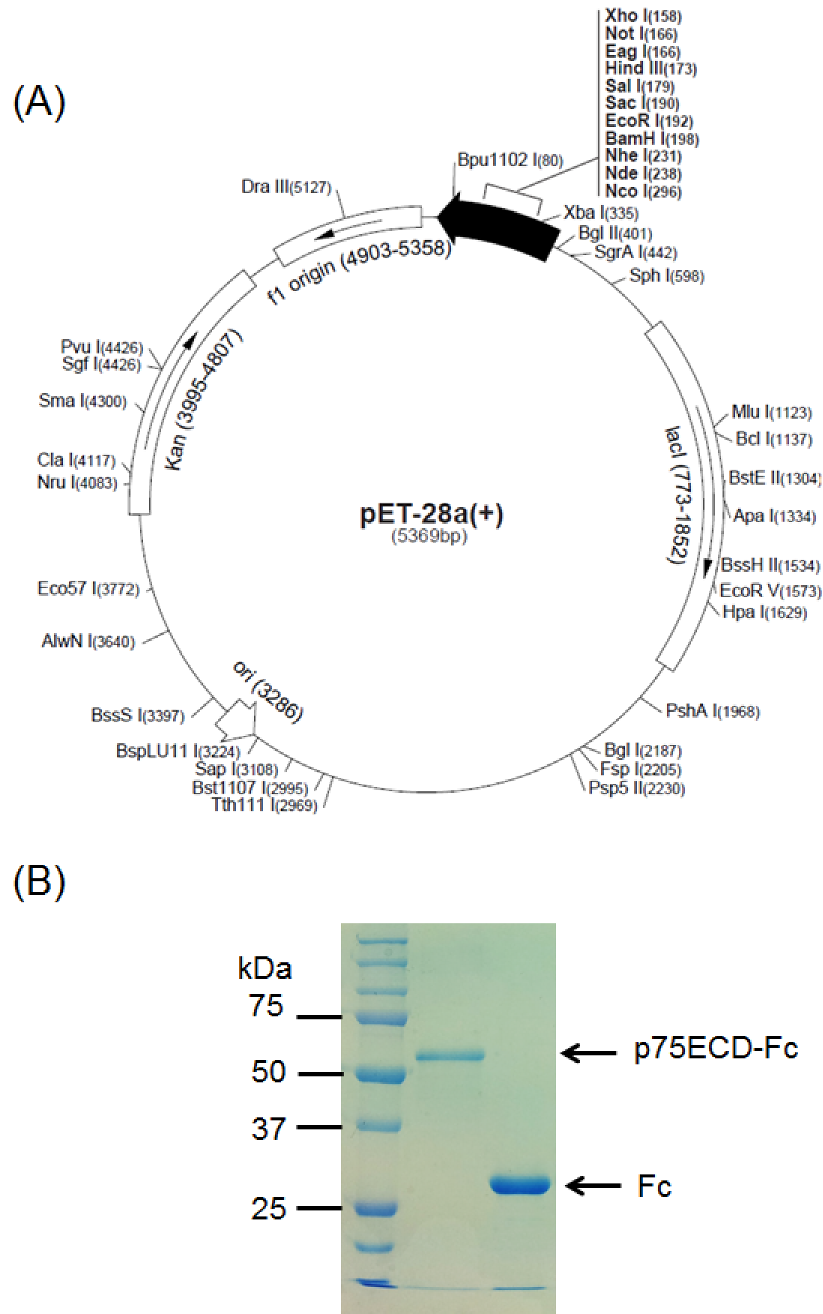


Figure 2-1: Presenting pET-28a vector, p75ECD-Fc and Fc recombinant proteins

A) Map showing pET-28a vector design; B) Coomassie blue staining of SDS-PAGE showing the expression of p75ECD-Fc and Fc recombinant proteins in E.coli

Preparation of $A\beta_{42}$ oligomer and $A\beta_{25-35}$

In the present study, the oligomer form of $A\beta_{42}$ was used. $A\beta_{42}$ oligomer and its scramble were prepared following the protocols described previously (Dahlgren, Manelli et al. 2002, Wang, Wang et al. 2011, Saadipour, Yang et al. 2013). In brief, the peptides were dissolved in HFIP solvent at 1 mg/ml concentration and then allocated in 1.7 ml tubes, following HFIP was allowed to evaporate and peptides were dried under fume hood overnight and pellets stored at -20°C until used. For oligomerization of $A\beta_{42}$, dried pellet was re-suspended in DMEM to a final concentration of 100 µM and incubated at 4°C for 24 hours. The oligomer form of $A\beta_{42}$ was tested by Western blot before use (Figure 2-2). $A\beta_{25-35}$ was prepared in the same manner of $A\beta_{42}$ and pellet was dissolved in DMEM just before use.

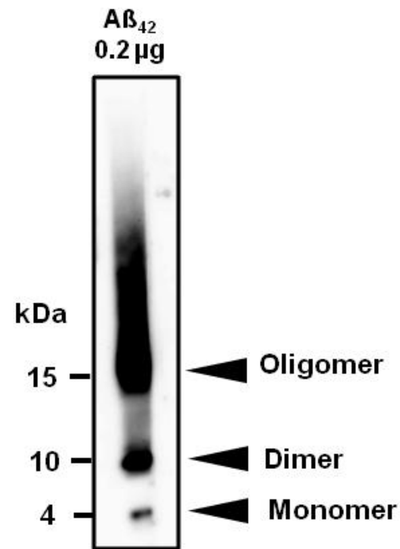


Figure 2-2: Western blot presenting the oligomer form of $A\beta_{42}$

$A\beta_{42}$ oligomer was prepared in DMEM and incubated at 4°C for 24 hours and then 0.2µg of $A\beta_{42}$ oligomer was subjected to Western blot and detected with anti-$A\beta_{42}$ antibody (6E10).

Primary neuron culture

One day old mice from different genotypes, C57bl, 129sv, p75$^{-/-}$, AD/p75$^{-/-}$ and AD/p75$^{+/+}$ were humanly killed and the cortical neurons were isolated and cultured for subsequent experiments (Wang, Valadares et al. 2010, Xu, Sun et al. 2011, Sun, Lim et al. 2012). In order to have a high purity of cortical neuron culture, we have developed and employed a complete removal of brain meninges (Figure 2-3) prior to isolating the neurons from the mice brains. After the brain meninges was removed, the cortex parts of brain from both hemispheres were separated and transferred into 15 ml falcon tubes including 2 ml DMEM medium and centrifuged at 2000 RPM for 2 min at 4°C and then DMEM was removed and tissues were incubated in 0.5% trypsin including 0.1% DNaseI (NEB, US) for 20 min at 37ºC with shaking every 5 min. Afterward digestion was stopped by adding 15% FBS and tissues were passed through a 9-inch Pasteur pipette with a fire polished tip to an opening of 0.5 mm for 10 times. After 10 min when pellets settled, supernatant was gently collected and transferred into a new 15 ml falcon tub and centrifuged with 2000 RPM for 2 min at 4°C and then the pellet was re-suspended in 1 ml Neurobasal medium supplemented with B27(2%), N2 (1%), penicillin/streptomycin (100 U), L-glutamine (2 mM) and FBS (2%). In order to harvest neurons after treatment for western blotting or for immunocytochemistry assay, cortical cells were placed into PDL and laminin-coated 6 well plate ($2x10^6$) and on PDL and laminin- coated coverslips in 4 well plates ($2.5x10^4$), respectively and after 72 hours incubation at 37ºC supplemented with 95% O2, 5% CO2 incubator, neurons were ready for further treatment and study.

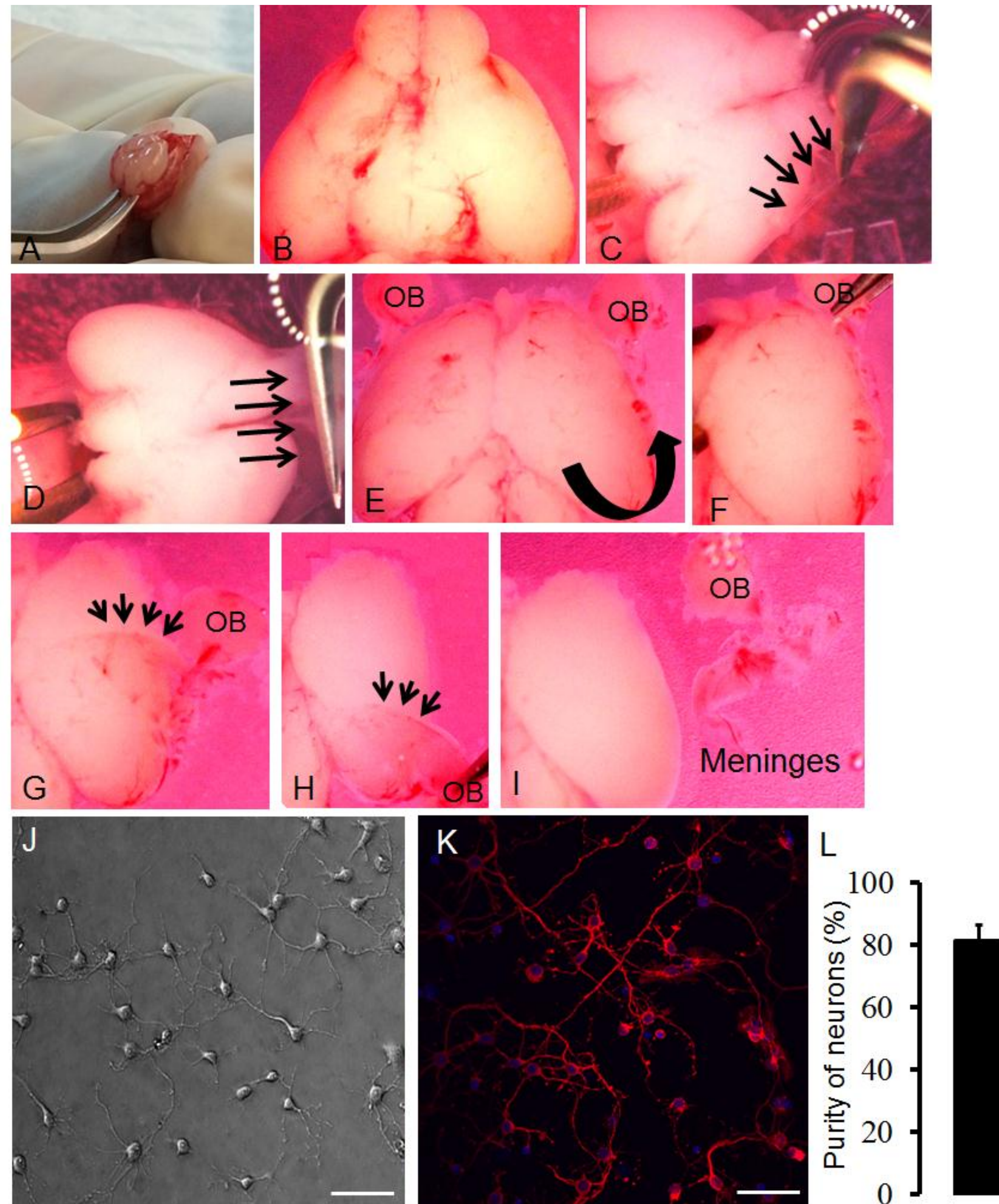


Figure 2-3: The meninges removal from neonatal mouse brain provides a high purity of mouse cortical neurons in culture

(A) Dissected neonatal mice brain tissue, (B) Brain position for the meninges removal, (C-H) representing pictures showing the meninges removal procedure, (I) Completion of the meninges removal (right-hand side in the picture), (J) Phase contrast bright field image of cortical neurons image was taken by Olympus IX71 microscope (K) Immunostaining for β-tubulin III (red) and DAPI (blue), image was taken by Zeiss confocal microscope (LSM710) (L) Plot presenting the purity of the cortical neuron; OB (olfactory bulb); image scale bar 50 μM.

Cell line culture

HEK-293T cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). CHO^{APP695} cells were kindly provided by Prof. Andrew Hill (Department of Biochemistry and Molecular Biology, University of Melbourne). Cells were grown in DMEM medium (Invitrogen, Australia) supplemented with 10% FBS and 2 mM L-glutamine and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Protein extraction from cell line and the brain tissue of mice

Total protein from cell lines, cortical neurons, and mice brain was obtained to subject to Western blotting by following method. Culture medium was removed and cells were gently washed twice with chilled PBS and lysed by suspending in RIPA buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 0.5% Sodium deoxycholate, pH7.4) containing protease inhibitors including 1 mM phenyl methane sulfonyl fluoride (PMSF), antipain, pepstatin, and leupeptin (Roche, Australia). Mouse brain tissue was also homogenised in RIPA buffer containing the protease inhibitors. The cells and brain lysates were sonicated and then centrifuged at 14,000 RPM for 10 min at 4°C and total protein concentration of supernatant was determined using BCA protein assay Kit (Thermo Scientific, Rockford, US). All lysates were stored at -80°C until use.

Western blot assay

Protein extracts (20 µg) from cell lines, cortical neurons and the brain of mice were separated by 8%, 10%, 12% or 4–12% precast SDS-PAGE gels (Bio-Rad, US) which were run at 80-120 V using Tris-glycine running buffer and transferred to nitrocellulose membrane (0.2 mm pore size, GE Healthcare, US). The membrane was then blocked for 1 hour at room temperature in PBS containing 5% skim milk and then incubated with primary antibodies with appropriate dilution in 2% skim milk in PBS overnight at 4°C on a shaker. This was followed respectively by incubation with HRP-linked secondary antibodies with 1:3000 dilution in 2% skim milk in PBST (Sigma, US) for 1 hour at room temperature (TBS and TBST were substituted for PBS and PBST, respectively when phosphorylated proteins were being detected). The membrane was developed by Image Quant LAS 4000 (GE Healthcare, US) and Image J software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html) was used for quantitative analysis (Saadipour, Yang et al. 2013).

Sucrose subcellular fractionation assay in mice brain

In order to elucidate distribution of APP in different subcellular organelles, the brain cortex including hippocampus of 9-month old p75KO and wild type mice where homogenized in homogenate buffer (0.25 M Sucrose, 1mM EDTA, 10 mM HEPES, pH7.4 and 1mM PMSF) and passed 5 times through a 27 gauge needle. The homogenate was then centrifuged at 1500 RPM for 10 min at 4°C and the supernatant was collected and the total protein concentration in supernatant was measured by BCA Kit (Thermo-Fisher science, Australia). Following this, 3 mg of protein from individual mouse were

loaded on top of a continuous 12.5-50% sucrose gradient containing 1mM EDTA, 10 mM HEPES, and 1mM PMSF (pH7.4) and centrifuged at 31,000 RPM for 3 hours at 4°C using a SW60 rotor (Beckman) and an equal volume of each fraction was taken and subjected to Western blot by using 4–12% precast SDS-PAGE. The Western blot membrane was then incubated with primary antibodies against APP and subcellular organelle markers (Widmann, Dolci et al. 1995, Uldry, Steiner et al. 2004).

DNA plasmid transfection into HEK-293T and CHO^{APP695} cells

Transfection technique was used to deliver DNA plasmids into cells in order to over-express target proteins. In this method recombinant plasmid constructs were transfected to cells using Lipofectamine 2000 (Invitrogen, Mulgrave, VIC, Australia). After overnight culture, when the cells proliferation was reached about 80–90% confluence, they were transfected with plasmid-Lipofectamine complex in an optimized ratio. For instance, in order to co-transfect APP-YFP/p75-CFP in HEK-293T cells, APP-YFP and p75-CFP constructs (~1µg plasmids) were taken in 50 µl fresh DMEM and 4 µl of Lipofectamine was added in another 50 µl DMEM and left at room temperature for 3-5 minutes. Later the medium containing Lipofectamine was mixed with medium containing plasmid DNA. The total transfection mixture (100 µl) was incubated at room temperature for 15-20 minutes following the entire volume was gently transferred into the well of culture plate and cells were then placed in the incubator. After 6 hours culture medium was changed and transfection process was completed in 24 hours. The transfection efficiency in cells was measured according to the following protocol. To evaluate the transfection efficiency, fluorescence and bright field microarray images

from 3 different areas of transfected cells were taken by fluorescence (Olympus IX71) microscope and images were then merged. The number of transfected and non-transfected cells within a diameter of 250 µm was counted and the transfection ratio was calculated according to the formula [Transfection ratio= (The number of total cells - number of non-fluorescence visualized cells) x100]. More than 80% transfection was used for subsequent studies.

Förster resonance energy transfer (FRET) assay

FRET was performed to investigate direct protein-protein interactions. In this experiment, HEK-293T cells were seeded (2.5x104) on coverslips for overnight and then co-transfected with either human p75-CFP/APP-YFP, p75-CFP/APP(1-542)-YFP, p75-CFP/APP(671-770)-YFP, or BACE1-CFP/APP-YFP plasmids and then the interaction between co-transfected molecules was measured by FRET acceptor bleach assay. In treatment groups, after 24 hours transfection, cells were incubated with different reagents, concentrations and times and cells were then fixed using 4% PF and coverslips mounted on glass slides. FRET experiments were accomplished on the cells to characterize the nature of the interaction between the two proteins. FRET is physical process wherein energy is transferred in a non-radioactive manner from one fluorophore (donor) to another (acceptor) by protein dipole-dipole interaction. Fundamental occurrence of FRET is a due to significant overlap of the donor emission spectrum and the acceptor absorption spectrum, the spatial proximity and orientation of two fluorophores is typically within a 10 nm distance range i.e. ~ (10 – 100 Å) (Gryczynski, Gryczynski et al. 1999). In this study we used Cyan Fluorescence Protein (CFP) and

Yellow Fluorescence Protein (YFP) for FRET experiments and these two fluorophores are well established FRET pairs (Pollok and Heim 1999). To explain in detail, CFP (Quencher fluorophore) emission spectrum is at (488 nm). The CFP emission energy is absorbed by YFP (Acceptor fluorophore) which has a spectral overlap to CFP emission. If the proteins we bound to these fluorophores are in proximal distance, then YFP (sensitized emission) emits energy at a higher wavelength (528nm). We employed the acceptor bleaching method to test the interaction. FRET acceptor bleaching is compares the donor fluorophore intensity in the same sample, before and after destroying the acceptor by photo bleaching. The YFP fluorophore was bleached completely by exposing 100% 514 nm line argon laser and therefore, the bleached YFP fluorophore can no longer acquire energy from the donor CFP fluorophore. If there is an occurrence of FRET, the intensity of the donor fluorescence will be altered after photo-bleaching the acceptor. This change in emission spectrum (donor de-quenching) within the Forster radius was measured to calculate the FRET efficiency (Figure 2-4).

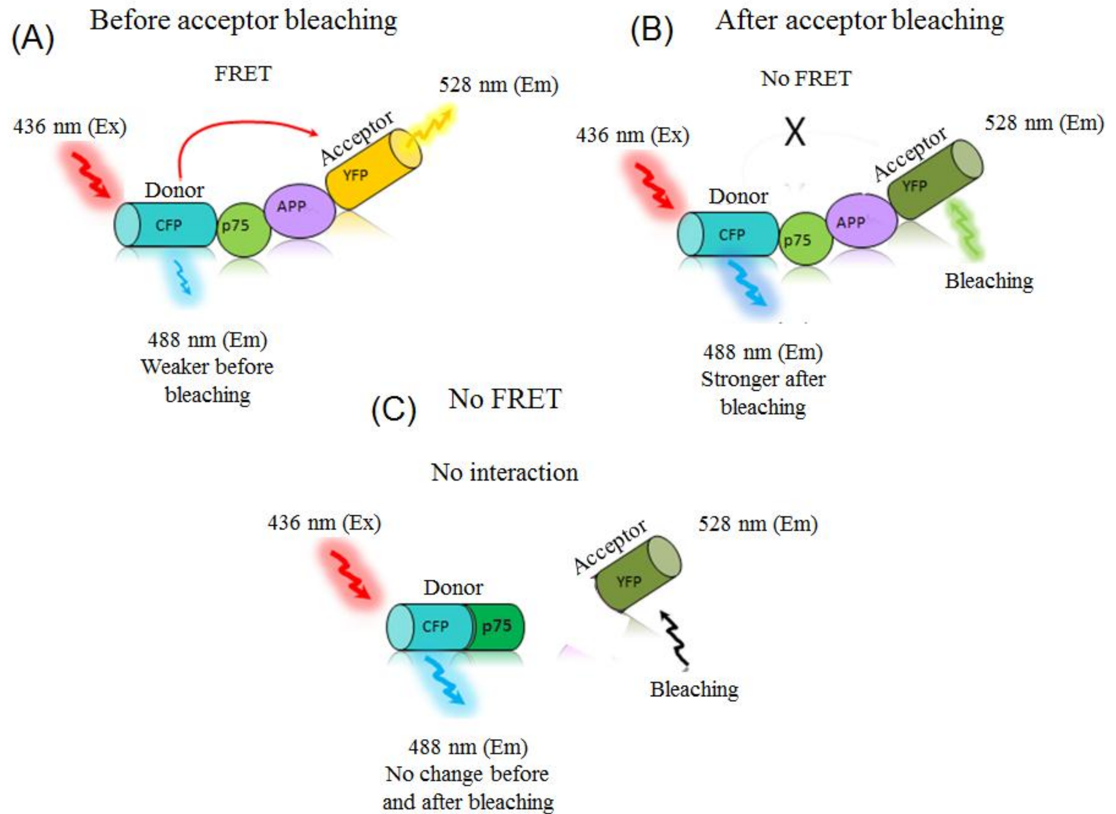


Figure 2-4: The diagram presenting FRET acceptor photo-bleaching signals from the APP/p75^{NTR} interaction

HEK-293T cells were co-transfected with either APP-YFP/p75-CFP or pEYFP/p75-CFP (negative control) plasmids and then were subjected to FRET acceptor bleaching assay in order to detect a direct interaction between APP and p75^{NTR}. A) The diagram showing the FRET signals from APP-YFP/p75-CFP interaction before acceptor (APP-YFP) photo bleaching. p75-CFP was excited and the CFP emission energy is absorbed by acceptor fluorophore, APP-YFP, which is in a close distance from p75-CFP therefore the CFP emission is weaker. B) In case of acceptor, APP-YFP, bleaching with argon laser, the CFP emission energy cannot be transferred to YFP consequently p75-CFP emission is going to be stronger in comparison with before acceptor bleaching. C) p75-CFP signals from pEYFP/p75-CFP (negative control) does not show any changes before and after bleaching due to lack of interaction between p75-CFP and pEYFP.

The efficiency of FRET is dependent on inverse sixth power of inter molecular separation. At the end, the FRET efficiency was calculated according to the formula; $FRET_{Eff}$.= (D_{post} -D_{pre}.)/ D_{post}X100 (FRET $_{Eff}$: FRET efficiency, D_{post}: Fluorescence intensity of the Donor after acceptor photo bleaching, D_{pre}: Fluorescence intensity of the Donor before acceptor photo bleaching)(Clegg 1992). To quantify FRET efficiency, a region of interest was selected for the YFP fluorophore and was bleached completely by exposure 100% of 514 nm line argon laser. If two proteins are together, energy transfer will be altered and it can be seen as yellow burned spot. For each cell under investigation more than five different yellow burned spot were selected as “Region of interests” (ROI). Therefore, FRET efficiency from ROI in 6 cells for each group was calculated and reported. A single transfection of p75-CFP-YFP fusion protein was employed as positive control. pEYFP/p75-CFP or pEYFP/BACE1-CFP were used as negative control as they don’t have any interaction. Leica SP5 Confocal microscopy (Leica Microsystems, Mannheim, Germany) was employed to determine the APP/p75^{NTR} and BACE1/APP interactions by FRET acceptor bleaching analysis software and the results were quantified by using SPSS software.

Immunocytochemistry (ICC) assay

In this experiment, cortical neurons from wild type (129sv) neonatal mouse were cultured on coverslips for 72 hours. Cells were fixed in 4% PF for 10 min and afterward subjected to immunostaining in order to quantify colocalization between endogenous p75^{NTR} and APP. Immunostaining was performed by overnight incubation in rabbit polyclonal anti-APP C-terminus (678-695aa) (6687) and mouse polyclonal anti-p75ECD (5B1D2) antibody at 4°C, following 1 hour incubation with secondary rabbit anti-Cy3 and anti-mouse-Alexa488 antibodies and DAPI as nuclei marker. The cells were mounted using polyvinyl alcohol mounting medium (sigma-Aldrich, US) on glass slides and images obtained sequentially using Zeiss confocal microscope (LSM710). Following this quantitative assessment of co-localization between Cy3 and Alexa 488 was performed by calculating the overlap coefficient (ranging from 0%, minimum colocalization, to 100%, maximum colocalization). An average of 12 cells from 2 different coverslips were analysed and the overlap coefficient (%) was then calculated. For exogenous colocalization between APP and p75^{NTR}, HEK-293T cells were seeded on coverslips and transfected with APP-YFP and p75^{NTR}-CFP for 24 hours. Cells were fixed by 4% PF for 10 minutes and subjected to Leica SP5 Confocal microscopy (Leica Microsystems, Mannheim, Germany) in order to quantify the co-localization between APP-YFP and p75-CFP (Yang, Lim et al. 2011).

Coimmunoprecipitation (Co-IP) assay

Co-IP assay was used to quantify the interaction between two proteins (Yang, Lim et al. 2011). In this study, HEK-293T cells were co-transfected with full length APP-YFP/p75-HA and pEYFP/p75-HA and cell lysates were prepared in RIPA buffer containing 1 mM PMSF, antipain, pepstatin, and leupeptin (Roche), following sonication and protein extraction. The protein concentration of the lysates was determined using BCA protein assay Kit (Thermo Scientific, Rockford, US) and the pre-cleared lysates (400µg) were then incubated with 2 µg primary (goat anti-GFP) antibody and goat IgG as a control for antibody specificity at 4°C overnight. Lysates were then incubated with protein G beads for two hours at 4°C in order to immobilize the primary antibody and the IgG. The beads were washed four times with PBST and boiled in 30 µl SDS page loading buffer and then subjected to Western blot to detect p75-HA using rabbit anti-HA antibody. At the same time 10 µg of sample lysate was loaded as a comparative (input) control.

Cell surface biotinylation assay

Cortical neurons from 1 day old 129sv (wild type) and p75$^{-/-}$ (p75 knockout) mice were isolated and cultured in 6-well plates and treated with 1 µM Aβ_{42} for 24 hours. On the day of experiment, culture plates were transferred on ice and gently rinsed three times with chilled PBS-CM (PBS containing 1mM $CaCl_2$, 0.5mM $MgCl_2$) before incubation with 0.5 mg/ml Biotin-S-S-NHS (Thermo Scientific, Australia) at 4°C for 60 min. The surface biotinylation reaction was quenched twice (10 min/each time) using 100 mM glycine and cells were then washed three times (5 min/each time) with chilled PBS-CM

and lysed in RIPA buffer containing 1 mM PMSF, antipain, pepstatin, and leupeptin and placed on ice for 30 min. Cell membranes were pelleted at 14,000 RPM for 10 min at 4°C and supernatant was collected and subjected to BCA protein assay (Thermo Scientific, Australia) to quantify total protein. 150 µg of total protein was incubated with streptavidin agarose beads (Sigma-Aldrich, US) overnight at 4°C. Beads were washed 5 times with RIPA buffer and mixed with 30 µl of SDS page buffer and then boiled and subjected to Western blot analysis in order to detect APP, BACE1 and p75^{NTR}using rabbit anti-APP C-terminal (678-695aa) (6687), mouse monoclonal anti-BACE1, (MAB931), and rabbit anti-p75^{NTR} (from Prof. Louis F. Reichardt) (Twelvetrees, Yuen et al. 2010, Fu, Yang et al. 2011).

Statistical analysis

All statistical analysis was performed using SPSS 22 software package (IBM, provided by Flinders University). Variables between groups were determined by either one-way ANOVA following Tukey's post-hoc test or Student's t-test and values of $p<0.05$ were considered statistically significant. Data is presented as mean±SEM.

2.4 Results

p75^{NTR} is co-localized with APP in mouse cortical neurons and HEK-293T cells

In order to investigate whether p75^{NTR} associates with APP, we first quantified the co-localization between endogenous p75^{NTR} and APP in cortical neurons from wild type mouse using mouse anti-p75^{NTR} (5B1D2), and rabbit anti-APP (6687) antibodies followed by anti-mouse Alexa488 and anti-rabbit-Cy3. The neurons were subjected to confocal microscope in order to detect the co-localization between p75^{NTR} and APP. The co-localization between over-expressed p75^{NTR} and APP also were measured in HEK-293T cells co-transfected with human full length APP-YFP and p75-CFF plasmids. The co-localization results indicated that p75^{NTR} associated with APP in both cortical neurons (74%±7) and HEK-293T cells (71%±3) (Figure 2-5, A-C).

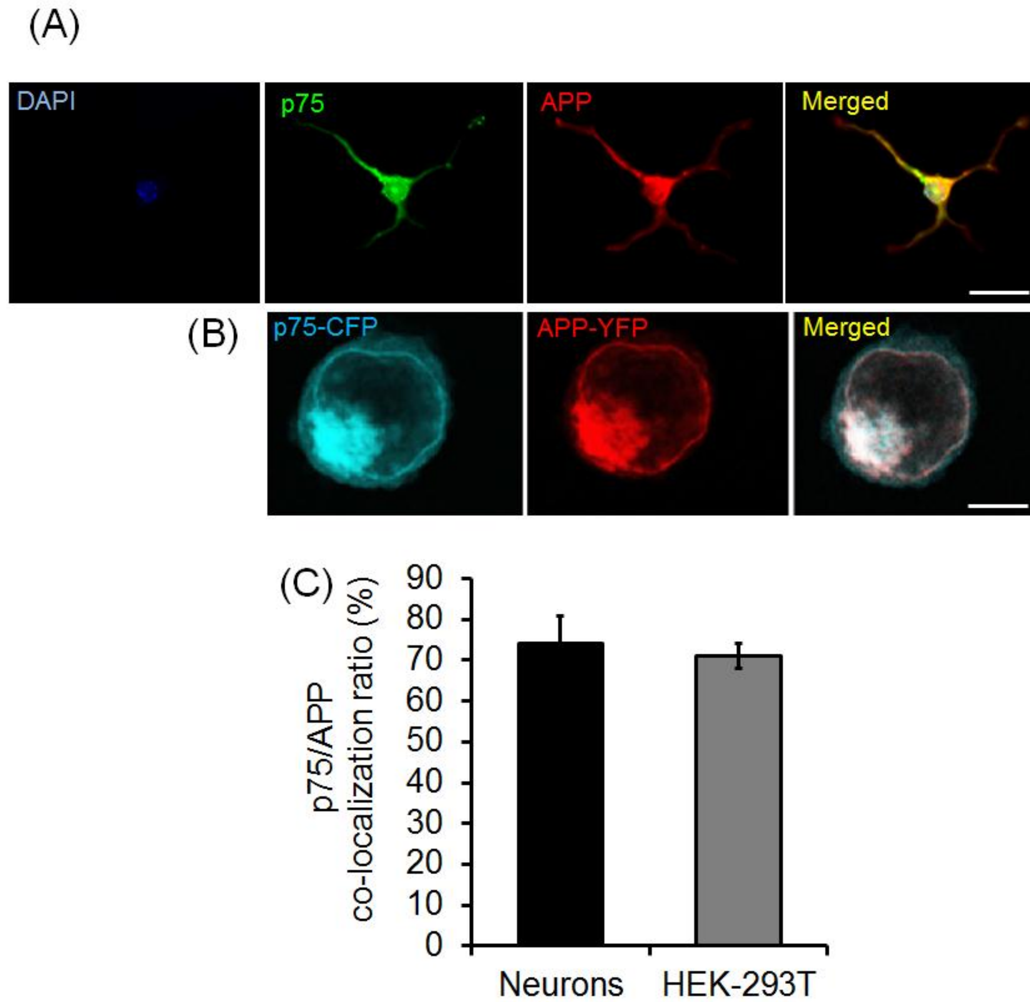


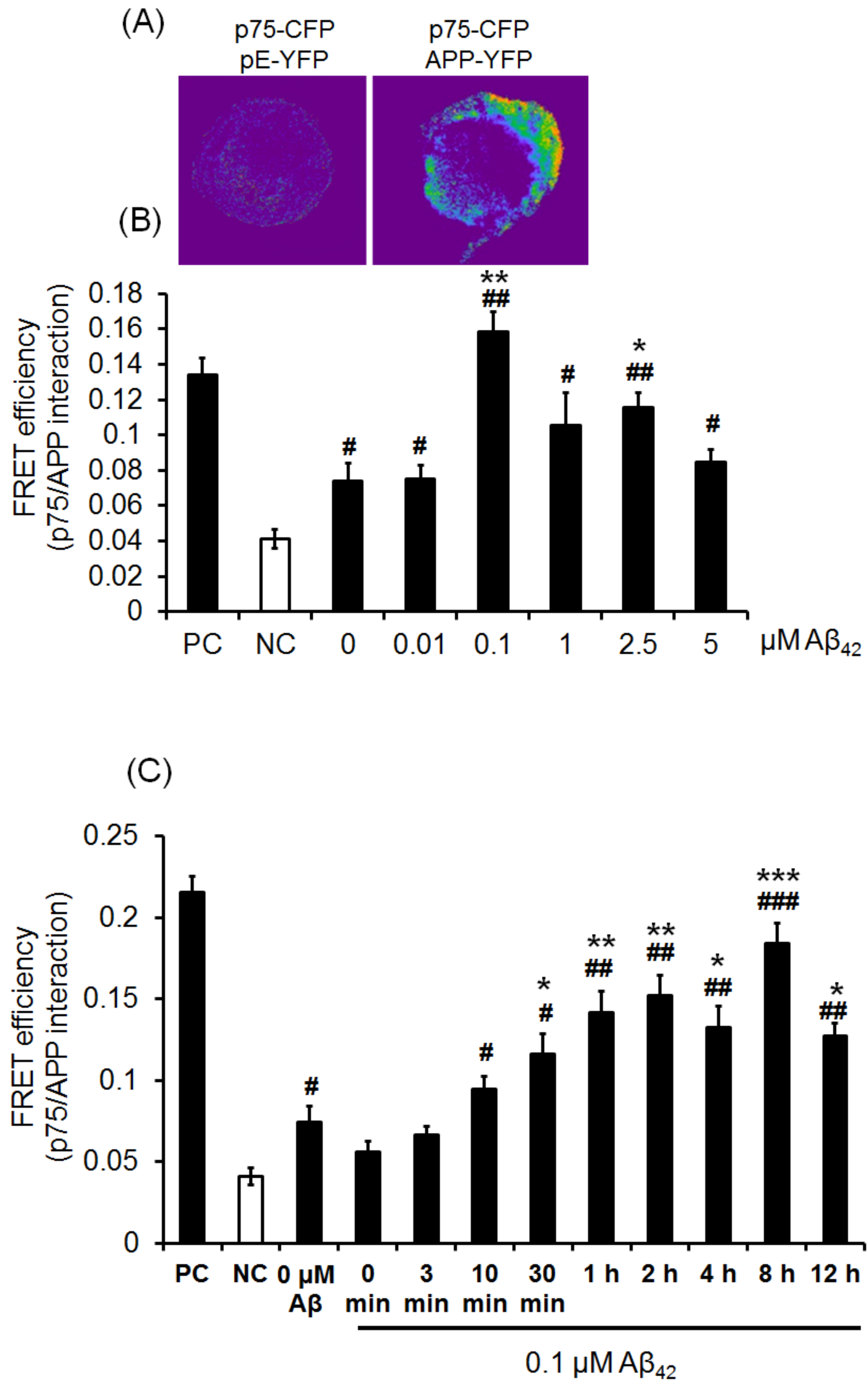
Figure 2-5: The co-localization ratio between p75^{NTR} and APP in mouse cortical neurons and HEK-293T cells

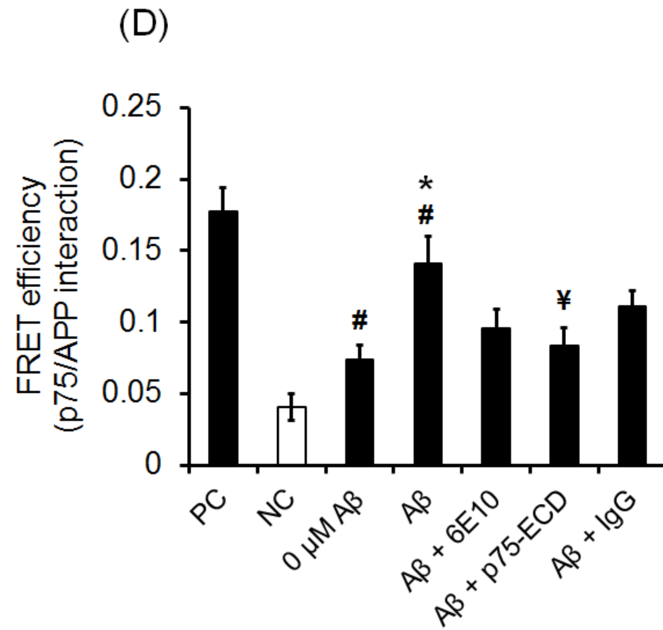
A) Endogenous p75^{NTR} co-localized with APP in a day old 129sv mouse cortical neurons B) p75^{NTR} associated with APP in HEK-293T cells co-transfected with full length p75-CFP and APP-YFP. C) Plot presenting the co-localization ratio in cortical neurons (74%±7) and HEK-293T cells (71%±3) (n=12 from two different coverslips, mean±SEM, Student's t-test).

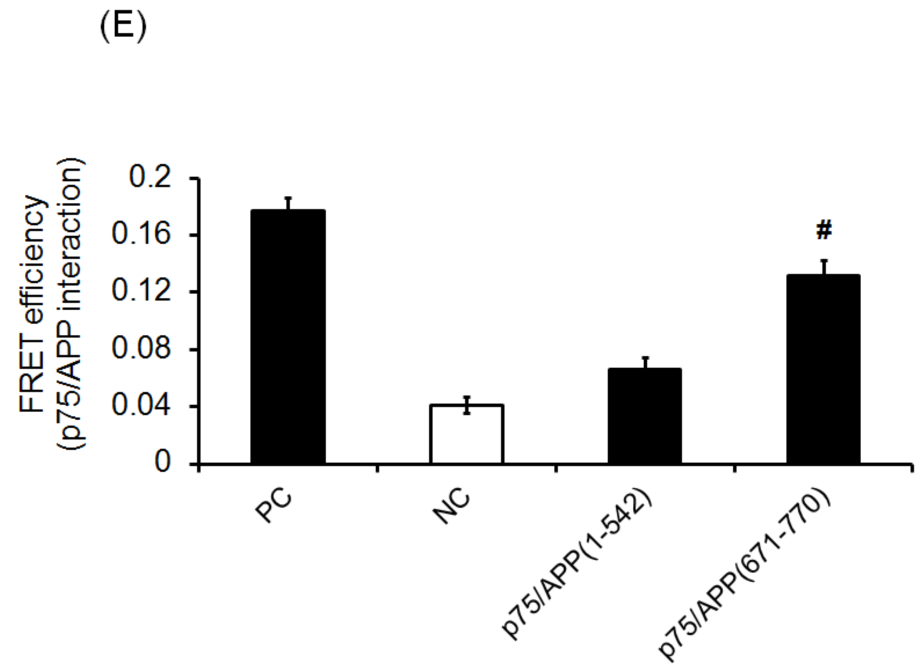
p75^{NTR} interacts with APP and Aβ increases the interaction

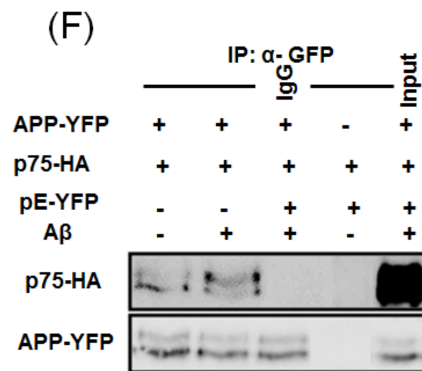
Next, we investigated whether p75^{NTR} interacts with APP and if Aβ acts as a ligand for p75^{NTR} to regulate the p75^{NTR}/APP interaction. HEK-293T cells were co-transfected with APP-YFP and p75-CFP for 24 hours and then treated with 0, 0.01, 0.1, 1, 2.5 and 5 μM of $A\beta_{42}$ overnight. Cells were subjected to FRET acceptor bleaching analysis to quantify the interaction between APP and p75^{NTR}. FRET signals showed that p75^{NTR} highly interacts with APP ($p<0.05$) compare to negative control (NC: co-transfected with pEYFP/p75-CFP plasmids) and that $A\beta_{42}$ increased the p75^{NTR}/APP interaction ($p<0.05$, $p<0.01$) (Figure 2-6 A and B). Next, the cells were treated with 0.1μM $A\beta_{42}$ as the most effective dose on the interaction for different time period. The results demonstrated that p75^{NTR}/APP interaction is consistently increased with time course incubation with Aβ ($p<0.05$, $p<0.01$, and $p<0.001$) (Figure 2-6 C). In order to confirm the effects of Aβ on p75^{NTR}/APP interaction, the transfected HEK-293T were incubated with 0.1μM $A\beta_{42}$ in conjunction with 2μg/ml 6E10 antibody and 10 μg/ml p75ECD recombinant protein which block the biological activity of Aβ. p75ECD recombinant protein attenuated the increased p75^{NTR}/APP interaction induced by Aβ and the effect of p75ECD recombinant protein on blocking this effect in comparison with sham control (Aβ+IgG) was significant ($p<0.05$) (Figure 2-6 D). Furthermore, we tested which domain of APP interacts with p75^{NTR}, HEK-293T cells were co-transfected with p75-CFP/APP (1-542)-YFP and p75-CFP/APP(671-770)-YFP and cells were subjected to FRET acceptor bleaching analysis. The FRET signals indicated that the intracellular domain of APP, APP(671-770), significantly interacts with full length p75^{NTR} ($p<0.05$) (Figure 2-6 E).

We employed Co-IP technique to confirm the baseline and Aβ-induced p75^{NTR}/APP interaction in HEK-293T cells. HEK-293T cells were co-transfected with p75-HA/APP-YFP and p75-HA/pEYFP as negative control and cells treated with 5 μM $A\beta_{42}$ overnight. APP-YFP was then pulled down using goat anti-GFP antibody. The associated p75^{NTR} protein was detected using Western blot by incubation with rabbit anti-HA antibody. The blotting result presented that p75^{NTR} interacts with APP. However, our Co-IP results did not show a significant increased APP/p75^{NTR} interaction induced by Aβ (Figure 2-6 F-G).


(A)
p75-CFP
pE-YFP
p75-CFP
APP-YFP
(B)
FRET efficiency
(p75/APP interaction)
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
PC
NC
0
0.01
0.1
1
2.5
5
μM Aβ42
(C)
FRET efficiency
(p75/APP interaction)
0.25
0.2
0.15
0.1
0.05
0
PC
NC
0 μM Aβ
0 min
3 min
10 min
30 min
1 h
2 h
4 h
8 h
12 h
0.1 μM Aβ42


(D)
FRET efficiency (p75/APP interaction)
0.25
0.2
0.15
0.1
0.05
0
*
#
#
¥
PC
NC
0 µM Aβ
Aβ
Aβ + 6E10
Aβ + p75-ECD
Aβ + IgG


(E)
FRET efficiency (p75/APP interaction)
0.2
0.16
0.12
0.08
0.04
0
#
PC
NC
p75/APP(1-542)
p75/APP(671-770)


(F)
IP: α- GFP
IgG
Input
APP-YFP
p75-HA
pE-YFP
Aβ
p75-HA
APP-YFP

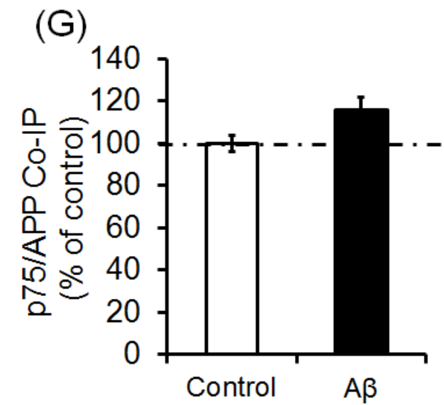

(G)
p75/APP Co-IP (% of control)
140
120
100
80
60
40
20
0
Control
Aβ

Figure 2-6: p75^{NTR} interacts with APP and Aβ_{42} enhances the p75^{NTR}/APP interaction in a dose dependent and time course manner

A) FRET signals from p75-CFP/APP-YFP and p75-CFP/pE-YFP interactions. B) Graph presenting FRET acceptor bleaching analysis for p75-CFP/APP-YFP interaction compare to NC (#$p<0.05$). Aβ_{42} increases the p75^{NTR}/APP interaction ($p<0.05$, $p<0.01$). 0.1 μM Aβ_{42} is the most effective dose in increasing the p75^{NTR}/APP interaction (#$p<0.05$, ##$p<0.01$ vs NC; *$p<0.05$, **$p<0.01$ vs 0 μM Aβ). C) Effects of 0.1μM Aβ_{42} on p75^{NTR}/APP association in a time dependent manner (#$p<0.05$, ##$p<0.01$, ###$p<0.01$ vs NC; *$p<0.05$, **$p<0.01$, ***$p<0.001$, vs 0 μM Aβ) D) p75ECD-Fc recombinant protein, but not 6E10 antibody reduced 0.1 μM Aβ-induced p75^{NTR}/APP interaction in comparison with "Aβ+IgG" group (#$p<0.05$ vs NC; *$p<0.05$ vs 0 μM Aβ; ¥$p<0.05$ vs "Aβ+IgG" group) E) FRET shows that the intracellular domain of APP, APP (671-770), interacts with p75^{NTR} (#$p<0.05$ vs NC) whereas the p75^{NTR}/APP(1-542) interaction was not significant (n=12, mean±SEM., ANOVA, Tukey's post-hoc test; PC: positive control: transfected with p75-CFP-YFP plasmid, NC: negative control: co-transfected with pE-YFP/p75-CFP plasmids). F) Presenting Western blot test for analysis the p75^{NTR}/APP interaction using Co-IP G) Graph demonstrating p75^{NTR}/APP interaction in the presence and absence of 0.1 μM Aβ_{42} on p75^{NTR}/APP interaction using Co-IP method (n=3, mean±SEM., Student's t-test; Control: No treatment).

Overexpression of p75^{NTR} enhances amyloidogenic processing of APP in CHO^{APP695} cells

So far we have shown that p75^{NTR} interacts with APP and Aβ increases p75^{NTR}/APP association. Here, we investigated the significance of this interaction in AD and tested whether p75^{NTR} overexpression increases APP processing. To examine this, CHO^{APP695} cells were transfected with either p75^{NTR} or BACE1 as positive control and empty vector as negative control for 24 hours and cell lysate was then subjected to Western blot to measure APP processing. Both p75^{NTR} and BACE1 significantly increased sAPPβ level ($p<0.01$). However, the effects of p75^{NTR} and BACE1 were not significant on CTFβ/CTFα and $A\beta_{42}$ oligomer generation in CHO^{APP695} cells (Figure 2-7, A and B). Our results suggest that p75^{NTR} mediates amyloidogenic processing of APP.

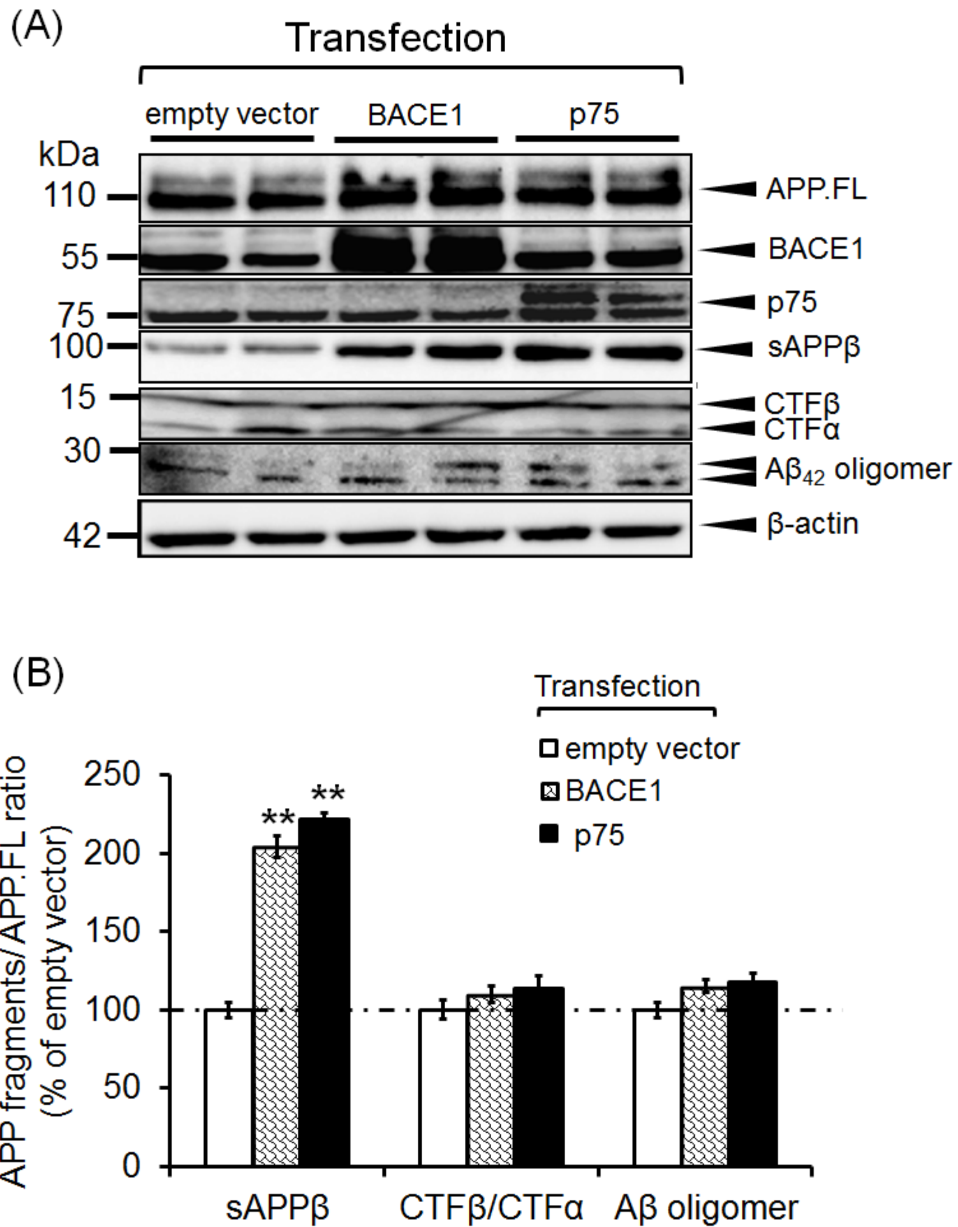
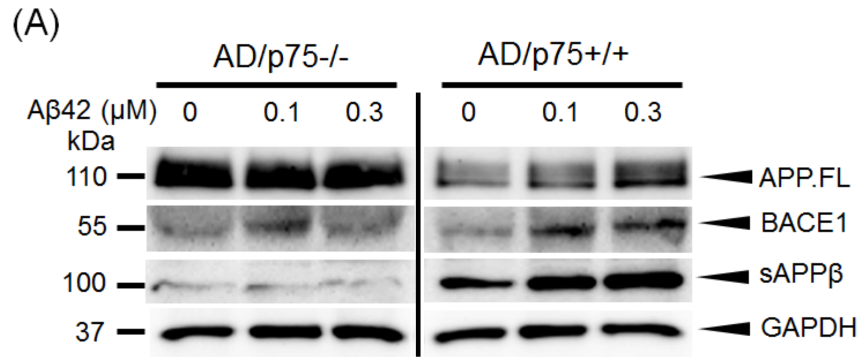


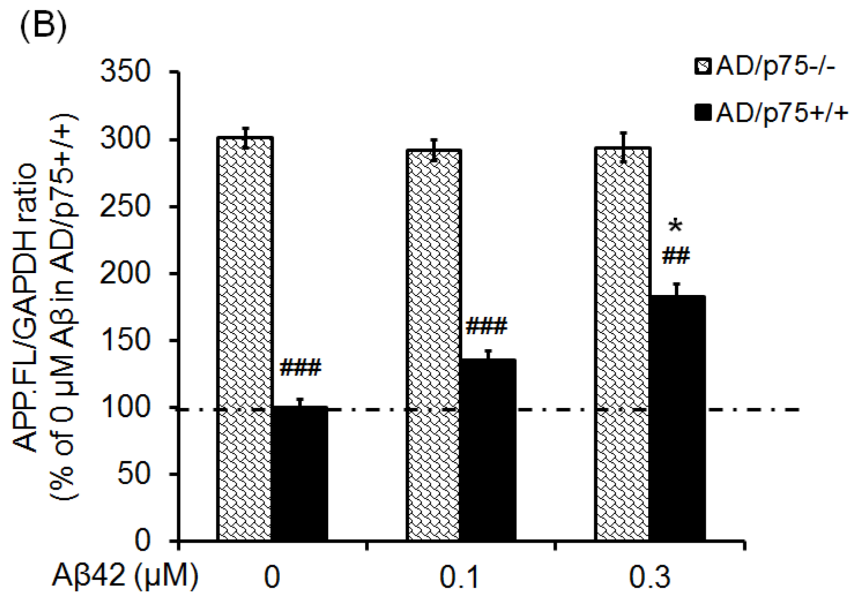
Figure 2-7: Overexpression of p75^{NTR} mediates amyloidogenic processing of APP in CHO^{APP695} cells

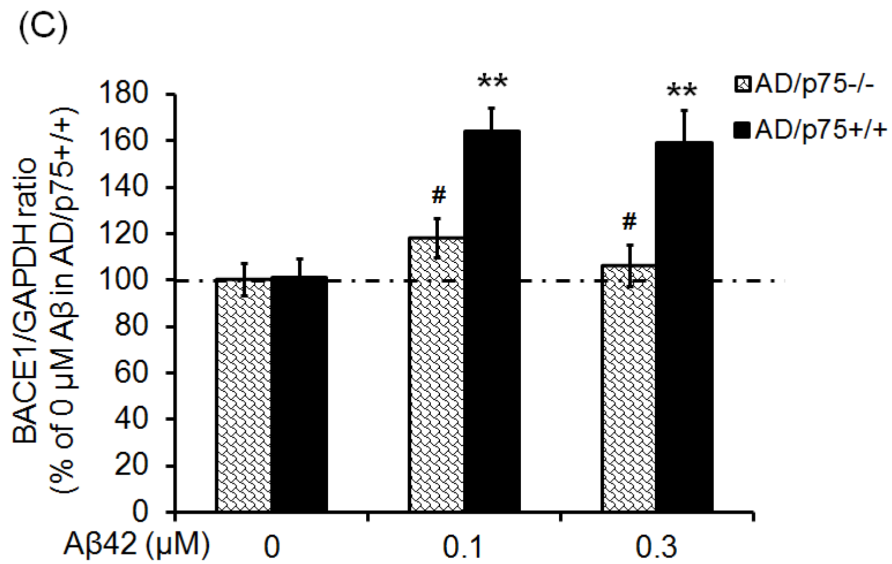
A) Western blot test was conducted to quantify sAPPβ, CTFβ/CTFα ratio and $A\beta_{42}$ oligomer in CHO^{APP695} cells transfected with p75^{NTR} and BACE1. B) Graph indicating the effects of p75^{NTR} and BACE1 transfection on sAPPβ, CTFβ/CTFα ratio and $A\beta_{42}$ oligomer production (**$p<0.01$ vs empty vector, n=4 per group; mean ±SEM, ANOVA, Tukey's post-hoc test; "empty vector" was normalized to 100%).

Aβ_{42} regulates APP processing in AD/$p75^{+/+}$, but not in AD/$p75^{-/-}$ mouse cortical neurons through upregulation of APP and BACE1 expression

After we found that transfection of $p75^{NTR}$ increased APP processing in the CHO^{APP695} cell line, we next investigated whether Aβ influences APP processing toward Aβ generation in mouse cortical neurons through $p75^{NTR}$. Cortical neurons were isolated from AD/$p75^{+/+}$ and AD/$p75^{-/-}$ neonatal mouse brain and treated with different concentrations (0.1 and 0.3 μM) of Aβ_{42} for 24 hours. Cell lysate was then subjected to Western blot to quantify the level of APP, sAPPβ, BACE1 and GAPDH (Figure 2-8 A). The Western blot analysis demonstrated that Aβ_{42} enhanced the level of APP and BACE1expression level in AD/$p75^{+/+}$, but not in AD/$p75^{-/-}$ ($p<0.05$, $p<0.01$) (Figure 2-8 B and C). Furthermore, Aβ_{42} in a dose dependent manner significantly increased the ratio of sAPPβ/APP and sAPPβ/GAPDH in AD/$p75^{+/+}$ but not in AD/$p75^{-/-}$ neurons ($p<0.05$, $p<0.01$, and $p<0.001$) (Figure 2-8 D and E). Our data suggest that Aβ_{42} induces APP and BACE1 expression and regulates amyloidogenic processing of APP via a $p75^{NTR}$-dependent mechanism.


(A)
AD/p75-/-
AD/p75+/+
Aβ42 (μM) 0 0.1 0.3 0 0.1 0.3
kDa
110
55
100
37
APP.FL
BACE1
sAPPβ
GAPDH


(B)
AD/p75-/-
AD/p75+/+
APP.FL/GAPDH ratio
(% of 0 μM Aβ in AD/p75+/+)
350
300
250
200
150
100
50
0
###
###
*
##
Aβ42 (μM) 0 0.1 0.3


(C)
AD/p75-/-
AD/p75+/+
BACE1/GAPDH ratio
(% of 0 μM Aβ in AD/p75+/+)
180
160
140
120
100
80
60
40
20
0
**
**
#
#
Aβ42 (μM) 0 0.1 0.3

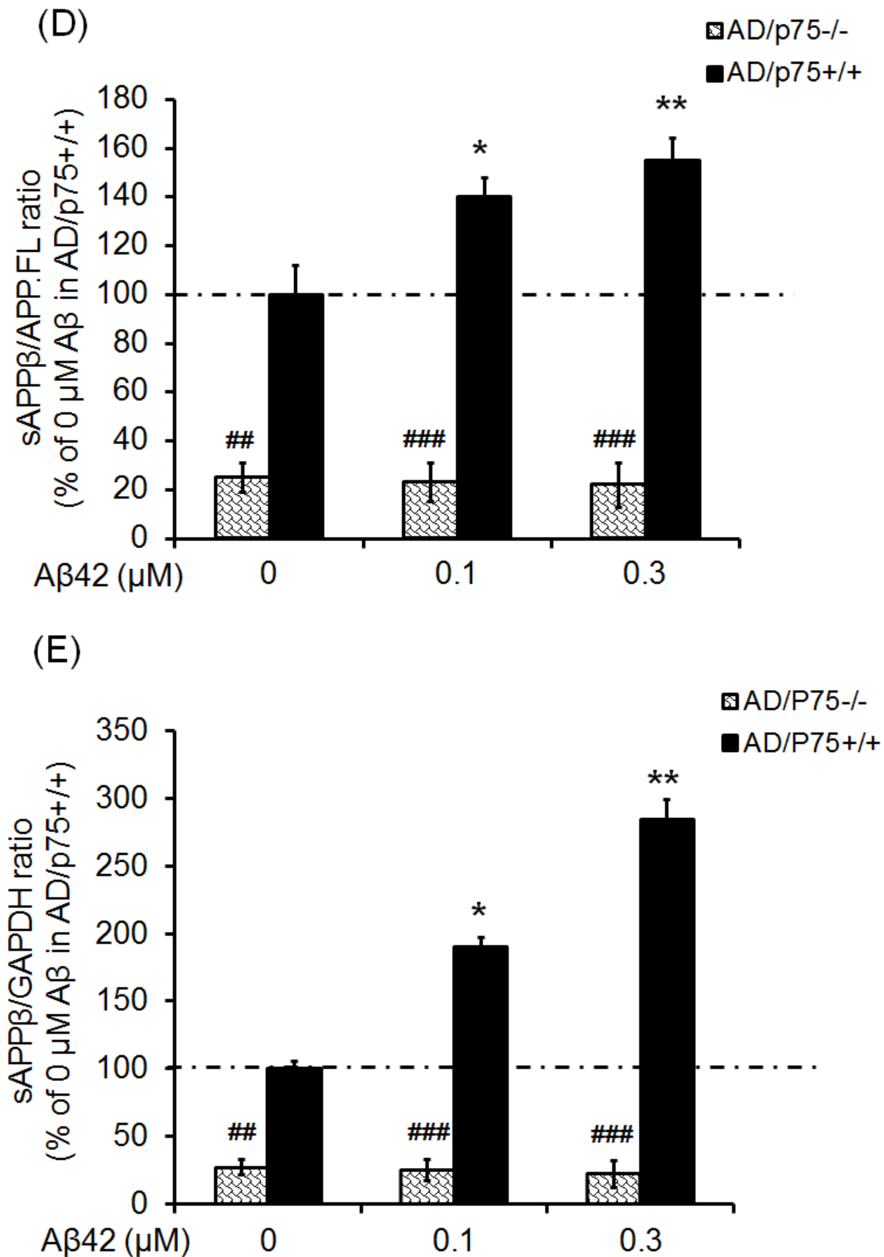


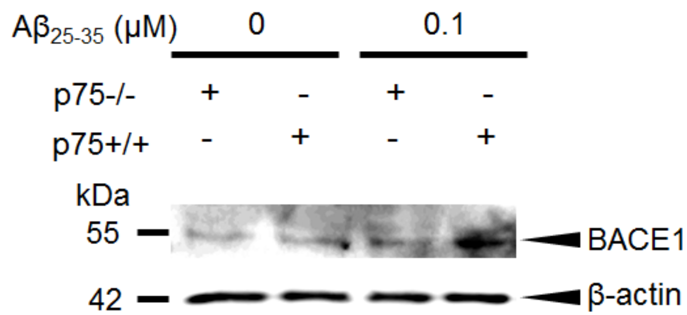
Figure 2-8: $A\beta_{42}$ increased APP processing in AD/p75$^{+/+}$, but not in AD/p75$^{-/-}$ mouse cortical neurons through upregulation of APP and BACE1 expression

A) Western blot presenting full length of APP and BACE1, and the processed fragments of APP in cultured primary cortical neurons from AD/p75$^{+/+}$ and AD/p75$^{-/-}$ treated with 0.1 and 0.3 μM $A\beta_{42}$. B) Effects of Aβ on APP expression in AD/p75$^{+/+}$ and AD/p75$^{-/-}$ cortical neurons in a dose dependent manner (*$p<0.05$, vs 0 μM $A\beta_{42}$ in AD/p75$^{+/+}$; ##$p<0.01$, ###$p<0.001$ vs AD/p75$^{-/-}$; "0 μM $A\beta_{42}$ AD/p75$^{+/+}$" was normalized to 100%). C) Effects of 0.1 and 0.3 μM $A\beta_{42}$ on BACE1 expression level in AD/p75$^{+/+}$ and AD/p75$^{-/-}$ mouse cortical neurons (**$p<0.01$ vs 0 μM $A\beta_{42}$ in AD/p75$^{+/+}$; #$p<0.05$ vs

AD/p75$^{+/+}$; "0 μM Aβ_{42} AD/p75$^{+/+}$" was normalized to 100%). Effects of 0.1 and 0.3 μM Aβ_{42} on the ratio of sAPPβ/APP.FL (D) and sAPPβ/GAPDH (E) in a dose dependent manner in AD/p75$^{+/+}$ and AD/p75$^{-/-}$ mouse cortical neurons (*$p<0.05$ and **$p<0.01$ vs 0 μM Aβ_{42} in AD/p75$^{+/+}$; ##$p<0.01$ and ###$p<0.001$ vs AD/p75$^{+/+}$; "0 μM Aβ_{42} AD/p75$^{+/+}$" was normalized to 100%). (n=4 per dose, mean±SEM, ANOVA, Tukey's post-hoc test).

$A\beta_{25\text{-}35}$ induces BACE1 upregulation in $p75^{+/+}$, but not in $p75^{-/-}$ mouse cortical neurons

In order to confirm the Aβ induced BACE1 upregulation in association with $p75^{NTR}$, cortical neurons from $p75^{+/+}$ and $p75^{-/-}$ mouse were treated with 0.1 μM $A\beta_{25\text{-}35}$ peptide for 24 hours. $A\beta_{25\text{-}35}$ fragment was generally considered as the biologically active region of $A\beta_{42}$. Cell lysate was subjected to Western blot to detect the BACE1 expression. $A\beta_{25\text{-}35}$ significantly enhanced the expression of BACE1 in $p75^{+/+}$ but did not change BACE1 expression in $p75^{-/-}$ mouse cortical neurons ($p<0.05$) (Figure 2-9).



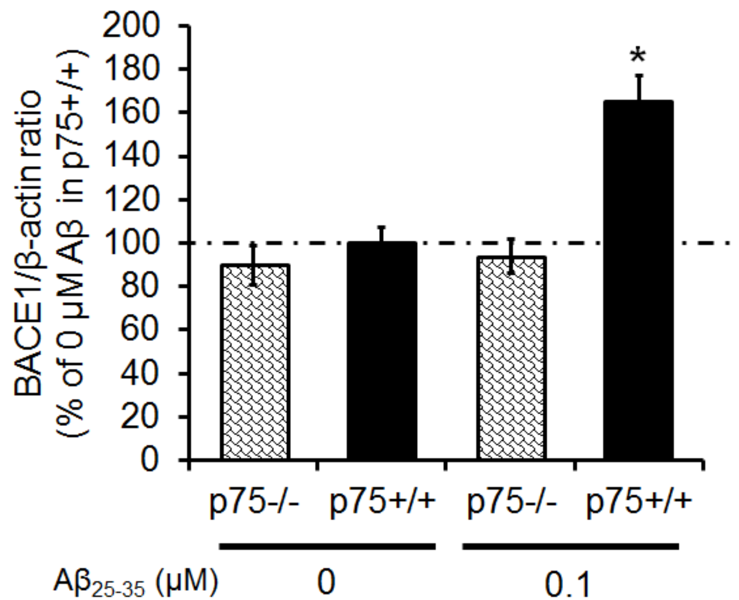
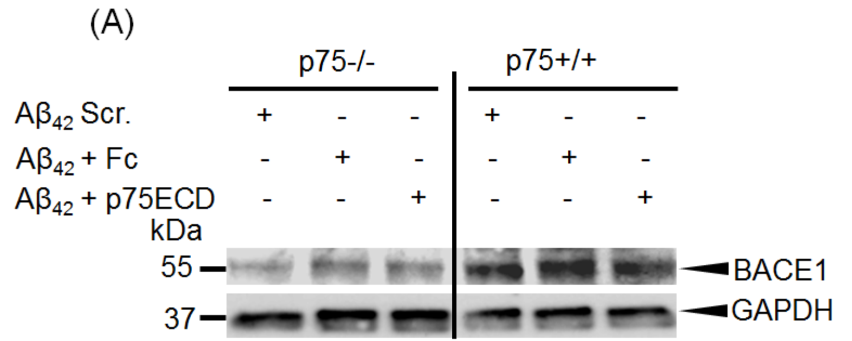


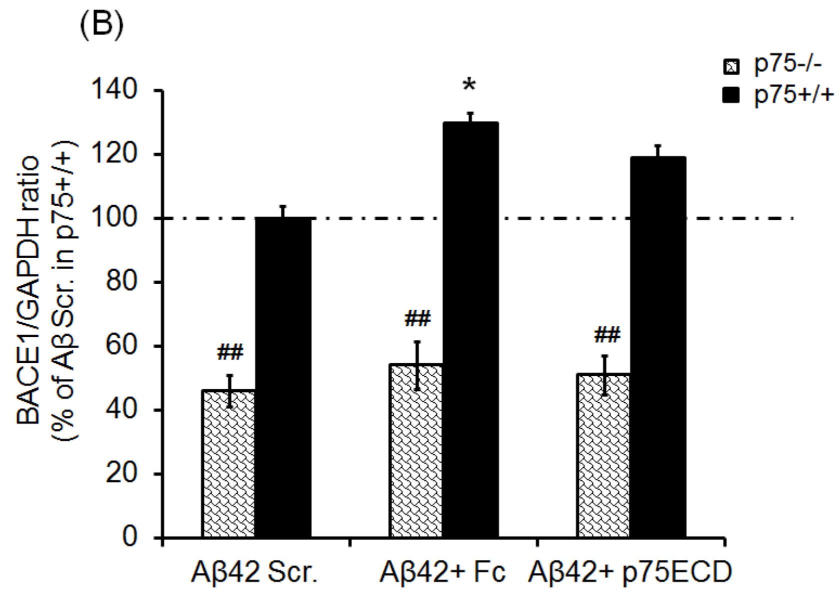
Figure 2-9: $A\beta_{25\text{-}35}$ stimulates BACE1 expression in p75$^{+/+}$, but not in p75$^{-/-}$ mouse cortical neurons

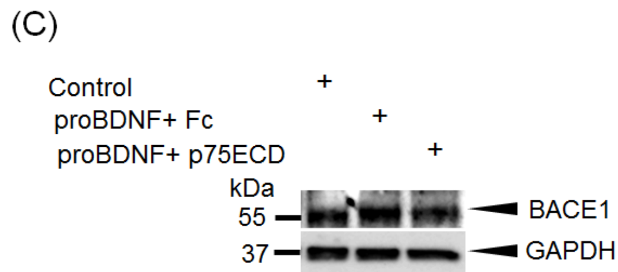
A) Western blot demonstrating BACE1 expression level in p75$^{+/+}$ and p75$^{-/-}$ cultured mouse primary cortical neurons treated with 0.1 µM $A\beta_{25\text{-}35}$. B) Graph presenting the statistical analysis of the effect of $A\beta_{25\text{-}35}$ on BACE1 expression in p75$^{+/+}$ and p75$^{-/-}$ (*$p<0.05$ vs 0 µM $A\beta_{25\text{-}35}$ in p75$^{+/+}$) (n=3 per group, mean±SEM, ANOVA, Tukey's post-hoc test; "0 µM Aβ p75$^{+/+}$" was normalized to 100%).

Effects of p75ECD recombinant protein on Aβ$_{42}$-induced BACE1 upregulation in mouse cortical neurons

Our study showed that p75ECD-Fc recombinant protein inhibited Aβ-induced Sortilin upregulation (Saadipour, Yang et al. 2013). Here, we tested whether p75ECD-Fc prevents Aβ-induced BACE1 upregulation. To address this, cortical neurons from p75$^{-/-}$ and p75$^{+/+}$ mice were cultured and then treated with 0.1 μM Aβ$_{42}$ in conjunction with 10 μg/ml p75ECD-Fc and 5 μg/ml Fc recombinant proteins for 24 hours. Cell lysate was then subjected to Western blot to quantify the expression level of BACE1. The result indicated that Aβ up-regulates BACE1 expression in p75$^{+/+}$, but not in p75$^{-/-}$ cortical neurons. The Western blot showed the effect of p75ECD-Fc on the Aβ-induced BACE1 overexpression was not significant (Figure 2-10 A and B). In our previous study, we found proBDNF increased gene and protein expression of Sortilin, here we tested whether proBDNF has any effect on BACE1 upregulation. We found proBDNF did up-regulate BACE1 expression and the effects of p75ECD-Fc peptide was not significant on proBDNF-induced BACE1 expression (Figure 2-10 C and D).


(A)
p75-/-
p75+/+
Aβ42 Scr.
Aβ42 + Fc
Aβ42 + p75ECD
kDa
55
37
BACE1
GAPDH


(B)
p75-/-
p75+/+
BACE1/GAPDH ratio
(% of Aβ Scr. in p75+/+)
140
120
100
80
60
40
20
0
*
##
##
##
Aβ42 Scr.
Aβ42+ Fc
Aβ42+ p75ECD


(C)
Control
proBDNF+ Fc
proBDNF+ p75ECD
kDa
55
37
BACE1
GAPDH

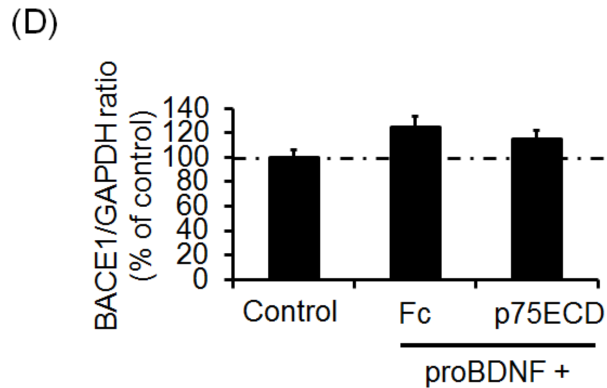

(D)
BACE1/GAPDH ratio
(% of control)
140
120
100
80
60
40
20
0
Control
Fc
p75ECD
proBDNF +

Figure 2-10: Effects of p75ECD-Fc recombinant protein on Aβ_{42}-induced BACE1 upregulation in mouse cortical neurons

A) Western blot presenting BACE1 expression in the presence of Aβ_{42} in conjunction with p75ECD-Fc and Fc recombinant proteins in p75$^{-/-}$ and p75$^{+/+}$ mice cortical neurons. B) Graph demonstrating effects of 0.1 μM Aβ_{42} in the presence of 10 μg/ml p75ECD-Fc and 5 μg/ml Fc recombinant proteins on BACE1 expression (*$p<0.05$ vs Aβ_{42} Scr in p75$^{+/+}$; ##$p<0.01$ vs p75$^{+/+}$; "0 μM Aβ p75$^{+/+}$" was normalized to 100%; Scr: Aβ_{42} scramble). C) Western blot for BACE1 expression in the presence of 50 ng/ml proBDNF in conjunction with 10 μg/ml p75ECD-Fc and 5 μg/ml Fc recombinant proteins in p75$^{+/+}$ neurons. D) Graph showing the effects of proBDNF in the presence of p75ECD-Fc on BACE1 expression in mouse neurons (n=3 per group, mean±SEM, ANOVA, Tukey's post-hoc test; "Control" was normalized to 100%).

Effect of p75^{NTR} on APP distribution in subcellular compartments

We have done subcellular fractionation assay by sucrose gradient in order to investigate whether p75^{NTR} participates on APP distribution in different subcellular organelles. The brain cortex including hippocampus of 9-month old p75$^{+/+}$ and p75$^{-/-}$ mouse was subjected to a high-speed centrifuge to isolate different fractions and Western blot was then conducted to detect APP and subcellular organelles. Our Western blot for sucrose gradient showed that APP appears to have more co-localization with the Early endosome, Golgi, and Lysosome in p75$^{+/+}$ in comparison with p75$^{-/-}$, however we are not able to statistically analyse this data due to insufficient number of repeat experiments, but it leads us for doing further subsequent experiments (

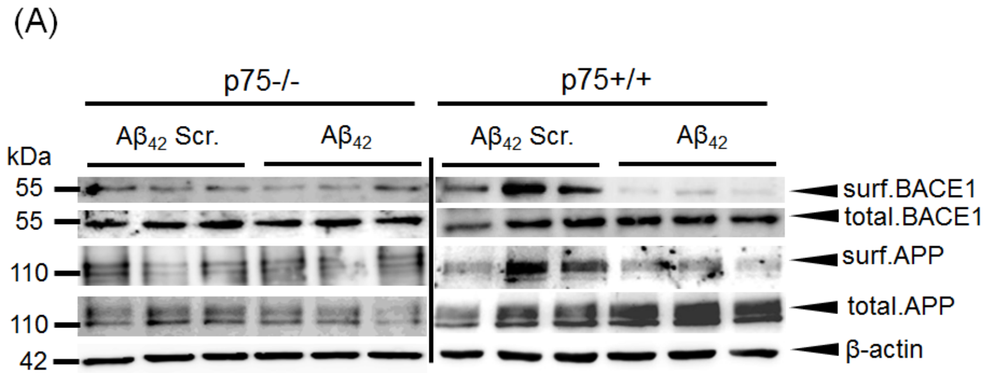
Figure 2-11).

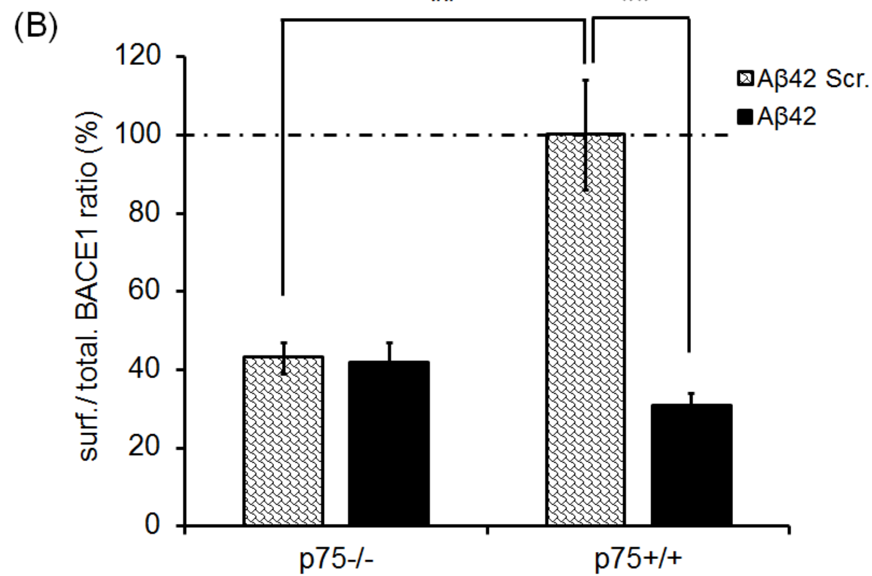
Figure 2-11: Effect of p75^{NTR} on APP distribution in subcellular compartments

A) The Western blot presenting the distribution of APP in subcellular compartments in a 9-month old p75$^{+/+}$ and p75$^{-/-}$ mouse brain isolated by sucrose gradient (Flotilin1: Lipid raft, GRP78: ER, LAMP1: Lysosome, Integrin-α: Plasma membrane, GM130: cis-Golgi complex, EEA1: Early endosome) B) Plot demonstrating the distribution of APP in subcellular compartments normalized to protein markers and GAPDH (n=1, no statistical analysis was applied due to due to insufficient number of the experiment).

Aβ_{42} regulates APP and BACE1 internalization through p75^{NTR} in mouse cortical neurons

Amyloidogenic processing of APP mostly occurs in intracellular organelles as BACE1 activity increases in the acidic environment (Daugherty and Green 2001). How BACE1 and APP converge into the acidic environment is not clear. We propose that p75^{NTR}/Aβ interaction regulates APP and BACE1 internalization. To test this hypothesis, mouse cortical neurons from p75$^{-/-}$ and p75$^{+/+}$ were treated with 0.1 μM Aβ_{42} and Aβ_{42} scramble for 24 hours. Cells were then subjected to a cell surface biotinylation assay in order to evaluate APP and BACE1 internalization. The results demonstrate that the baseline BACE1 protein on the cell surface in p75$^{+/+}$ was significantly less than p75$^{-/-}$ cortical neurons ($p<0.01$), whereas the baseline expression of APP on the cell surface was unchanged in p75$^{+/+}$ versus p75$^{-/-}$ cortical neurons. Aβ reduced the cell surface APP and BACE1 in p75$^{+/+}$ cortical neurons ($p<0.01$) whereas the cell surface expression of APP and BACE1 level were not changed in the presence of Aβ in p75$^{-/-}$ neurons (Figure 2-12) indicating Aβ induces APP and BACE1 internalization through a p75^{NTR}-dependent mechanism.


(A)
p75-/-
p75+/+
Aβ42 Scr.
Aβ42
Aβ42 Scr.
Aβ42
kDa
55
55
110
110
42
surf.BACE1
total.BACE1
surf.APP
total.APP
β-actin


(B)
##
**
120
100
80
60
40
20
0
surf./total. BACE1 ratio (%)
Aβ42 Scr.
Aβ42
p75-/-
p75+/+

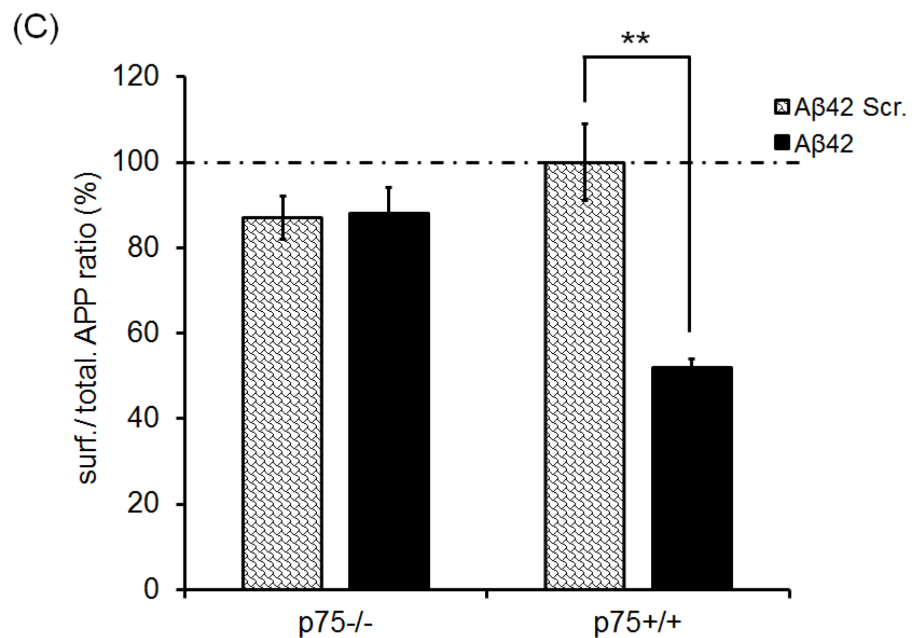

(C)
**
120
100
80
60
40
20
0
surf./total. APP ratio (%)
Aβ42 Scr.
Aβ42
p75-/-
p75+/+

Figure 2-12: Aβ_{42} induces APP and BACE1 internalization in p75$^{+/+}$, but not in p75$^{-/-}$ mouse cortical neurons

A) Western blot for BACE1 and APP internalization assay in p75$^{-/-}$ and p75$^{+/+}$ mouse cortical neurons treated with 0.1 μM Aβ_{42} and Aβ_{42} scramble for 24 hours. Graphs presenting the statistical analysis of the baseline and Aβ-induced BACE1 (B) and APP (C) internalization in p75$^{-/-}$ and p75$^{+/+}$ neurons (**$p<0.01$ and ##$p<0.01$.) (n=3 per group, mean±SEM, ANOVA, Tukey's test; "Aβ_{42} Scr. p75$^{+/+}$" was normalized to 100%).

Aβ_{42} does not mediate p75^{NTR} internalization in mouse cortical neurons

We found that Aβ_{42} induces APP and BACE1 internalization via p75^{NTR}, but there is a question whether p75^{NTR} internalization is mediated by Aβ_{42}. To address this the cultured mouse cortical neurons from p75$^{+/+}$ and p75$^{-/-}$ were treated with 0.1µM Aβ_{42} and Aβ_{42} scramble for 24 hours. Following this, the cell surface proteins were subjected to biotinylation assay and the cell surface p75^{NTR} was measured by Western blot. The results indicated that Aβ_{42} did not significantly change the level of p75^{NTR} on the cell surface in p75$^{+/+}$ (Figure 2-13) indicating p75^{NTR} internalization is not influenced by Aβ in neurons. In this experiment, p75$^{-/-}$ neurons were used as a control indicating specificity of the p75^{NTR} antibody.

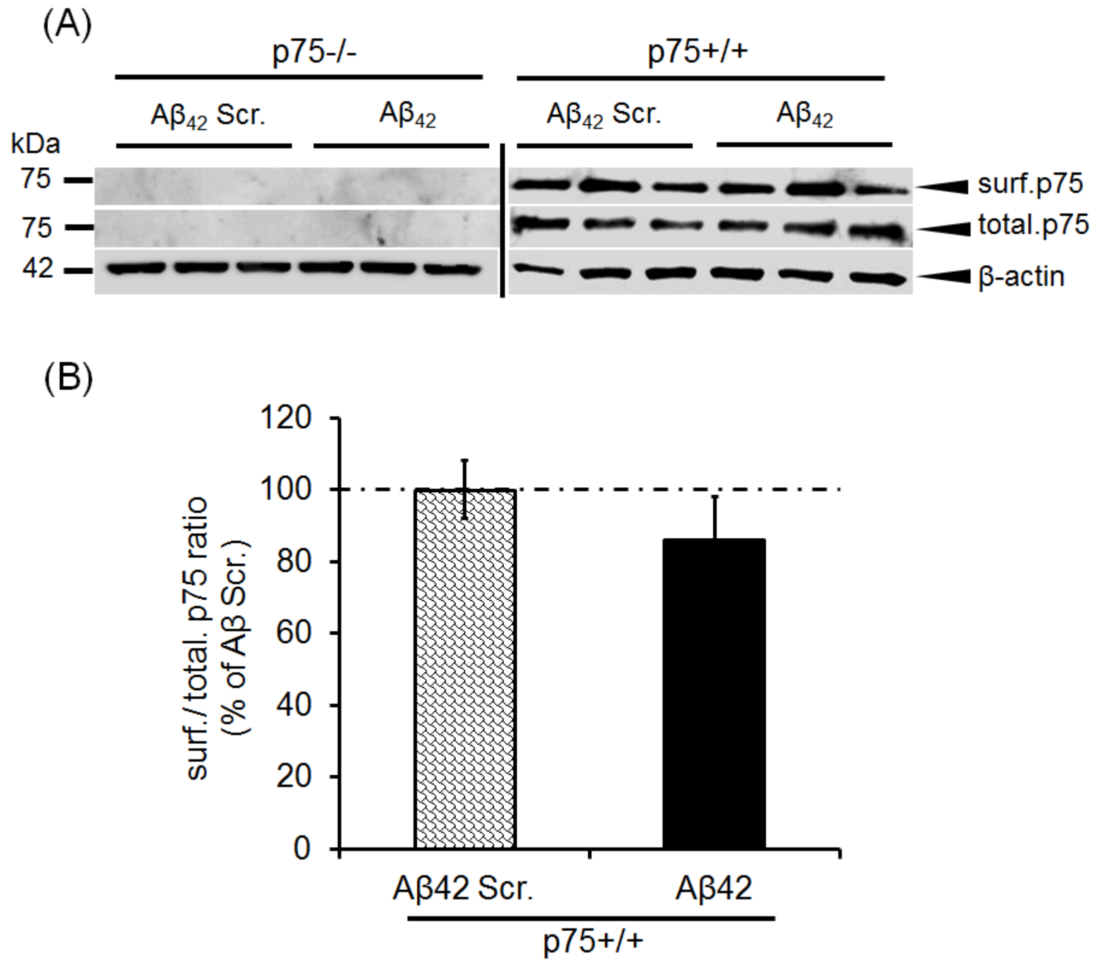


Figure 2-13: Aβ_{42} did not regulate p75^{NTR} internalization in mouse cortical neurons

A) Western blot image for p75^{NTR} internalization assay indicating the cell surface and total expression of p75^{NTR} in p75$^{+/+}$ mouse cortical neurons. p75$^{-/-}$ neurons were used as a control for specificity of the p75^{NTR} antibody. B) Plot presenting statistical analysis in Western blot image from 3 indipendent experimnet (mean±SEM, Student's t-test; "Aβ_{42} Scr. p75$^{+/+}$" was normalized to 100%).

Aβ$_{42}$ and proNGF enhance APP/BACE1 interaction

As Aβ induces APP and BACE1 upregulation, APP and BACE1 internalization and APP processing through interaction with p75^{NTR}, it is possible that Aβ$_{42}$ and proNGF play a key role to regulate APP/BACE1 interaction and therefore increase Aβ production. To test this hypothesis, HEK-293T cells were co-transfected with APP695-YFP and BACE1-CFP 24 hours. Following this, cells were treated with 0.1 µM Aβ$_{42}$ and 100 ng/ml proNGF for overnight and subsequently were subjected to FRET acceptor bleaching analysis. The results present that Aβ and proNGF both significantly increased the APP/BACE1 interaction ($p<0.01$) (Figure 2-14).

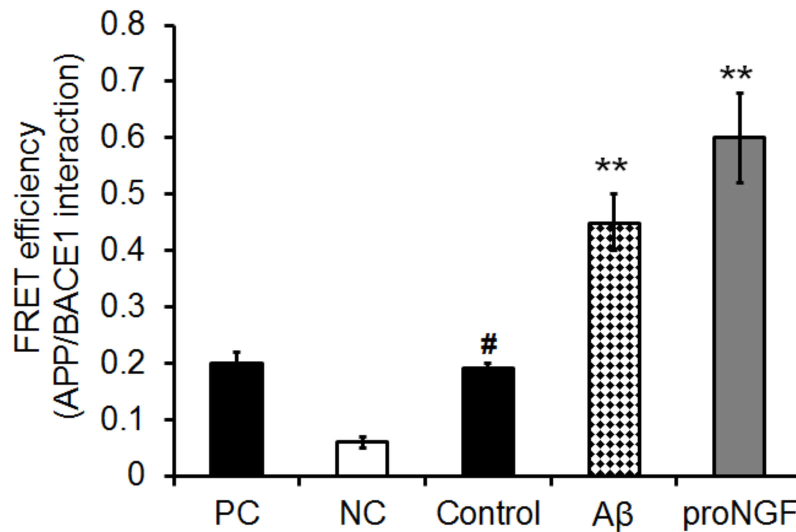


Figure 2-14: Aβ$_{42}$ and proNGF enhanced APP/BACE1 interaction

Plot presenting FRET efficiency of the interaction between APP695-YFP and BACE1-CFP in HEK-293T cells (#$p<0.05$ vs NC, **$p<0.01$ vs Control) (n=12 per group; mean±SEM, ANOVA, Tukey's post-hoc test; PC: positive control: transfected with p75-CFP-YFP plasmid, NC: negative control: co-transfected with pE-YFP/BACE1-CFP plasmids, Control: no treatment).

$A\beta_{42}$ increased phosphorylation state of APP at threonine-668 through $p75^{NTR}$ in mouse cortical neurons

It was previously reported that phosphorylation of APP at Thr668 site is critical for the neuronal functions of APP and plays a critical role in APP metabolism and Aβ generation by mediating the BACE cleavage of APP (Lee, Kao et al. 2003). We propose that Aβ plays a critical role to regulate APP-Thr668 phosphorylation through interaction with $p75^{NTR}$ therefore facilitates BACE1 cleavage of APP. To test our hypothesis, cortical neurons from AD/$p75^{-/-}$ and AD/$p75^{+/+}$ mouse were cultured and treated with 1 μM $A\beta_{42}$ and $A\beta_{42}$ scramble 24 hours. Cells were then subjected to Western blot to quantify phosphorylated APP-Thr668 level. Our data showed that $A\beta_{42}$ significantly increased APP-Thr668 phosphorylation in AD/$p75^{+/+}$, but not in AD/$p75^{-/-}$ cortical neurons ($p<0.05$) (Figure 2-15). Our data suggest that $A\beta_{42}$ regulates the phosphorylation of APP-Thr668 through a $p75^{NTR}$-dependent signalling pathway.

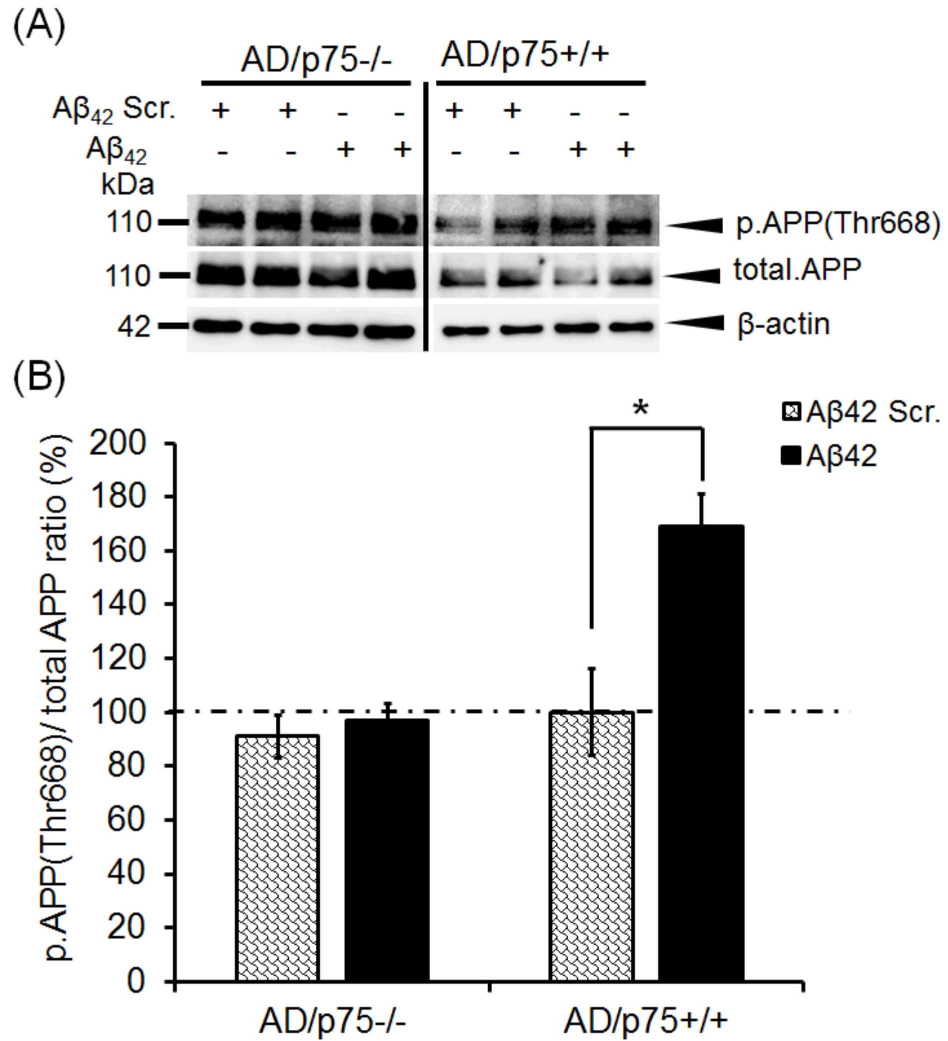


Figure 2-15: $A\beta_{42}$ induces APP-Thr668 phosphorylation in AD/p75$^{+/+}$, but not in AD/p75$^{-/-}$ mouse cortical neurons

A) Western blot demonstrating the level of phosphorylated APP-Thr668 and total APP in the presence of 1 μM $A\beta_{42}$ and $A\beta_{42}$ scramble in AD/p75$^{-/-}$ and AD/p75$^{+/+}$ mouse cortical neurons B) Graph showing the statistical analysis of p.APP-Thr668/total APP in Western blot image from 3 indipendent experimnet ($p<0.05$) (mean±SEM, ANOVA, Tukey's post-hoc test; "$A\beta_{42}$ Scr. AD/p75$^{+/+}$" was normalized to 100%).

Aβ_{42} induced Tau-phosphorylation through p75^{NTR} in mouse cortical neurons

Hyper-phosphorylation of Tau results in formation of NFTs in neurons and it is considered a hallmark of AD. As it is reported that Aβ induces Tau phosphorylation and promotes degeneration of cholinergic neurons in rat primary septal cultures (Zheng, Bastianetto et al. 2002), here we investigated whether an increased phosphorylated Tau by Aβ occurs via a p75^{NTR} dependent mechanism in primary cortical neurons. To address this, cortical neurons were isolated from AD/p75$^{-/-}$ and AD/p75$^{+/+}$ mouse brain and treated with 1 μM Aβ_{42} and Aβ_{42} scramble for 24 hours. The cells were subjected to Western blot analysis in order to detect phosphorylated Tau using mouse phospho-PHF-Tau pSer202+Thr205 monoclonal (AT8) antibody and total Tau as reference. The data indicated that Aβ_{42} significantly enhanced the level of phosphorylated Tau in AD/p75$^{+/+}$ cortical neurons ($p<0.05$) whereas Tau phosphorylation was not changed in AD/p75$^{-/-}$ in the presence of Aβ_{42} (Figure 2-16). Our study suggests p75^{NTR} plays a critical role in Aβ-induced Tau hyper-phosphorylation in neurons.

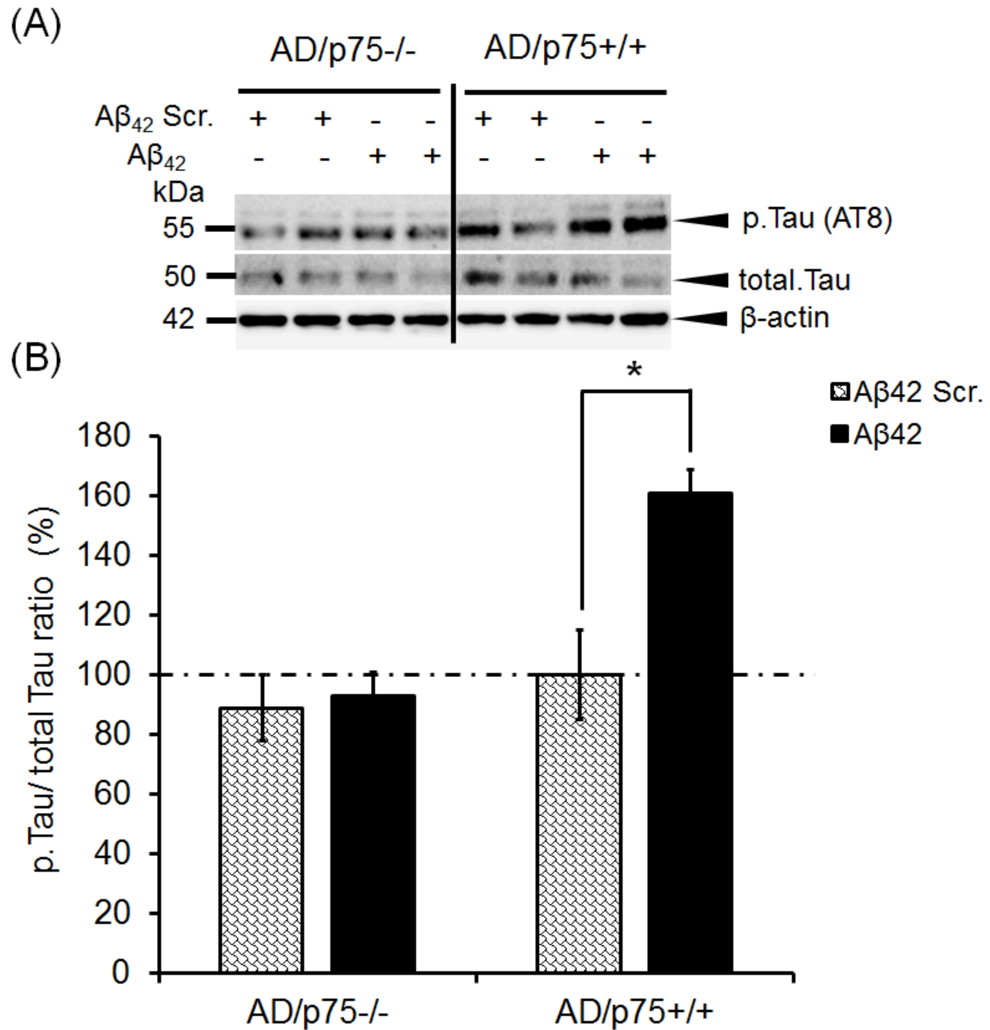


Figure 2-16: Aβ_{42} increased Tau phosphorylation in AD/p75$^{+/+}$, but not in AD/p75$^{-/-}$ mouse cortical neurons

A) Western blot presenting the level of phosphorylated Tau in the presence of 1 μM Aβ_{42} and Aβ_{42} scramble in AD/p75$^{-/-}$ and AD/p75$^{+/+}$ mouse cortical neurons using mouse phospho-PHF-Tau pSer202+Thr205 monoclonal (AT8) antibody. B) Plot demonstrating the statistical analysis of phosphorylated/total Tau in Western blot image from 3 indipendent experimnet ($p<0.05$.) (mean±SEM, ANOVA, Tukey's post-hoc test; "Aβ_{42} Scr. AD/p75$^{+/+}$" was normalized to 100%)

2.5 Discussion

In the current study, we discovered that p75^{NTR} associates with APP. We first evaluated endogenous p75^{NTR}/APP co-localization in mouse cortical neurons. We also quantified p75^{NTR}/APP co-localization in HEK-293T cells co-transfected with p75^{NTR} and APP. Although, HEK-293T cells are not a neuronal cell line and may not be a suitable cell line for study on neuronal functions, it is widely used to test over-expressed protein co-localization and interaction studies due to an easy delivery of recombinant plasmid into these cells (Baeza-Raja, Li et al. 2012, Skeldal, Sykes et al. 2012). Before using HEK-293T cells, we tested human neuroblastoma SH-SY5Y cells for the interaction assay, but the transfection efficiency was very low (<10%) in this cell line. Our FRET results indicated that p75^{NTR} interacts with APP and $A\beta_{42}$ increased the p75^{NTR}/APP interaction. In this study, we have used the oligomer form of $A\beta_{42}$ and it was confirmed by Western blot using 6E10 antibody. However, the Western blot for $A\beta_{42}$ oligomer confirmation shows some other minor bands indicating monomer and dimer forms of $A\beta_{42}$, we have experienced it is impossible to have pure $A\beta_{42}$ oligomer and this is consistent with previous reports (Nielsen, Ek et al. 2013). The effect of 0.1 µM $A\beta_{42}$ was the most significant effect on the interaction. We found that a high concentration (5 µM) of $A\beta_{42}$ did not change the p75^{NTR}/APP interaction. We do not know the exact reason for this, but we have already demonstrated that the cell viability of SH-SY5Y cells was reduced in the presence of different concentrations of $A\beta_{42}$, in a dose-dependent manner and 5 µM significantly reduced the cell viability (Saadipour, Yang et al. 2013). Thus, we assume a low effect of 5 µM $A\beta_{42}$ on the p75^{NTR}/APP interaction may be due to

apoptotic effects 5 µM Aβ_{42} and that may be the reason why our Co-IP result is not consistent with our FRET data regarding to Aβ_{42} induced p75^{NTR}/APP interaction.

To test the significant of the APP/p75^{NTR} interaction in AD, we found that p75^{NTR} regulated APP processing and significantly increased the sAPPβ production in CHO cells which constantly expressed APP695 (CHO^{APP695}). However, our Western blot data illustrated that p75^{NTR} did not change the level of Aβ_{42} oligomer and the ratio of CTFβ/CTFα. Overexpression of BACE1 as a positive control, also was not able to alter the Aβ_{42} and CTFβ/CTFα levels. We do not know what might be the reason, but at least over production of sAPPβ in the presence of p75^{NTR} and BACE1 leads us to have more investigation on the role of p75^{NTR} on APP processing in mouse cortical neurons. We have found the baseline of sAPPβ expression in AD/p75$^{+/+}$ is significantly higher than AD/p75$^{-/-}$ whereas the total APP protein in AD/p75$^{+/+}$ is decreased compared with AD/p75$^{-/-}$ mouse cortical neurons. This indicates that p75^{NTR} mediates amyloidogenic processing of APP to generate sAPPβ and it is consistent with our CHO^{APP695} results. Furthermore, our data showed that Aβ increases sAPPβ production in AD/p75$^{+/+}$ cortical neurons in a dose dependent manner whereas it did not change APP processing in AD/p75$^{-/-}$ neurons. Although, sAPPβ production is a well-known indicator for the amyloidogenic processing pathway for APP, it would be better to quantify CTFβ, CTFα and Aβ in neurons. We also have tried to detect these fragments of APP using Western blot, but our experiments failed and it is thought Western blot was not sensitive enough to detect CTFβ, CTFα and Aβ in mouse cortical neurons. We examined how p75^{NTR} may promote APP processing in response to Aβ. Our data indicated that Aβ induced APP and BACE1 expression in a p75^{NTR}–dependent mechanism and therefore increased

sAPPβ production. The sAPPβ production in AD/p75$^{+/+}$ and AD/p75$^{-/-}$ neurons in the presence and absence of Aβ was presented as sAPPβ/APP.FL and sAPPβ/GAPDH ratio. The sAPPβ/APP.FL ratio indicates the contribution of shared factors except APP upregulation, activated by p75^{NTR}±Aβ on APP processing. The sAPPβ/GAPDH ratio illustrates the contribution of all mechanisms caused by p75^{NTR}±Aβ in sAPPβ generation. Although we found p75^{NTR} mediates APP processing in neurons through up-regulation of APP and BACE1, we did not examine which p75^{NTR}-dependent downstream signalling pathway involved in these phenomena and it is lacking in our study. In addition, we did not test the effect of p75^{NTR} ligands including NGF and proNGF on APP processing. We also did not investigate whether p75^{NTR} promotes non-amyloidogenic pathway of APP processing in neurons.

To further confirm that Aβ-induced BACE upregulation occurs in a p75^{NTR}-dependent mechanism, we discovered that Aβ$_{25-35}$, an active fragment of Aβ$_{42}$, significantly increased BACE1 in p75$^{+/+}$, but not p75$^{-/-}$ cortical neurons. p75ECD-Fc recombinant protein was used to block BACE1 upregulation caused by Aβ in p75$^{+/+}$. Our data showed that p75ECD-Fc was not able to restore the BACE1 upregulation. We already found that 50 ng/ml proBDNF increased Sortilin upregulation in human neuroblastoma SH-SY5Y cells (Saadipour, Yang et al. 2013), so here we tested whether 50 ng/ml proBDNF increases BACE1 upregulation in p75$^{+/+}$ neurons in the presence of p75ECD-Fc and Fc. The data demonstrated that the effect of proBDNF on BACE1 upregulation was not significant. We investigated whether p75^{NTR} mediates APP distribution in subcellular fractions extracted from a 9-month old p75$^{+/+}$ and p75$^{-/-}$ mouse brain. The data indicated a co-localization between APP and the Lysosome, early endosome and Golgi in p75$^{+/+}$ is

higher than $p75^{-/-}$; however, we only did this experiment one time and we were not able to statistically analyse the result. Following subcellular fractionation, we conducted a biotinylation assay to test whether $p75^{NTR}$ in the presence and absence of Aβ mediates APP and BACE1 internalization. Our results demonstrated that the cell surface of APP and BACE1 in $p75^{+/+}$ is higher than $p75^{-/-}$ neurons. In addition, we found Aβ significantly increased APP and BACE1 internalization in $p75^{+/+}$, but not $p75^{-/-}$ neurons whereas Aβ did not change $p75^{NTR}$ internalization. This result suggests Aβ mediates APP and BACE1 trafficking to subcellular compartment through $p75^{NTR}$. Following this, $p75^{NTR}$ re-cycles back to the cell surface to mediate more APP and BACE1 internalization. A live imaging technique would be helpful to address whether APP and BACE1 internalization results in APP/BACE1 interaction and convergence in subcellular compartments in a $p75^{NTR}$-dependent mechanism. Unfortunately, the live imaging system was not installed on our confocal microscope and that is why we were not able to do this experiment. Our FRET data indicates that Aβ and proNGF which are highly expressed in AD brain, increase APP/BACE1 interaction in HEK-293T cells transfected with APP-YFP and BACE1-CFP plasmids. Although, this data does not directly indicate that Aβ-induced APP/BACE1 interaction occurs through $p75^{NTR}$, it suggests that the APP and BACE1 internalization mediated by Aβ/$p75^{NTR}$ may result in APP/BACE1 convergence in subcellular compartments. Moreover, we found Aβ induced APP phosphorylation at Thr668 via association with $p75^{NTR}$ in mouse cortical neurons. In addition, Aβ increased the phosphorylation of Tau protein at Ser202+Thr205 in AD/$p75^{+/+}$, but not in AD/$p75^{-/-}$ mouse cortical neurons, suggesting $p75^{NTR}$ is a key molecule participating in AD pathogenesis. Although, phosphorylation of Tau at Ser202

and Thr205 is a well-known biomarker for AD (Rankin, Sun et al. 2005), the phosphorylation of Tau at other regions such as Ser262 and Ser422, promotes Aβ_{42}-induced Tau toxicity in AD (Biernat, Gustke et al. 1993, Zheng, Bastianetto et al. 2002, Ma, Yang et al. 2009, Iijima, Gatt et al. 2010). We have not tested whether Aβ regulates the phosphorylation of Tau at Ser262 and Ser422 regions through p75^{NTR} which could be important for a better understanding of the function of p75^{NTR} in Tau phosphorylation.

In sporadic AD brain, Aβ increases BACE1 expression which promotes Aβ generation, creating a vicious cycle that drives Aβ over-production (Buggia-Prevot, Sevalle et al. 2008, Guglielmotto, Monteleone et al. 2011, Chami, Buggia-Prevot et al. 2012). Aβ is both an initiating factor and a product of this cycle. However, the exact mechanism underlying the cycle is not clear. Accumulating evidence shows that neurodegenerative signalling receptors such as p75^{NTR} and Sortilin are activated by Aβ and proneurotrophins and are upregulated in sporadic AD (Mufson and Kordower 1992, Selkoe 2001, Hu, Zhang et al. 2002, Costantini, Weindruch et al. 2005, Ito, Menard et al. 2012, Saadipour, Yang et al. 2013, Tiveron, Fasulo et al. 2013), whereas BDNF/TrkB signalling is downregulated in AD brain (Peng, Wuu et al. 2005, Cattaneo and Calissano 2012). These changes lead to an imbalance between neurotrophic and neurodegenerative signalling in sporadic AD (Zeng, Lu et al. 2011). For example, it is reported that Aβ mediates p75^{NTR} upregulation in SH-SY5Y human neuroblastoma cells and AD mice brain (Chakravarthy, Gaudet et al. 2010). Our laboratory also showed that the level of p75^{NTR} in APPswe/PS1dE9 transgenic mouse is higher than wild type (Wang, Wang et al. 2011). In addition, an increased level of membrane-associated p75^{NTR} in human AD

hippocampus was reported (Chakravarthy, Menard et al. 2012). Furthermore, there are some reports to indicate p75^{NTR} is up-regulated in response to pathological stimuli such as neurotrauma, inflammation and epilepsy (Zhou, Rush et al. 1996, Dechant and Barde 2002, Ibanez and Simi 2012).

Recently, it was reported that p75^{NTR} interacts with APP and attenuates non-amyloidogenic processing of APP and sAPPα generation (Fombonne, Rabizadeh et al. 2009). Thus, the APP processing balance shifts toward the amyloidogenic pathway and Aβ generation. Furthermore, NGF and Aβ reduce the interaction between p75^{NTR} and APP and decrease Aβ generation. In this study, we also found that p75^{NTR} interacts with APP consistent with the previous studies. Moreover, Aβ increased p75^{NTR}/APP association in a time and dose dependent manner. To confirm this, it already has been shown that the p75^{NTR} antagonistic peptide (CATDIKGAEC) including KGA motif, significantly attenuated Aβ toxicity in brain (Yaar, Arble et al. 2008). We also used p75ECD-Fc recombinant protein to block the biological activity of Aβ and investigate whether Aβ-induced p75^{NTR}/APP interaction is inhibited by p75ECD-Fc. Our FRET data confirms that p75ECD recombinant protein could decrease Aβ-induced p75^{NTR}/APP interaction which suggests Aβ regulates p75^{NTR}/APP interaction most likely through activation of its receptor, p75^{NTR}.

$A\beta_{42}$ increases endogenous Aβ generation in cultured hippocampal neurons of adult rats and therefore promotes AD (Majd, Rastegar et al. 2007), however the exact mechanism is not known. Here we proposed the interaction of APP and p75^{NTR} promotes APP processing. Our data showed that overexpression of p75^{NTR} in CHO^{APP695} cell line increased APP processing. To confirm this, we found the sAPPβ in AD/p75$^{+/+}$ is higher

than AD/p75$^{-/-}$ mouse cortical neurons. In addition, our data demonstrated that Aβ_{42} enhanced amyloidogenic processing of APP in a dose dependent manner in AD/p75$^{+/+}$ mouse cortical neurons whereas APP processing was unchanged in AD/p75$^{-/-}$. In the amyloidogenic pathway in AD, APP is sequentially cleaved firstly by BACE1 and then by γ-secretase to generate Aβ peptides. Aging is a risk factor for AD (Jorm, Korten et al. 1987) and the biological activity of BACE1 increases with aging in human, monkey and mouse brain to promote APP processing and Aβ generation (Fukumoto, Rosene et al. 2004). In addition, the expression and activity of BACE1 is increased in association with elevated Aβ in different regions of sporadic and familial AD brain (Fukumoto, Cheung et al. 2002, Holsinger, McLean et al. 2002, Tyler, Dawbarn et al. 2002, Yang, Lindholm et al. 2003, Li, Lindholm et al. 2004). It was reported that Aβ stimulates the expression of BACE1 through the JNK pathway (Piccini, Borghi et al. 2012). However, it is not known which receptor mediates the Aβ-JNK-BACE1. Since JNK is a common signalling pathway downstream of p75^{NTR} and contributes to neuronal apoptosis when p75^{NTR} bound to Aβ (Casaccia-Bonnefil, Carter et al. 1996, Yoon, Casaccia-Bonnefil et al. 1998, Friedman 2000, Yaar, Zhai et al. 2002). We propose that p75^{NTR} is a critical molecule to regulate BACE1 upregulation. Our data confirmed this hypothesis and showed that Aβ_{42} induces BACE1 expression in AD/p75$^{+/+}$ mouse cortical neurons, but not in AD/p75$^{-/-}$. In addition, we found that A$\beta_{25\text{-}35}$ stimulates BACE1 expression in p75$^{+/+}$ mouse cortical neurons whereas A$\beta_{25\text{-}35}$ was not effective on the level of BACE1 in p75$^{-/-}$. These results indicate that p75^{NTR} is the key receptor which mediates the Aβ-driven vicious cycle in which BACE1 is up-regulated and the amyloidogenic pathway is activated.

Our study indicates that Aβ increased APP expression in AD/p75$^{+/+}$ mouse cortical neurons, but not AD/p75$^{-/-}$. Consistent with our results, others demonstrate that Aβ up-regulates the expression of APP expression in cultured astrocytes (Moreno-Flores, Salinero et al. 1998). However, we do not know the exact mechanism of Aβ-induced APP over expression in cortical neurons; our data suggest that p75^{NTR} plays a crucial in the APP upregulation phenomena. The processing of APP is accomplished through secretory pathway as well as the endocytic pathway. It is widely reported that the ER; the Golgi apparatus; early, late, or recycling endosomes, and the lysosome contribute to Aβ production due to localization of γ-secretase in these compartments. In comparison, the non-amyloidogenic processing pathway of APP by α-secretase occurs mainly at the plasma membrane and lipid raft (Lammich, Kojro et al. 1999, Small and Gandy 2006, Thinakaran and Koo 2008). The endocytic pathway plays an important role in APP processing as inhibition of APP endocytosis by expression of dominant-negative mutant dynamin or by mutation of the endocytic sorting signal of APP, significantly decrease Aβ generation (Perez, Soriano et al. 1999, Carey, Balcz et al. 2005). Moreover, there are some evidence indicating the first step of APP processing that is mediated by BACE1, mostly occurs in the early endosome compartment due to a required acidic environment for the biological activity of BACE1 (Huse, Pijak et al. 2000, Kinoshita, Fukumoto et al. 2003). However, an opposite evidence showing Aβ reduces the expression of dynamin in neurons and therefore reduces APP processing (Kelly, Vassar et al. 2005). Most consistent finding in the literature is that amyloidogenesis requires activity dependent endocytosis of APP (Koo and Squazzo 1994, Perez, Soriano et al. 1999, Daugherty and Green 2001, Schneider, Rajendran et al. 2008, Choy, Cheng et al. 2012) which regulates

the convergence of APP and BACE1 in endosomes (Huse, Pijak et al. 2000, Kinoshita, Fukumoto et al. 2003, Das, Scott et al. 2013). We found that Aβ stimulates the endocytosis of APP and BACE in p75$^{+/+}$ mouse primary neurons. In addition, our data showed that the reduction in the level of cell surface APP and BACE1 induced by Aβ did not occur in p75$^{-/-}$ neurons. These data clearly indicate that the endocytosis of APP and BACE1 triggered by Aβ is regulated by p75^{NTR}. Aβ did not change the level of p75^{NTR} on the cell surface, suggesting that Aβ induces p75^{NTR} to the recycling endosomal pathway. Our results suggest that p75^{NTR} is a key receptor to modulate APP and BACE1 internalization in response to Aβ. Thus, we have revealed a key mechanism by which p75^{NTR} promotes amyloidogenesis by facilitating the endocytosis and co-localization of APP and BACE in endosomes. Since Aβ increases APP and BACE1 internalization through p75^{NTR}, it needs to be investigated whether Aβ modulates APP/BACE1 interaction. FRET analysis from others demonstrated that the APP interacts with BACE1 at the cell surface and in early endosomes (Kinoshita, Fukumoto et al. 2003). Our FRET analysis indicated that Aβ and proNGF enhanced APP/BACE1 interaction. Although, we have not addressed yet whether Aβ regulates APP/BACE1 convergence in subcellular compartments, there is evidence showing the convergence of APP and BACE1 is increased within acidic subcellular organelles in AD brain and promote Aβ generation (Das, Scott et al. 2013).

Threonine 668 (Thr668) is on the cytoplasmic domain of APP and phosphorylated by a multiple protein kinases, including GSK3β, SAPK1b/JNK3, Cdc2, and Cdk5 (Suzuki, Oishi et al. 1994, Aplin, Gibb et al. 1996, Iijima, Ando et al. 2000, Standen, Brownlees et al. 2001). The phosphorylation of APP-Thr668 is enhanced in AD and it mediates

APP processing and Aβ generation through mediating APP/BACE1 association (Lee, Kao et al. 2003). However, there is evidence indicating APP-Thr668 phosphorylation is not enhanced in AD, but its co-localization with BACE1 is increased in AD brain (Lee, Kao et al. 2003). Here we found that Aβ increased APP-Thr668 phosphorylation of in AD/p75$^{+/+}$, but not AD/p75$^{-/-}$cortical neuron. This suggests p75^{NTR} is required for APP-Thr668 phosphorylation in AD pathogenesis. Aβ is able to enhance the activity of several different protein kinases such as protein kinase C, casein kinases I and II and Tau protein kinase I and therefore contribute to the phosphorylation of Tau (Chauhan, Chauhan et al. 1991, Chauhan, Chauhan et al. 1993, Takashima, Noguchi et al. 1993). Tau phosphorylation induces cell death by disrupting microtubules structure and the neuronal cytoskeleton. Aβ induces Tau phosphorylation (Ser422) in rat hippocampal (Ma, Yang et al. 2009) and septal cholinergic neurons (Zheng, Bastianetto et al. 2002) to promote neuronal death and the development of AD. The phosphorylation of Tau at Ser202 and Thr205 induces PHF formation and subsequently contributes to AD pathogenesis (Selkoe 1991, Avila, Lucas et al. 2004, Rankin, Sun et al. 2005). Our data demonstrates that Aβ stimulates hyper-phosphorylation of Tau at Ser202+Thr205 in AD/p75$^{+/+}$, but not in the AD/p75$^{-/-}$ mouse cortical neurons. Antisense oligonucleotides against Tau protein kinase I prevent Aβ neurotoxicity on Tau phosphorylation (Takashima, Noguchi et al. 1993). Aβ is also known to bind to p75^{NTR} and promote neuronal apoptosis (Yaar, Zhai et al. 1997) and a p75^{NTR} antagonistic peptide (CATDIKGAEC) including KGA motif, significantly inhibited Aβ toxicity in mouse brain (Yaar, Arble et al. 2008). p75ECD-Fc was recently shown to reduce the oligomerization and fibrillation of synthetic $A\beta_{42}$ peptide *in vitro*, and abolish local Aβ

plaques after hippocampal injection *in vivo* (Wang, Wang et al. 2011). Based on our findings and published reports, we propose that p75^{NTR} is a critical regulatory receptor in Tau phosphorylation and contributes to Tau phosphorylation through Aβ binding.

In conclusion, our study reveals that the Aβ/p75^{NTR} association induces APP and BACE1 upregulation, increases APP and BACE1 internalization, and potentially exacerbates APP processing. Furthermore, Aβ stimulates Tau and APP-thr668 phosphorylation in a p75^{NTR}-dependent mechanism in neurons and therefore contributes to the progression AD pathogenesis (Figure 2-17).

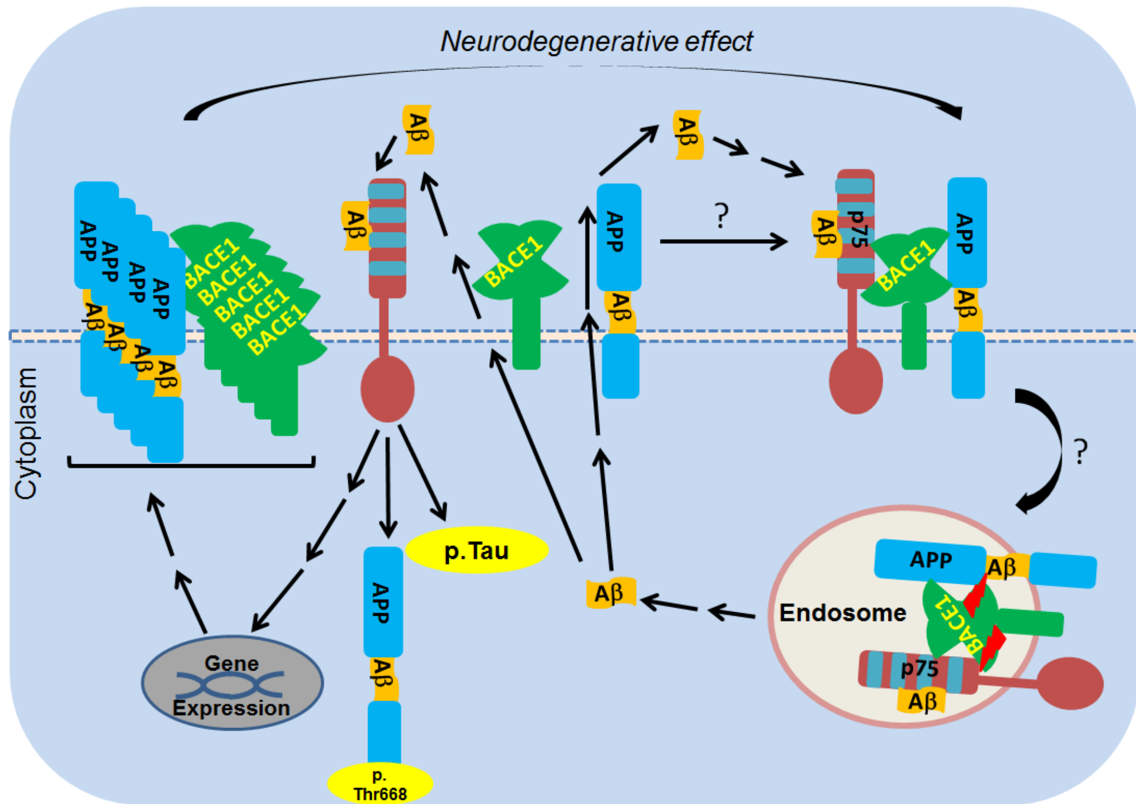


Figure 2-17: Graphical summary presenting how p75^{NTR} contributes to APP processing in AD pathogenesis.

Aβ mediates APP and BACE1 expression and internalization through a p75^{NTR}-dependent signalling pathway to produce more Aβ generation. Moreover, Aβ promotes the phosphorylation of APP-Thr668 and Tau through p75^{NTR}.

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CHAPTER 3: BACE1 REGULATES THE PROTEOLYTIC PROCESSING OF P75^{NTR} AND MITIGATES NEURODEGENERATIVE SIGNALS IN THE BRAIN

3.1 Abstract

Background: Aβ and proNGF mediate synaptic dysfunction and neurodegeneration through interaction with $p75^{NTR}$/Sortilin in AD. p75ECD, which is physiologically cut from the cell surface, has neuroprotective functions against neuronal toxicity caused by Aβ and proNTs. Recently, it was shown that Sortilin interacts with BACE1 and mediates retrograde trafficking of BACE1 and promotes Aβ generation (Finan, Okada et al. 2011). In this study, we have elucidated that BACE1, the rate limiting enzyme processing APP, interacts with $p75^{NTR}$, as a Sortilin co-receptor, and regulates its proteolytic processing and releases p75ECD. **Methods:** HEK-293T cells were co-transfected with human full length BACE1 and different fragments of $p75^{NTR}$ plasmid including FL, ECD, and ICD constructs. Cells were then treated with Aβ, proNGF and NGF. FRET and Co-IP were employed to quantify the intensity and region of BACE1/$p75^{NTR}$ interaction. Because BACE1 possesses proteolytic functions, we next investigated whether $p75^{NTR}$ is a substrate for BACE1. We quantified the endogenous $p75^{NTR}$ fragments including ECD, ICD and CTF in $BACE^{+/+}$ and $BACE1^{-/-}$ mouse brain by Western blot. To further investigate, we transfected full length BACE1 plasmid in the presence and absence of full length $p75^{NTR}$ in CHO^{APP695} and HEK-293T cells, respectively in order to quantify $p75^{NTR}$ fragments. To elucidate the effects of p75ECD on neurite outgrowth, we isolated and cultured cortical neurons from neonatal wild type (129sv) mouse and treated with 5 and 10 μM of $Aβ_{42}$ and 50 ng/ml proNGF and proBDNF in the presence and absence of 10 and 5 μg/ml p75ECD-Fc and Fc recombinant proteins for 24 hours. Neurons were fixed in 4% PF and stained by DAPI and a MAP-2 antibody. Images were taken by Olympus (CX40) florescence and Zeiss

confocal (LSM710) microscopes and the neurite length was measured by Image J software. All results are analysed and data presented as mean±SEM and $p<0.05$ considered statistically significant. **Results:** FRET and Co-IP results indicate that $p75^{NTR}$ associates with BACE1. In addition, CoIP showed that p75ECD interacts with BACE1. Our data revealed that Aβ and proNGF, but not NGF, significantly enhanced the $p75^{NTR}$/BACE1 interaction ($p<0.05$ and $p<0.01$). $p75^{NTR}$ and BACE1 are co-localized in dendrites and axons of cortical neurons and the $p75^{NTR}$/BACE1 co-localization is increased in response to Aβ ($p<0.05$). Next, we quantified the ratio of p75ECD/p75FL production in $BACE1^{-/-}$ and age matched $BACE1^{+/+}$ mouse brain. The p75ECD/p75FL ratio in $BACE1^{+/+}$ was significantly higher than $BACE1^{-/-}$ brain ($p<0.05$). The expression of TACE which physiologically processes $p75^{NTR}$ and generates p75ECD, was unchanged in $BACE1^{+/+}$ and $BACE1^{-/-}$ mice, suggesting BACE1 regulates $p75^{NTR}$ processing. Transfection of BACE1 with and without $p75^{NTR}$ in HEK-293T and CHO^{APP695} cells showed both fragments of p75ECD (26 and 50 kDa) are increased in response to BACE1 transfection in a dose dependent manner ($p<0.05$ and $p<0.01$). In contrast, p75ECD secretion is significantly reduced in HEK-293T cells which were co-transfected with BACE1/$p75^{NTR}$ compared to Empty vector/$p75^{NTR}$ ($p<0.01$). Finally, we addressed the role of p75ECD in AD and find that p75ECD significantly restored Aβ and proNT-induced impairment of neurite outgrowth in cortical neurons ($p<0.05$ and $p<0.01$). **Conclusion:** BACE1 interacts with $p75^{NTR}$ in the extracellular domain and subsequently processes $p75^{NTR}$ and generates p75ECD. Our data revealed that p75ECD attenuates neurotoxicity effects of Aβ and proNTs on neurite outgrowth and suggesting BACE1 may play a negative feedback role by shedding the $p75^{NTR}$ ectodomain in AD pathogenesis.

Keywords: p75^{NTR}, BACE1, Aβ, neurotrophins and Alzheimer's disease

3.2 Introduction

AD is the most common cause of dementia (Katzman 1986, Selkoe 2002). It leads to a marked impairment of cognition, loss of learning and memory and contributes to a significant social and economic burden (Katzman 1986, Selkoe 2002, Blennow, de Leon et al. 2006). AD is pathologically characterized by the progressive accumulation of amyloid plaques in the brain and blood vessels and neurofibrillary tangles in neurons (Glenner and Wong 1984). Aβ is generated by sequential cleavage of APP by β- and γ-secretases in sub-cellular compartments through the amyloidogenic pathway (Selkoe 2001, Vassar 2004).

BACE1 is a type I transmembrane aspartic protease with a large extracellular domain, single transmembrane domain, and a short cytoplasmic tail. The extracellular domain of BACE1 contains the catalytically active sites and plays a critical role in the pathogenesis of AD by cleaving APP at the amino terminus between residues 671 and 672 (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999). BACE1 initiates APP processing and generates sAPPβ and CTFβ which subsequently were cleaved by γ- secretase complex (PS1 or PS2) (Wolfe, De Los Angeles et al. 1999, Wolfe, Xia et al. 1999) or nicastrin (Yu, Nishimura et al. 2000) to generate pathogenic Aβ in the brain. In contrast to β-secretase enzymes, α-secratases like TACE1 (ADAM17) (Buxbaum, Liu et al. 1998), ADAM-9 and ADAM-10 prevent the production of Aβ via processing of APP in the non-amyloidogenic pathway in neurons (Lammich, Kojro et al. 1999). p75^{NTR} is a type-I receptor transmembrane protein and interacts with a many of signalling molecules. It can mediate apoptosis, Schwann cell migration, myelination, axonal growth, and regeneration (Frade,

Rodriguez-Tebar et al. 1996, Kalb 2005, Kenchappa, Zampieri et al. 2006). The p75^{NTR} is highly expressed by BFCNs which widely project to the hippocampus and neocortex and contribute to normal cognitive and psychomotor functions in the brain (Dechant and Barde 2002, Yan and Fan 2004, Wu, Thal et al. 2005). p75^{NTR} interacts with Aβ and proNTs and likely plays a critical role in mediating apoptosis of BFCNs in AD (Sotthibundhu, Sykes et al. 2008). The level of p75^{NTR} in cortical neurons and BFCNs is elevated in AD (Mufson and Kordower 1992, Yeo, Chua-Couzens et al. 1997, Volosin, Song et al. 2006). p75^{NTR} is physiologically processed by ADAM17 and γ-secretase similar to the processing of APP (Jung, Tan et al. 2003, Gowrishankar, Zeidler et al. 2004, Zampieri, Xu et al. 2005). p75^{NTR}/Sortilin interaction results in neuronal apoptosis in AD (Nykjaer, Lee et al. 2004, Skeldal, Sykes et al. 2012). Recently, it has been reported that Sortilin interacts with BACE1 and subsequently regulates BACE1 retrograde trafficking and Aβ generation in AD (Finan, Okada et al. 2011). p75^{NTR} is up-regulated by Aβ (Chakravarthy, Gaudet et al. 2010) and acts as a positive regulator in Aβ production (Costantini, Weindruch et al. 2005, Costantini, Scrable et al. 2006, Wang, Wang et al. 2011). Recent studies show that the p75ECD interacts with APP (Fombonne, Rabizadeh et al. 2009) and promotes sAPP-induced neurite growth (Hasebe, Fujita et al. 2013). A small molecule antagonist of p75^{NTR} can ameliorate the pathogenesis of AD (Knowles, Simmons et al. 2013). However, how p75^{NTR} mediates Aβ production and aggravates AD pathogenesis is not fully understood. Additionally, I already (Chapter 2) found that p75ECD attenuates Aβ/p75^{NTR} toxicity through inhibition of Aβ-induced APP/p75^{NTR} interaction. In this study, we investigate whether BACE1 interacts with p75^{NTR}, as a co-receptor for Sortilin, and regulates p75^{NTR} proteolytic

processing to generate p75ECD. In the case of BACE1/p75^{NTR} interaction and p75^{NTR} processing, we elucidate the significance of the effect of p75ECD in the brain.

3.3 Materials and methods

Antibodies, reagents and plasmids

In the current study, in addition to the antibodies, reagents and plasmids which were cited in Chapter 2, rabbit polyclonal anti-MAP-2 (OSM00030W, Osenses, Australia) and rabbit polyclonal anti-TACE (Cat.No.RB-1660-P0, Thermo Scientific, Australia) were used.

Preparation of oligomer form of $A\beta_{42}$

In the present study, the oligomer form of $A\beta_{42}$ was used. Synthetic $A\beta_{42}$ and scrambled $A\beta_{42}$ were prepared following the protocols described previously (Wang, Wang et al. 2011, Saadipour, Yang et al. 2013) and in methodology section of Chapter 2 of the thesis.

The brain tissue of mice

Five month old $BACE1^{-/-}$ and age matched $BACE1^{+/+}$ mouse brain tissue were kindly provided by Prof. Robert Vassar (Department of Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, US). The brain tissues were homogenized in RIPA buffer containing 1 mM PMSF, antipain, pepstatin, and leupeptin (Roche, Australia). Homogenate was centrifuged at 14000 g for 10 min at 4°C and supernatant was taken and subjected to a BCA protein assay (Thermo Scientific, Rockford, US) to quantify total protein amount. 25 µg supernatant was subjected to SDS-PAGE for Western blot analysis.

Primary neuron culture

Primary cortical neurons from a day old 129sv neonatal mouse were isolated and cultured on coverslips and in 4-well plates according to the protocol explained in Chapter 2 (Wang, Valadares et al. 2010, Xu, Sun et al. 2011, Sun, Lim et al. 2012).

FRET assay

FRET assay was performed to test the direct interaction between human BACE1-CFP and p75-YFP (Yang, Lim et al. 2011, Yang, Yang et al. 2012). HEK-293T cells were seeded on coverslips overnight and cells were then co-transfected with human BACE-CFP/p75-YFP, BACE-CFP/pEYFP (negative control), and a single transfection with p75-CFP-YFP plasmids (positive control) using Lipofectamin 2000 (Invitrogen, Australia). After 24 hours transfection, the cells were treated with Aβ_{42}, proNGF and NGF and fixed using 4% PF and subjected to FRET acceptor bleaching analysis according to FRET assay instructions explained in Chapter 2.

The co-localization assay

Cortical neurons from 1 day old wild type (129sv) mouse were isolated and cultured on PDL/Laminin coated coverslips. After 72 hours incubation at 37°C, cells were treated with 0.1 µM Aβ_{42} or Aβ_{42} scramble (control) for 15 min and cells were then fixed in 4% PF. Neurons were incubated in primary antibodies; rabbit monoclonal anti-BACE1 (D10E5, cell signalling, US) and mouse polyclonal anti-p75ECD (5B1D2), overnight at 4°C. They were then incubated for 1 hour in secondary anti-rabbit-Cy3 and anti-mouse-Alexa488 antibodies and DAPI. The coverslip was mounted using polyvinyl alcohol mounting medium (sigma-Aldrich, US) on glass slides and images of fluorochromes (Cy3/Alexa488/DAPI) were obtained sequentially using the Nikon confocal microscope

EZ-C1. Quantitative assessment of co-localization between Cy3 and Alexa 488 was performed by calculating the overlap coefficient (ranging from 0%, minimum colocalization, to 100%, maximum colocalization), using the EZ-C1 3.90 software. An average of 12 cells was analysed in each experiment and the overlap coefficient (%) was then calculated.

Neurite outgrowth assay

Cortical neurons from 1 day old wild type (129sv) mouse were seeded on PDL/Laminin coated coverslips and after 24 hours, neurons were treated with 5 and 10 μM Aβ_{42}, 50 ng/ml proNGF and proBDNF in presence and absence of 10 and 5 μg/ml p75ECD-Fc and Fc recombinant proteins for 24 hours. The cells were then fixed in 4% PF and incubated in rabbit anti-MAP-2 antibody overnight at 4°C. They were then incubated for 1 hour in secondary anti-rabbit Alexa488 antibody and DAPI. Images were taken from 35 neurons on two different coverslips for each individual group using an Olympus (CX40) microscope. Representative neuron images were obtained using a Zeiss confocal (LSM710) microscope. The length of dendrites was measured by Image J software and the data was reported as mean±SEM.

Co-IP assay

Co-IP assay was employed following the protocol explained in Chapter 2 to evaluate the interaction between p75FL, ECD, and ICD with BACE1. HEK-293T cells were co-transfected with different fragments of p75^{NTR} plasmid and full length BACE1 and cells were then lysed in RIPA buffer including containing 1 mM PMSF, antipain, pepstatin, and leupeptin (Roche, Australia). The cell lysate was subsequently subjected to sonication and protein extraction. The protein concentration of the lysates was

determined using BCA protein assay Kit (Thermo Scientific, Rockford, US). The pre-cleared lysates (400μg) were then incubated overnight at 4°C with 2 μg primary antibodies and appropriate species IgG as control for primary antibodies specificity and then incubated with protein G beads to immobilize primary antibody at 4°C for two hours. The beads were washed four times with PBST and boiled in 40 μl loading buffer before being subjected to Western blot to detect the interacted proteins. At the same time 10 μg of sample lysis was loaded as a comparative control for each sample.

Western Blot assay

Western blot analysis was performed exactly following the instructions which described in Chapter 2.

Enzyme-linked immunosorbent assay (ELISA) for p75ECD

In order to investigate whether BACE1 cuts p75^{NTR} on the cell surface, HEK-293T cells were co-transfected with 1.5 μg p75^{NTR} plasmid with either BACE1 construct or Empty vector for 24 hours. Culture medium was then collected and subjected to ELISA assay using NGF R/TNFRSF16 Kit (Cat.No.DY367, R&D Systems, US) according to manufacturer's instruction.

Statistical analysis

All statistical analysis was performed using SPSS 22 software package (IBM, provided by Flinders University). Variables between groups were determined by either one-way ANOVA following Tukey's post-hoc test or Student's t-test and values of $p<0.05$ were considered statistically significant. Data is presented as mean±SEM.

3.4 Results

BACE1 interacts with p75^{NTR} in mouse cortical neurons and HEK-293T cells

As Sortilin interacts with BACE1 (Finan, Okada et al. 2011), it is likely that p75^{NTR}, a co-receptor for Sortilin, associates with BACE1 and possibly participates in AD pathogenesis. To examine whether BACE1 co-localizes with p75^{NTR}, cortical neurons from wild type (129sv) mouse were cultured and incubated overnight with rabbit monoclonal anti-BACE1 (D10E5, cell signalling, US) and mouse polyclonal anti-p75ECD (5B1D2) antibody at 4°C. These neurons were then incubated with secondary anti-rabbit-Cy3 and anti-mouse-Alexa488 antibodies and DAPI as nuclei marker. The double staining result shows that BACE1 was significantly co-localized with p75^{NTR} in neurons (co-localization rate: 71±9 %) (Figure 3-1 A-B). Next, in order to see whether these proteins interact together, we co-transfected FL.p75-YFP/BACE1-CFP in HEK-293T and then subjected to FRET acceptor bleaching analysis. BACE1 significantly interacted with p75^{NTR} compared to negative group (NC: co-transfected with pECFP/p75-YFP plasmids) ($p<0.01$) (Figure 3-1 C-D).

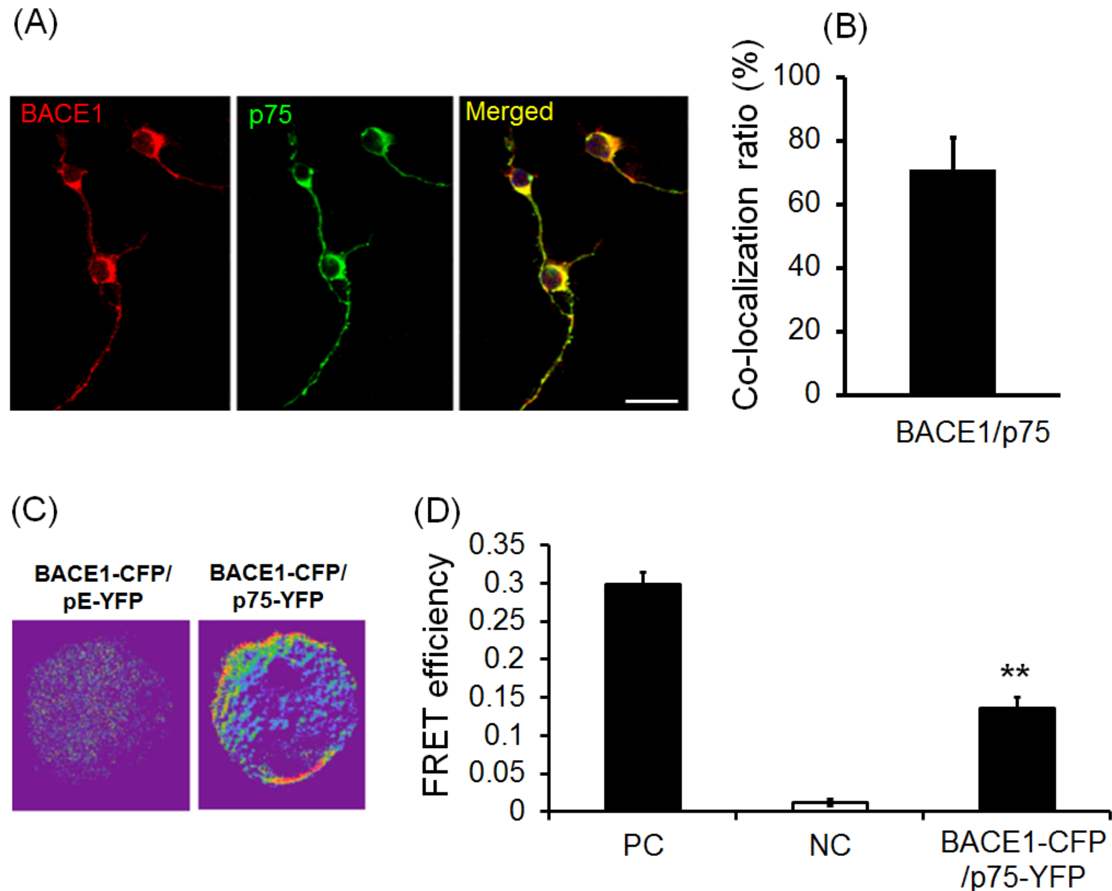


Figure 3-1: BACE1 co-localized and interacted with p75^{NTR} in mouse cortical neurons and HEK-293T cells, respectively

A) Image presenting the co-localization between endogenous BACE1 and p75^{NTR} in 129sv mouse cortical neurons B) Plot showing the co-localization ratio of BACE1/p75^{NTR} in neurons (co-localization ratio: 71±9 %, n=12, mean±SEM, Image scale 50 μm). C) FRET acceptor bleaching analysis for quantification BACE1/p75^{NTR} interaction in HEK-293T cells co-transfected with BACE1-CFP and p75-YFP D) Plot demonstrating FRET efficiency value for the BACE1/p75^{NTR} interaction (**$p<0.01$ vs NC) (n=12 cells per group, mean±SEM, Student's t-test; NC: Negative control: co-transfected with pECFP/p75-YFP plasmids, PC: Positive control: transfected with p75-CFP-YFP plasmid; Image scale 50 μm).

BACE1 interacts with p75ECD, but not p75ICD

Next, we employed the Co-IP assay in order to confirm our double staining and FRET results and also to detect which domain of p75^{NTR} interacts with BACE1. In this experiment, HEK-293T cells were co-transfected with BACE1-CFP and full length p75-HA plasmids and then subjected to Co-IP assay. Our Co-IP results showed that BACE1 interacted with p75^{NTR} (Figure 3-2 A and B) which is consistent with our FRET and co-localization results. Further, we investigated which fragments of p75^{NTR} interacts with BACE1. To address this question, HEK-293T cells were co-transfected with either p75ICD-HA or p75ECD-Myc with BACE1 and subjected to Co-IP assay. The result indicate that only p75ECD interacts with BACE1 whereas p75ICD does not interact with BACE1 (Figure 3-2 C and D).

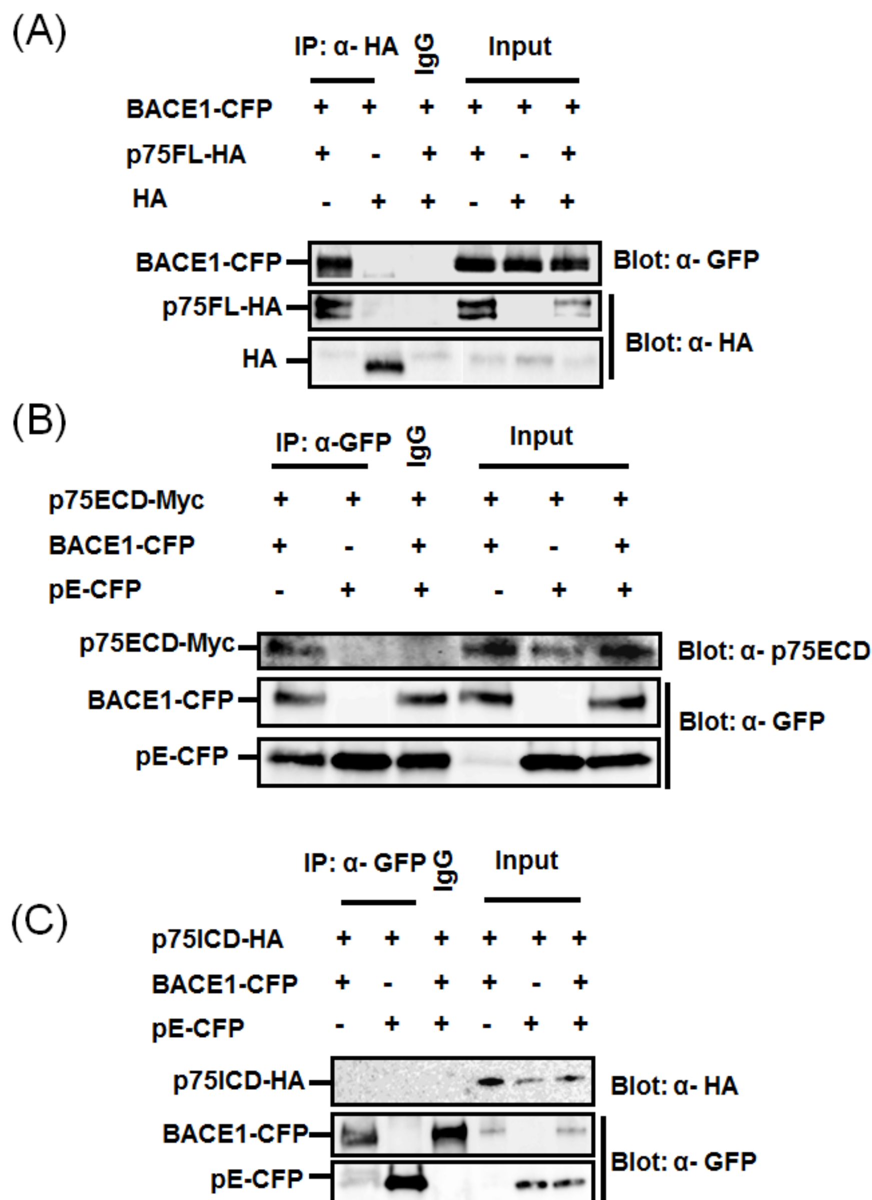


Figure 3-2: BACE1 interacts with p75ECD, but not p75ICD

A) Western blot signals from Co-IP demonstrating the interaction between BACE1 and p75FL using rabbit anti-HA antibody to immobilize p75FL-HA following anti-GFP to detect BACE1-CFP in transfected HEK-293T cells B) Western blot presenting the interaction between BACE1 and p75ECD using goat ant-GFP to immobilize BACE-CFP following rabbit anti-p75ECD (9650) to detect p75ECD-Myc in transfected HEK-293T cells C) Western blot showing the interaction between BACE1 and p75ICD using goat ant-GFP to immobilize BACE-CFP following rabbit anti-HA to detect p75ICD-HA in transfected HEK-293T cells (n=3 for each experiment)

Effects of $A\beta_{42}$ on BACE1/$p75^{NTR}$ co-localization in mouse cortical neurons

To investigate whether $A\beta_{42}$ influences the BACE1/$p75^{NTR}$ interaction, we treated cortical neurons with 0.1 μM $A\beta_{42}$ for short time, 15 min. Cells were then subjected to double staining to quantify the co-localization between BACE1 and $p75^{NTR}$. The result indicated that $A\beta_{42}$ significantly increased the co-localization between endogenous BACE1 and $p75^{NTR}$ in both soma and dendrites in mouse cortical neurons ($p<0.05$) (Figure 3-3)

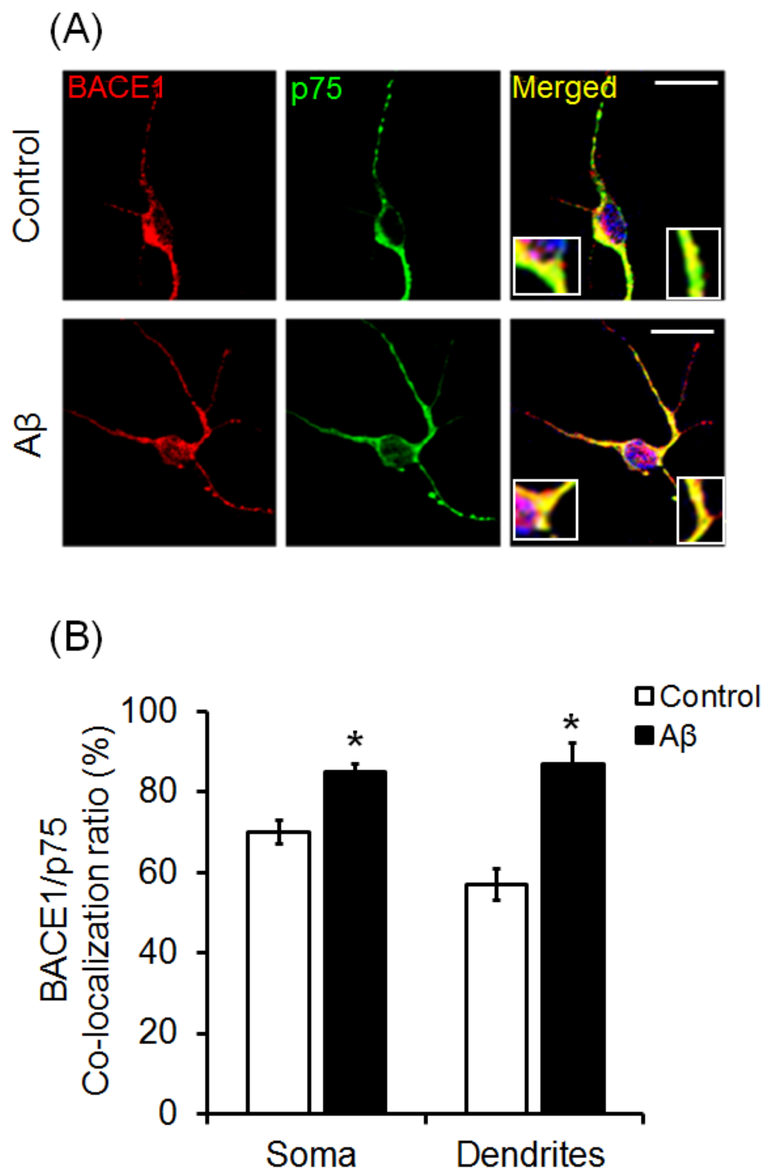


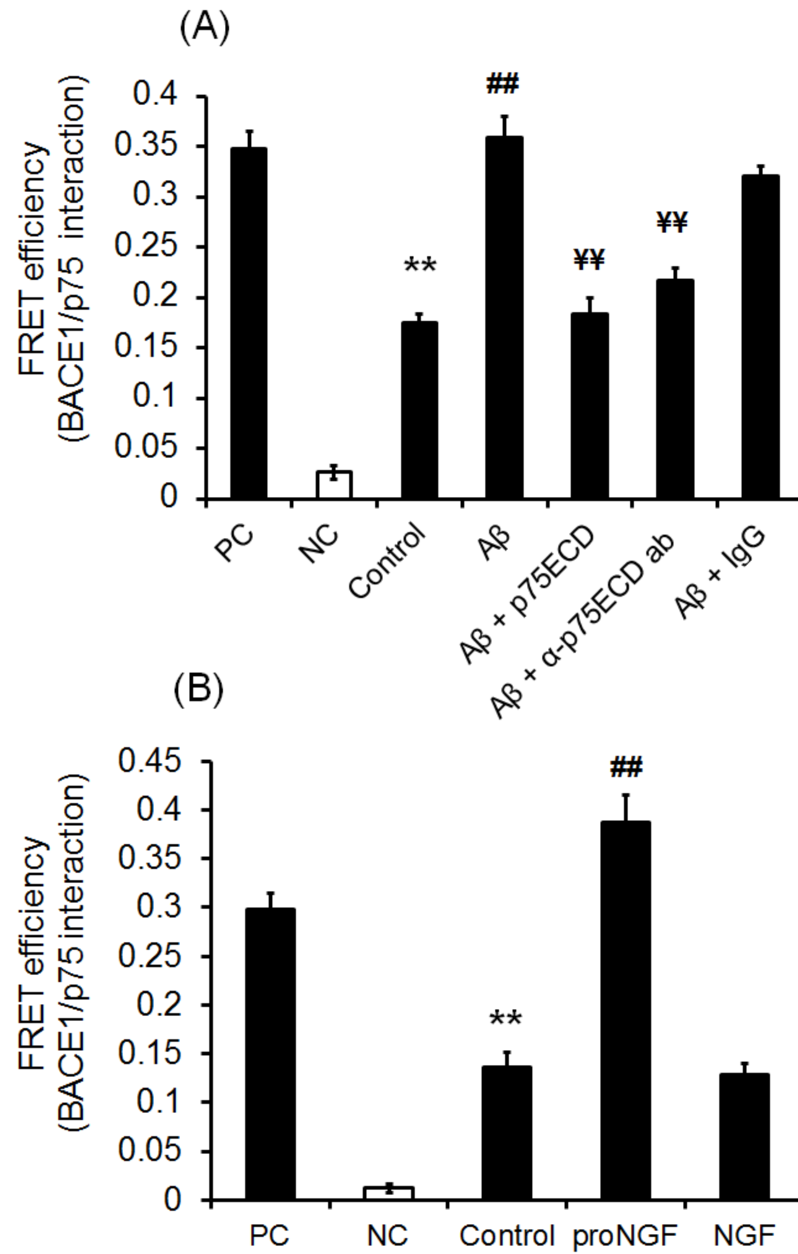
Figure 3-3: Aβ_{42} enhanced BACE1/p75^{NTR} co-localization in mouse cortical neurons

A) Confocal microscopy image from 129sv mouse cortical neurons presenting the co-localization ratio between endogenous BACE1 and p75^{NTR} in the presence and absence of 0.1 µM Aβ_{42} for 15 min in cell soma and dendrites B) Plot showing the statistical analysis of the co-localization between BACE1 and p75^{NTR} in cell soma and dendrites (*$p<0.05$ vs Control) (n=12 cells per group, mean±SEM, Student's t-test; Control: 0.1 µM Aβ_{42} Scramble; Image scale 100 µm).

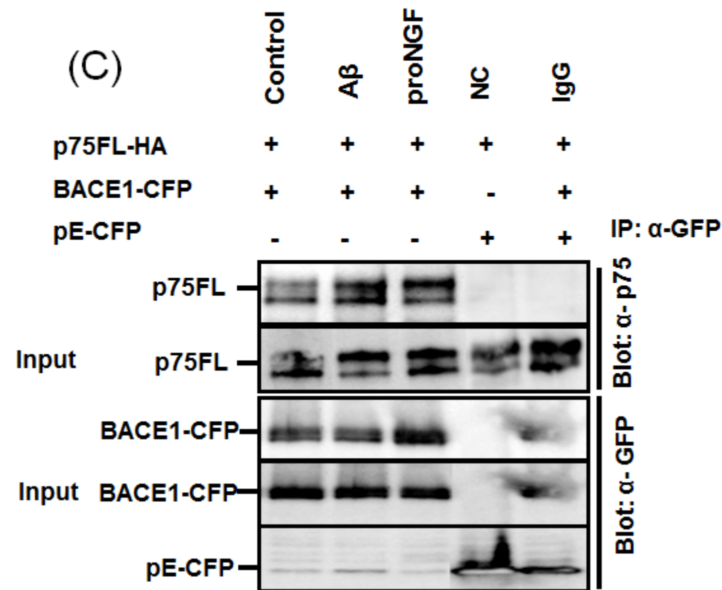
BACE1/p75^{NTR} interaction is increased in the presence of Aβ and proNGF, but not NGF.

So far we have confirmed that BACE1 interacts with p75^{NTR}. Here, we investigate whether the BACE1/p75^{NTR} interaction is regulated by p75^{NTR} ligands including Aβ, proNGF, and NGF. Our FRET results show that 1 μM $A\beta_{42}$ overnight increases the interaction between BACE1 and p75^{NTR} (Figure 3-4 A). To confirm the effects of Aβ on the BACE1/p75^{NTR}, we find that 10 μg/ml p75ECD-Fc recombinant protein and 2 μg/ml anti-p75ECD antibody which block the biological function of Aβ and its receptor, p75^{NTR}, respectively inhibit Aβ-induced BACE1/p75^{NTR} interaction ($p<0.01$) (Figure 3-4 A). In addition, our FRET data demonstrates that proNGF, but not NGF, enhances BACE1/p75^{NTR} association (Figure 3-4 B).

To confirm our FRET data, we tested the effects of 1 μM $A\beta_{42}$ and 50 ng/ml proNGF on the BACE1/p75^{NTR} interaction by Co-IP assay. Our Co-IP results demonstrate that $A\beta_{42}$ and proNGF significantly enhance the interaction between BACE1 and p75^{NTR} which is consistent with our FRET and ICC results ($p<0.05$) (Figure 3-4 C and D).



(A)
FRET efficiency
(BACE1/p75 interaction)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
##
**
¥¥
¥¥
PC
NC
Control
Aβ
Aβ + p75ECD
Aβ + α-p75ECD ab
Aβ + IgG
(B)
FRET efficiency
(BACE1/p75 interaction)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
##
**
PC
NC
Control
proNGF
NGF



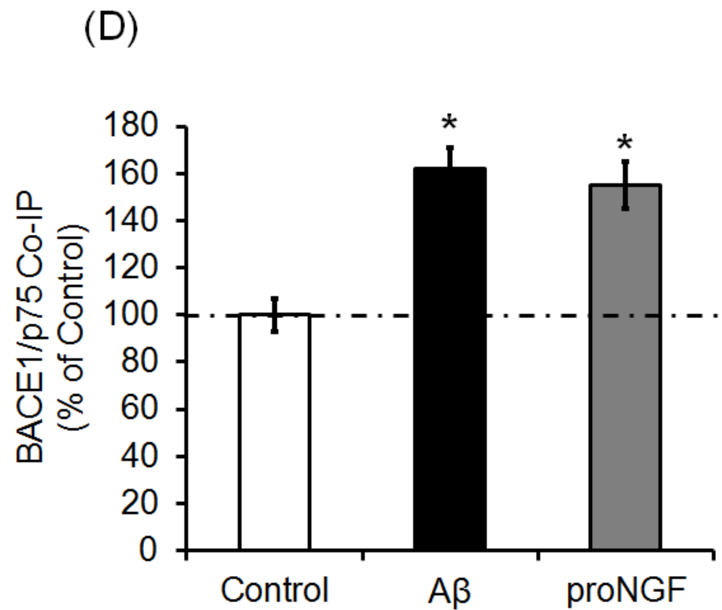


Figure 3-4: Aβ_{42} and proNGF, but not NGF, increases the BACE1/p75^{NTR} interaction

A) FRET demonstrating the interaction between BACE1 and p75^{NTR} in the presence and absence of Aβ_{42} and in conjunctions with p75ECD-Fc recombinant protein and anti-p75ECD antibody (**p<0.01 vs NC, ##p<0.01 vs control, ¥¥p<0.01 vs "Aβ+IgG"). B) FRET data presenting the effects of proNGF and NGF on the BACE1/p75^{NTR} interaction (**p<0.01 vs NC, ##p<0.01 vs Control) (n=12 cells per group, mean±SEM, ANOVA,

Tukey's post-hoc test; NC: Negative control: co-transfected with pECFP/p75-YFP plasmids, PC: Positive control: transfected with p75-CFP-YFP plasmid. Control: 1 μM Aβ_{42} Scramble). C) Western blot for Co-IP indicating the effects of Aβ_{42}, proNGF and NGF on the BACE1/p75^{NTR} interaction D) Plot presenting the statistical analysis of Co-IP data for the BACE1/p75^{NTR} interaction (*$p<0.05$ vs Control) (n=3, mean±SEM, ANOVA, Tukey's post-hoc test; Control: no treatment; "Control" was normalized to 100%).

BACE1 regulates the proteolytic processing of p75^{NTR} and generates p75ECD in the brain.

The interaction between BACE1 and p75ECD was a cue for further study to elucidate the significance of the interaction in AD pathogenesis. We hypothesized that BACE1 may regulate p75^{NTR} processing. In order to address this question, we quantified the level p75ECD, ICD, and CTF in 5-month old BACE1$^{-/-}$ and age-matched BACE1$^{+/+}$ mouse brain homogenate by Western blot. The results indicate that the level of p75ECD fragment present in BACE1$^{+/+}$ is significantly higher than BACE1$^{-/-}$ mouse brain (Figure 3-5). In addition, we quantified p75ICD and p75CTF levels in the brain homogenates and we found p75ICD as well as p75CTF were not altered in BACE1$^{+/+}$ vs BACE1$^{-/-}$ brains. As p75^{NTR} is physiologically processed by TACE (ADAM17) (Weskamp, Schlondorff et al. 2004, Zampieri, Xu et al. 2005), we then quantified the TACE level in the brain lysate and we found that TACE level was unchanged in BACE1$^{-/-}$ and BACE1$^{+/+}$ mouse brain, suggesting unchanged TACE1 may not be responsible for the increased p75ECD in BACE1$^{-/-}$ and BACE1$^{+/+}$ mouse brain (Figure 3-5). Our results suggest that BACE1 contributes to p75^{NTR} processing in the brain.

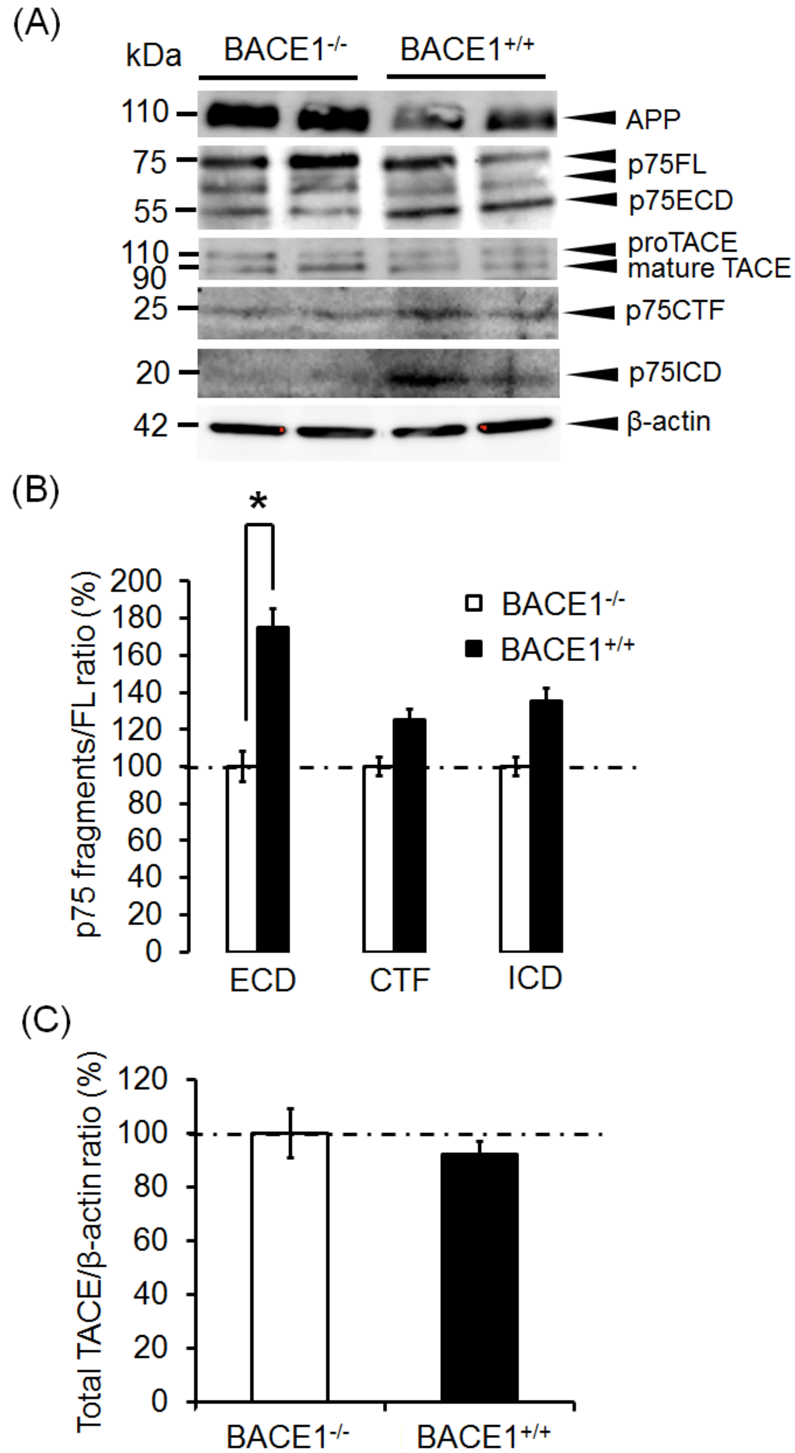


Figure 3-5: BACE1 processes p75^{NTR} and generates p75ECD in mouse brain

A) Western blot presenting the expression of APP, p75FL, p75ICD, p75CTF, and TACE in BACE1$^{-/-}$ and BACE1$^{+/+}$ mouse brain B) Plot demonstrating the statistical analysis of p75fragments/FL ratio in BACE1$^{-/-}$ and BACE1$^{+/+}$ mouse brain ($p<0.05$). C) The

expression of total TACE (pro- and mature TACE) in $BACE1^{-/-}$ and $BACE1^{+/+}$ mouse brain (n= 5 per group, mean±SEM, Student's t-test; "$BACE1^{-/-}$" was normalized to 100%).

Effects of over expression BACE1 on p75^{NTR} processing of in CHO^{APP695} cell line.

So far, our data showed that the ratio of p75ECD/FL in BACE1$^{+/+}$ is higher than BACE1$^{-/-}$ mouse brain. In order to further investigation the effects of BACE1 on p75^{NTR} processing CHO^{APP695} cell line was used to test the effect of over-expressed BACE1 on the processing of endogenous p75^{NTR}. We first transfected 1 µg BACE1 plasmid in CHO^{APP695} cell line to evaluate whether BACE1 regulates p75^{NTR} processing in this cell line by quantifying p75ECD level using Western blot analysis. The results showed that the level of 55 kDa p75ECD was increased in BACE1 transfected cells ($p<0.05$) (Figure 3-6 A and B). Next we tested effects of 0, 0.25 and 0.5 µg of BACE1 transfection on p75^{NTR} processing by quantifying both fragments (26, 55 kDa) of p75ECD in cell lysate after 24 hours transfection. The results indicated that the 26 and 55 kDa fragments of p75ECD are increased in the presence of BACE1 in a dose dependent manner (Figure 3-6 C and D) ($p<0.05$, $p<0.01$). In addition, we quantified the expression of p75ICD and CTF in the cells. The statistical analysis indicate the ratio of p75ICD/FL and CTF/FL were not significantly changed in the presence of BACE1 in CHO^{APP695} cells (Figure 3-6 C and D).

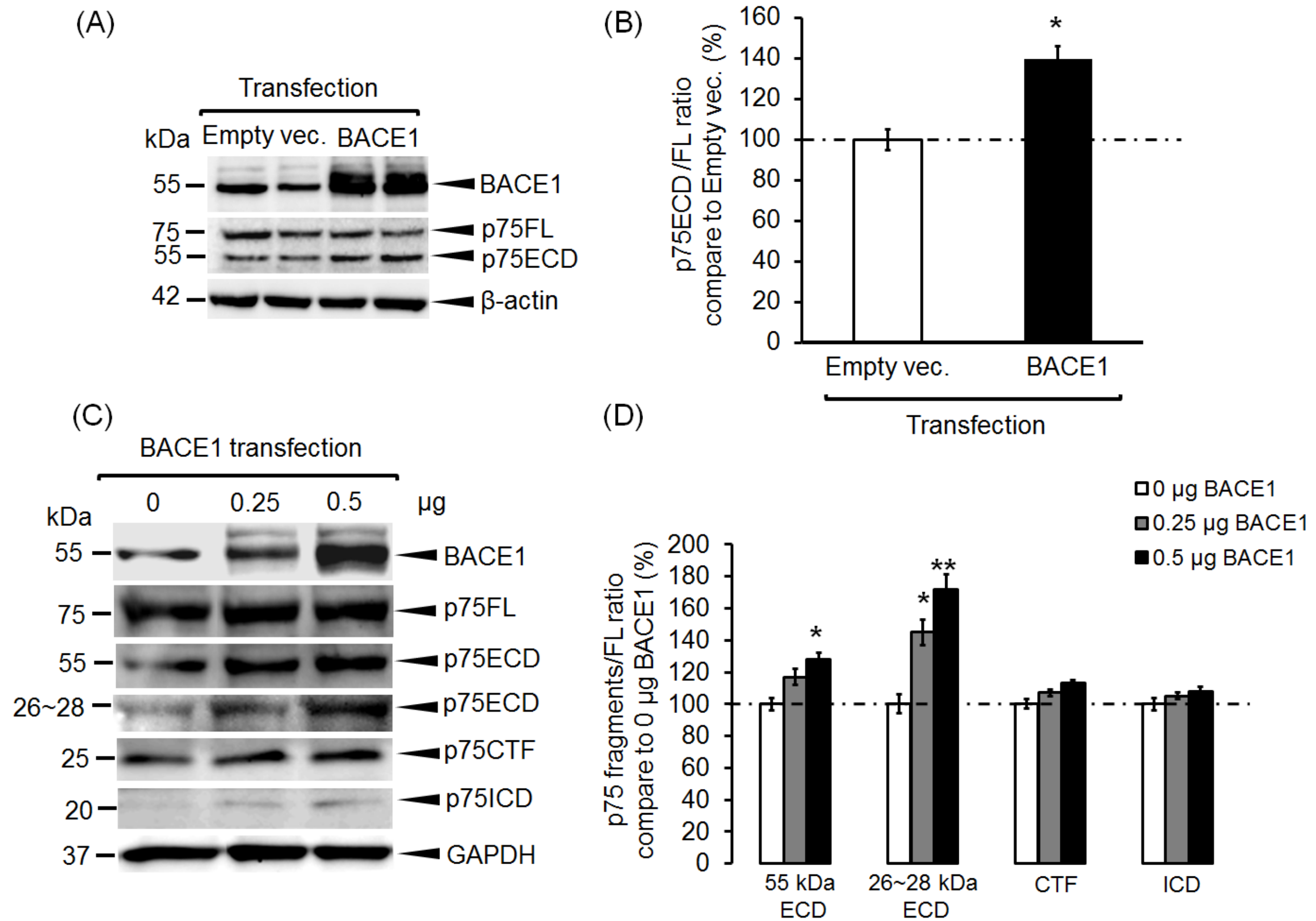
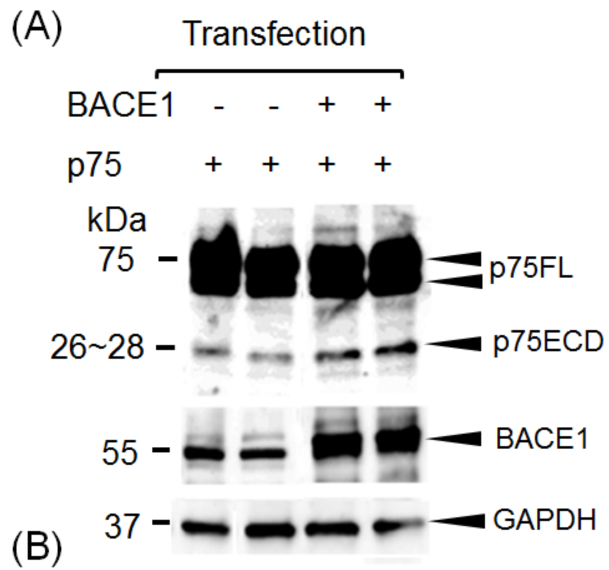

(A)
Transfection
kDa Empty vec. BACE1
55
75
55
42
BACE1
p75FL
p75ECD
β-actin
(B)
p75ECD/FL ratio
compare to Empty vec. (%)
160
140
120
100
80
60
40
20
0
*
Empty vec.
BACE1
Transfection
(C)
BACE1 transfection
kDa
0 0.25 0.5 µg
55
75
55
26~28
25
20
37
BACE1
p75FL
p75ECD
p75ECD
p75CTF
p75ICD
GAPDH
(D)
0 µg BACE1
0.25 µg BACE1
0.5 µg BACE1
p75 fragments/FL ratio
compare to 0 µg BACE1 (%)
200
180
160
140
120
100
80
60
40
20
0
*
*
**
55 kDa ECD
26~28 kDa ECD
CTF
ICD

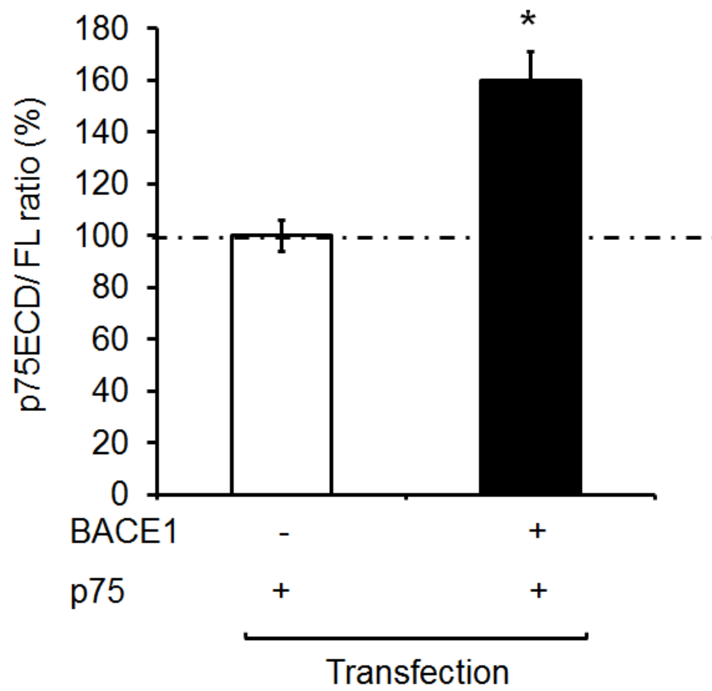
Figure 3-6. Effects of over expression BACE1 on endogenous p75^{NTR} processing in CHO^{APP695} cell line.

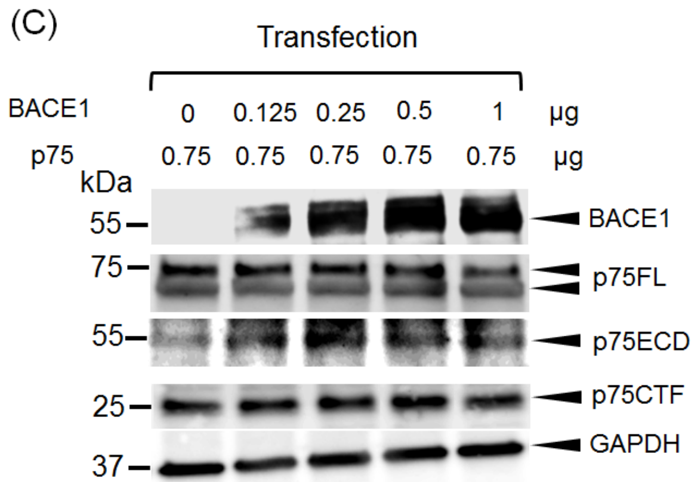
A) Western blot presenting effect of 1 µg BACE1 transfection on endogenous ratio of p75ECD/FL in CHO^{APP695} cells B) The statistical analysis for the effect of 1 µg BACE1 on p75ECD/FL in CHO^{APP695} cells (*$p<0.05$ vs Empty vec) (n=3, mean±SEM, Student's t test; "Empty vec." was normalized to 100%) C) Western blot for effects of 0, 0.25 and 0.5 µg of BACE1 on the level of p75ECD (26 and 55 kDa), CTF and ICD in CHO^{APP695} D) Plot demonstrating the statistical analysis of p75ECD (26 and 55 kDa), CTF and ICD expression in the presence of BACE1 in CHO^{APP695} cells (*$p<0.05$, **$p<0.01$ vs 0 µg BACE1) (n=3, mean±SEM, ANOVA, Tukey's post-hoc test; "0 µg BACE1" and was normalized to 100%).

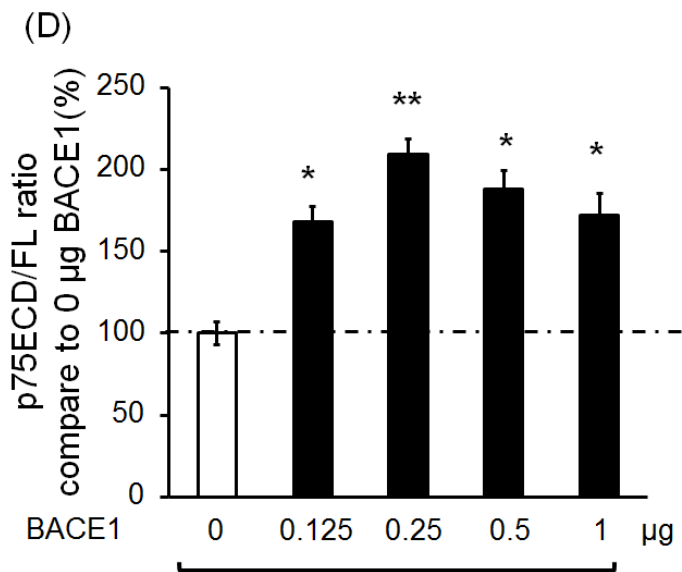
Effects of BACE1 on p75^{NTR} processing in HEK-293T cells

In order to further investigate whether BACE1 regulates p75^{NTR} processing, HEK-293T cells were co-transfected with 2 µg BACE1 and p75^{NTR} 24 hours and the 26 kDa p75ECD was quantified by Western blot. The data indicated that BACE1 significantly increased the level of the 26 kDa band, suggesting p75^{NTR} processing is increased by BACE1 in HEK-293T ($p<0.05$) (Figure 3-7 A-B). In addition, we assessed the effects of a dose dependent BACE1 transfection on p75^{NTR} processing. Here, HEK-293T cells were co-transfected with 0, 0.125, 0.25, 0.5 and 1 µg of BACE1 in conjunction with 0.75 µg p75^{NTR} plasmid and Western blot was then conducted to measure the 55 kDa fragment of p75ECD in cell lysate. Statistical analysis showed that BACE1 significantly increased p75^{NTR} processing and generated p75ECD in a dose dependent manner ($p<0.05$ and $p<0.01$) (Figure 3-7 C-D). Moreover, our data demonstrate that the level of p75CTF significantly increased in the presence of 0.5 µg BACE1 ($p<0.05$) (Figure 3-7 E).


(A)
Transfection
BACE1 - - + +
p75 + + + +
kDa
75
p75FL
26~28
p75ECD
55
BACE1
37
GAPDH


(B)
p75ECD/ FL ratio (%)
180
160
140
120
100
80
60
40
20
0
*
BACE1 - +
p75 + +
Transfection


(C)
Transfection
BACE1 0 0.125 0.25 0.5 1 µg
p75 0.75 0.75 0.75 0.75 0.75 µg
kDa
55
75
55
25
37
BACE1
p75FL
p75ECD
p75CTF
GAPDH


(D)
p75ECD/FL ratio
compare to 0 µg BACE1(%)
250
200
150
100
50
0
*
**
*
*
BACE1 0 0.125 0.25 0.5 1 µg
Transfection

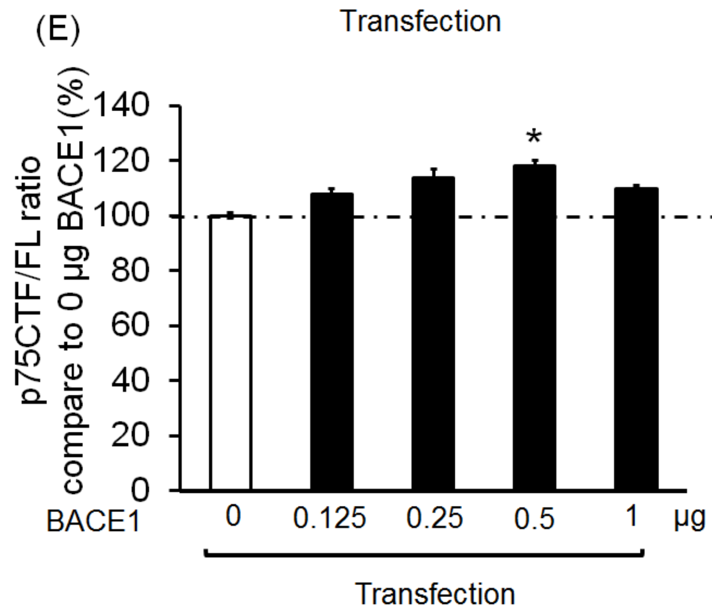

(E)
p75CTF/FL ratio
compare to 0 µg BACE1(%)
140
120
100
80
60
40
20
0
*
BACE1 0 0.125 0.25 0.5 1 µg
Transfection

Figure 3-7: BACE1 mediates p75^{NTR} processing in HEK-293T cells

A) Western blot demonstrating BACE1, p75FL and p75ECD (26 kDa) expression in HEK-293T cells co-transfected with 2µg BACE1 and p75^{NTR} B) Graph showing the statistical analysis for the ratio of p75ECD/FL in HEK-293T cells co-transfected with 2µg of BACE1 and p75^{NTR} (*$p<0.05$) (n=3, mean±SEM, Student's t-test; "0 µg BACE1" was normalized to 100%). C) Western blot presenting the expression of BACE1, p75FL, ECD (55 kDa) and CTF in HEK-293T co-transfected with 0, 0.125, 0.25, 0.5 and 1 µg BACE1 and 0.75 µg p75^{NTR}. Graphs demonstrating the ratio of p75ECD/FL (D) and p75CTF/FL (E) in HEK-293T co-transfected with different concentrations BACE1 and 0.75 µg p75^{NTR} (*$p<0.05$ and **$p<0.01$) (n=3, mean±SEM, ANOVA, Tukey's post-hoc test; "0 µg BACE1" was normalized to 100%).

BACE1 reduced the processing of p75^{NTR} at the cell surface

We have found that BACE1 regulates p75^{NTR} processing and increases p75ECD in cell lysate. Here we tested whether BACE1 cuts p75^{NTR} at the cell curface and secretes soluble p75ECD. To address this question, HEK-293T cells were cotransfected with 1.5 μg BACE1/p75^{NTR} and empty vector/p75^{NTR} constructs for 24 hours and culture medium was then collected and subjected to ELISA to detect p75ECD. The p75ECD level significantly reduced in culture medium of BACE1/p75^{NTR} in comparison with Empty vector/p75^{NTR} group ($p<0.01$) (Figure 3-8).

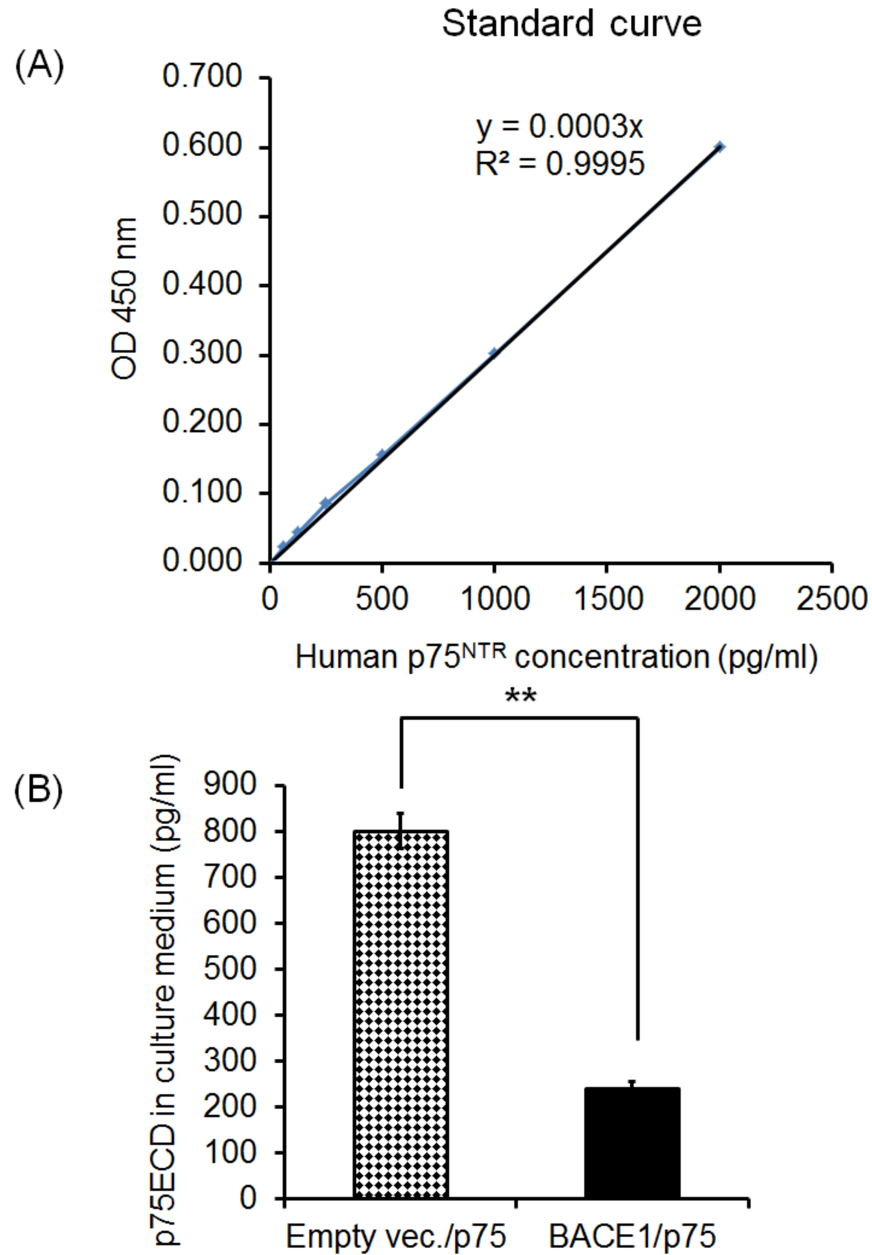


Figure 3-8: p75ECD is decreased in culture medium of HEK-293T cells co-transfected with BACE1/p75^{NTR} vs Empty vector/p75^{NTR}

A) Graph presenting a standard curve for p75ECD ELISA using NGF R/TNFRSF16 Kit
B) Plot demonstrating the statistical analysis for p75ECD measured by ELISA in culcure medium of HEK-293T cells transfected with BACE1/p75^{NTR} and Emptay vector/p75^{NTR} (**p<0.01) (n=3, mean±SEM, Student's t-test; pg: picogram).

p75ECD-Fc recombinant protein restores Aβ and proNT-induced neurite outgrowth impairment in mouse cortical neurons

In the current chapter, we have found that BACE1 interacts with p75^{NTR} and proteolytically processed p75^{NTR} and produced p75ECD. Now we test the significance of p75ECD in AD. In order to address this question, cortical neurons were isolated from 1 day old wild type (129sv) mouse brain and cultured on coverslips. After overnight seeding, the neurons were treated with 5 or 10 µM $A\beta_{42}$, 50 ng/ml proNGF or 50 ng/ml proBDNF in the presence of 10 µg/ml p75ECD-Fc or or 5 µg/ml Fc recombinant proteins for 24 hours. The neurons were then fixed and stained with a MAP-2 antibody and DAPI. Images were taken by a fluorescence (Olympus CX40) and a Zeiss confocal (LSM710) microscopes. Neurite length was measured by Image J software from 35 neurons on two different coverslips for each individual group. Data analysis showed that 5 and 10 µM $A\beta_{42}$ stimulate neurite outgrowth impairment in cortical neurons ($p<0.01$) whereas incubation $A\beta_{42}$ with 10 µg/ml p75ECD-Fc at the same time significantly restored Aβ-induced neurite outgrowth impairment (##$p<0.01$) (Figure 3-9). A comparison between "Aβ+p75ECD" and "Aβ scarmble" shows that p75ECD-Fc could completly rescue effect of 5 µM Aβ on neurite outgrowth impairments. p75ECD-Fc was not able to completely restore effect of 10 µM Aβ on neurite outgrowth impairment as there is a significant difference between "Aβ+p75ECD" and "Aβ scarmble" ($p<0.05$) (Figure 3-9). We found that 50 ng/ml proNGF (Figure 3-10) and 50 ng/ml proBDNF (Figure 3-11) induced neurite outgrowth impairment (**$p<0.01$ vs Control). p75ECD-Fc recombinant protein was able to rescue the effects of proNGF and proBDNF on neurite outgrowth impairments (Figure 3-10 and Figure 3-11). A comparison between "proNGF+p75ECD" and "control" indicate that p75ECD-Fc was able to entirely abolish

effects of proNGF on neurite outgrowth impairments. A significant difference between "proBDNF+p75ECD" and "control" ($p<0.01$) indicates that p75ECD-Fc could not completly restore proBDNF-induced neurite outgrowth impairment (Figure 3-11).

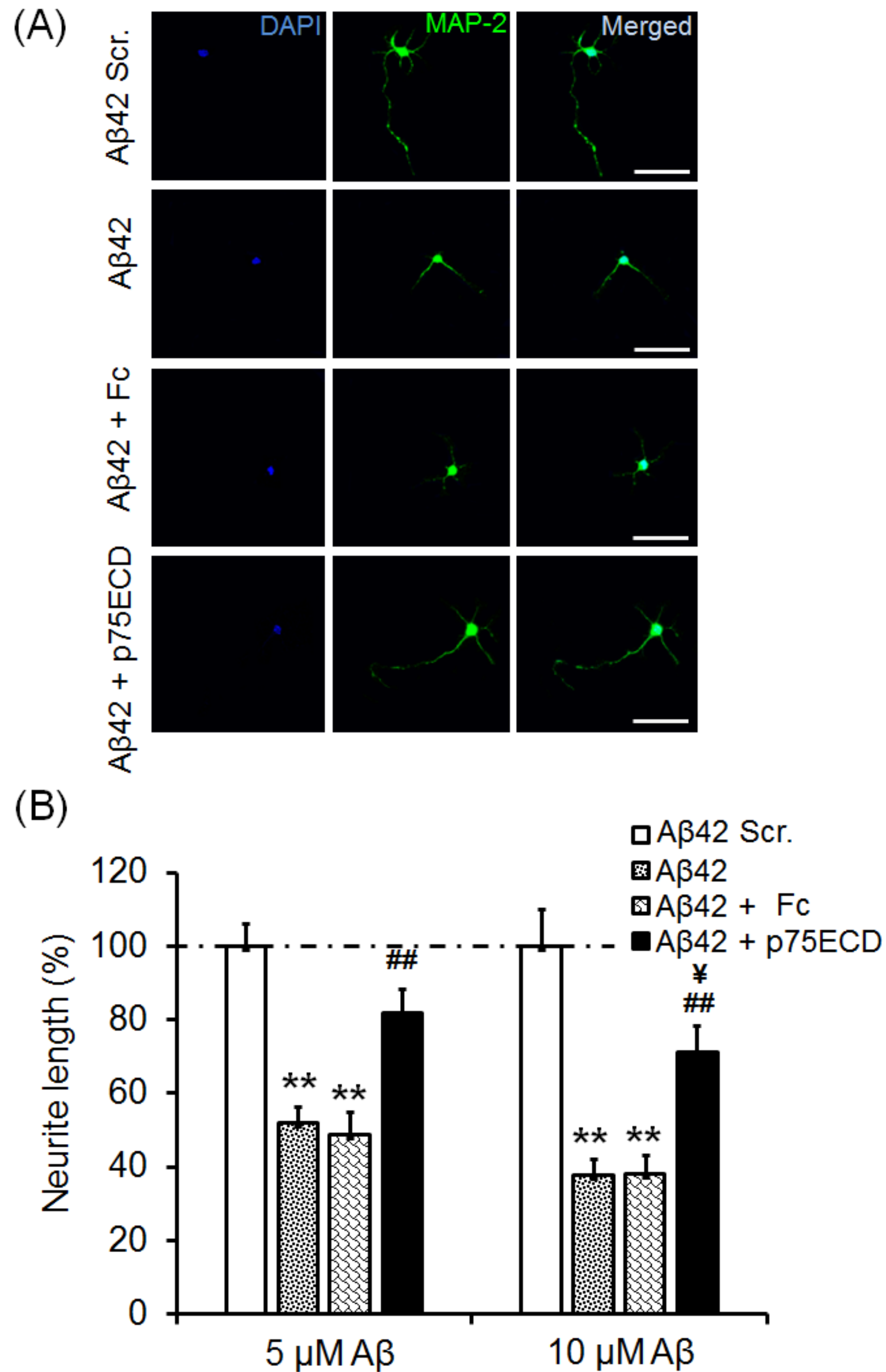


Figure 3-9: Effects of p75ECD-Fc on Aβ-induced neurite outgrowth impairment in mouse cortical neurons

A) Image demonstrating mouse cortical neurons stained with MAP-2 and DAPI from different groups treated with 5 µM Aβ_{42} or Aβ_{42} scramble in the presence 10 µg/ml p75ECD-Fc or 5 µg/ml Fc recombinant protein (Image for 10 µM Aβ_{42} was not shown).

B) Plot presenting the effect of p75ECD-Fc on neurite impairments induced by 5 and 10 μM $A\beta_{42}$ in mouse cortical neurons (**$p<0.01$ vs $A\beta_{42}$ Scr, ##$p<0.01$ vs "$A\beta_{42}$+Fc", ¥$p<0.05$ vs $A\beta_{42}$ Scr.)(n=35, mean±SEM, ANOVA, Tukey's post-hoc test; "$A\beta_{42}$ Scr." was normalized to 100%; Scr. Scrample).

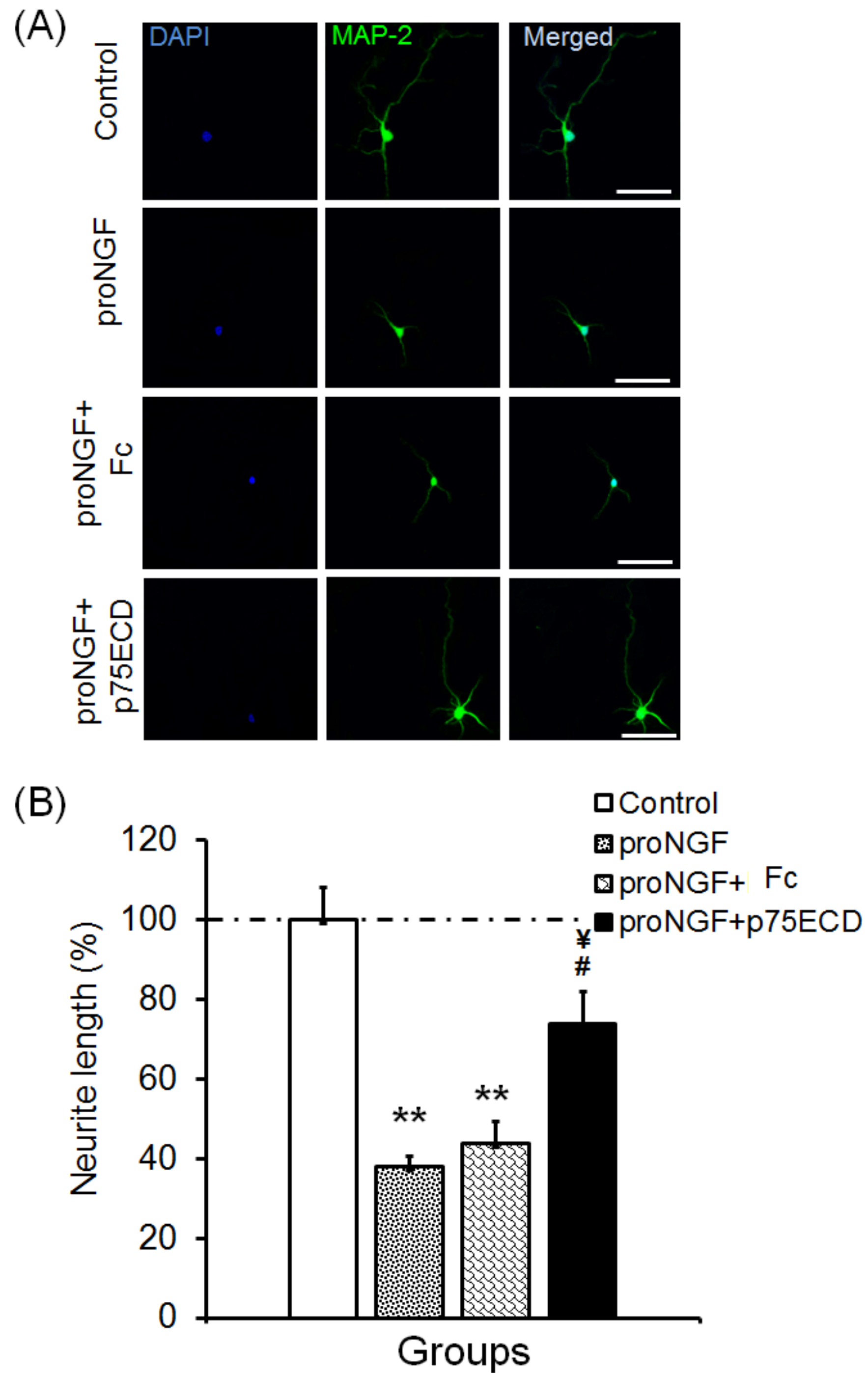


Figure 3-10: Effects of p75ECD-Fc on proNGF-induced neurite outgrowth impairment in mouse cortical neurons

A) Image indicating mouse cortical neurons stained with MAP-2 and DAPI from different groups treated with 50 ng/ml proNGF in the presence 10 µg/ml p75ECD-Fc or 5 µg/ml Fc recombinant protein. B) Graph showing the effect of p75ECD-Fc on neurite

impairments induced by proNGF in mouse cortical neurons (**p<0.01 vs Control, #p<0.05 vs “proNGF+Fc”, ¥p<0.05 vs Control)(n=35, mean±SEM, ANOVA, Tukey’s post-hoc test; “Control” was normalized to 100%; Control group: treated with BSA).

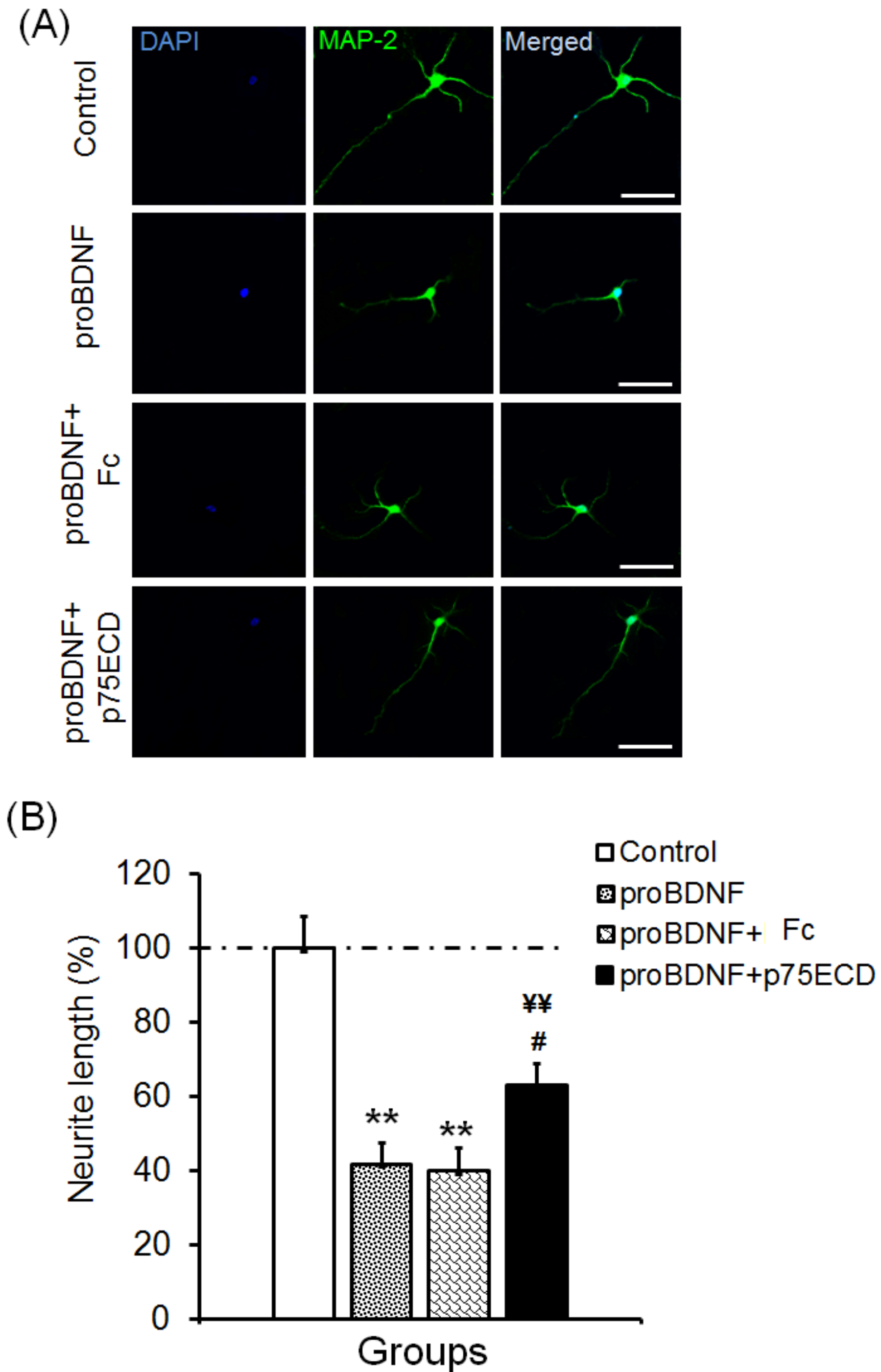


Figure 3-11: Effects of p75ECD-Fc on proBDNF-induced neurite outgrowth impairment in mouse cortical neurons

A) Representative image presenting mouse cortical neurons stained with MAP-2 and DAPI from different groups treated with 50 ng/ml proBDNF in the presence 10 µg/ml p75ECD-Fc or 5 µg/ml Fc recombinant protein. B) Plot indicating the effect of

p75ECD-Fc on neurite impairments induced by proBDNF in mouse cortical neurons (**p<0.01 vs Control, #p<0.05 vs "proBDNF+Fc", ¥¥p<0.01 vs Control)(n=35, mean±SEM, ANOVA, Tukey's post-hoc test; "Control" was normalized to 100%; Control group: treated with BSA)

3.5 Discussion

We have reported in Chapter 2 that p75^{NTR} regulates APP processing through Aβ-induced BACE1 and APP upregulation. In addition, we reported that Aβ and proNGF enhance the interaction between APP and p75^{NTR}. Moreover, p75^{NTR} is required for the BACE1/APP interaction. p75^{NTR} contributes to Aβ-induced BACE1 and APP endocytosis and plays a critical role in amyloidogenesis. In the current study, we have found that BACE1 associates with p75^{NTR} in mouse cortical neurons. A direct BACE1/p75^{NTR} interaction was seen in co-transfected HEK-293T cells with BACE1 and p75^{NTR} plasmids. Next we mapped which domain of p75^{NTR} interacts with BACE1. Our Co-IP results indicated that BACE1 interacts with p75ECD, but not with p75ICD. Aβ increases the co-localization between p75^{NTR} and BACE1 in the soma and dendrites of primary cortical neurons. Our FRET data also demonstrated that p75ECD-Fc recombinant protein and an antibody against p75ECD could inhibit BACE1/p75^{NTR} interaction increased by Aβ. Our FRET and Co-IP data showed that proNGF but not NGF increased the BACE1/p75^{NTR} interaction. Next, we test the significance of BACE1/p75^{NTR} association in the brain. We found that the level of p75ECD in BACE1$^{+/+}$ is higher than BACE1$^{-/-}$ mice brain whereas expression of TACE, a major enzyme that cuts p75^{NTR}, was unchanged. This suggests that p75^{NTR} is a substrate for BACE1. To further investigation we found that BACE1 overexpression intracellularly processed endogenous and over-expressed p75^{NTR} in CHO^{APP695} and HEK-293T cells, respectively. In contrast, our ELISA data for p75ECD in culture medium showed that BACE1 did not cut p75^{NTR} at the cell surface. However, we have not detected the cleavage site of p75^{NTR} by BACE1 and it is planned to be done in future to confirm our

finding. As Aβ and proNGF increased BACE1/$p75^{NTR}$ interaction, we have not shown whether Aβ and proNGF promotes the processing of $p75^{NTR}$ by BACE1. Our *in vitro* study presented that p75ECD prevents neurite outgrowth impairments caused by Aβ, proNGF and proBDNF in cortical neurons. Our data indicated that Aβ and proNTs decreased neurite length in comparison with Aβ scramble and control, respectively. p75ECD-Fc was able to restore neurite length impairment induced by Aβ and proNTs. "Aβ scramble+p75ECD" and "Control+p75ECD" groups were needed to be designed in our experiment to indicate whether p75ECD could completely rescue neurite length impairment caused by Aβ and proNTs. Although, our experiment does not have these controls, a comparison "Aβ+p75ECD" vs "Aβ scramble" and "proNTs+p75ECD" vs "Control" roughly indicate whether p75ECD could completely inhibit the neurite length impairment. The statistical analysis did not show a significant alteration between "5 µM Aβ+p75ECD" and 5 µM Aβ scramble. This means p75ECD-Fc could completely rescue the effect of 5 µM Aβ on neurite impairment, whereas p75ECD-Fc was not able to completely restore effects 10 µM Aβ and proNTs on neurite impairments. Although, our data reinforces the neuroprotective function of p75ECD in AD consistent with our previous findings (Chapter 2), we have not yet undertaken an *in vivo* study to elucidate the physiological function of BACE1/$p75^{NTR}$ interaction in mice brain.

$p75^{NTR}$ is a neurotrophin receptor and critically regulates neuron survival, apoptosis, innervation, migration and differentiation via binding with pro- and mature neurotrophins. For example, NGF improves survival of BFCN via binding TrkA and $p75^{NTR}$ (Bothwell 1995, Chao and Hempstead 1995, Kaplan and Miller 1997), but the main function of $p75^{NTR}$ in cooperation with Sortilin is to induce cell death in the brain

including BFCN. Aβ and proneurotrophins, which are abundant in the AD brain, bind $p75^{NTR}$/Sortilin independent of Trk receptors, and promote BFCN apoptosis and the progress of AD (Nykjaer, Lee et al. 2004, Sotthibundhu, Sykes et al. 2008). In addition, $p75^{NTR}$ interacts with several transmembrane proteins such as APP or the Nogo receptor and participates in neurodegenerative processing in AD and other disorders (Wang, Kim et al. 2002, Mi, Lee et al. 2004, Fombonne, Rabizadeh et al. 2009). We also found (Chapter 2) that $p75^{NTR}$ in conjunction with Aβ contributes to APP and BACE1 upregulation and promotes amyloidogenic processing of APP to play a critical role in AD pathogenesis.

In the present study, we found that $p75^{NTR}$ interacts with BACE1. This finding is consistent with a recent study showing that Sortilin, a close co-receptor of $p75^{NTR}$, interacts with BACE1 and mediates BACE1 retrograde trafficking and promotes Aβ generation (Finan, Okada et al. 2011). Our FRET and CoIP results show that BACE1 interacts with p75ECD, but not p75ICD. We also found that BACE1 and $p75^{NTR}$ association is increased in dendrites and axons of cortical neurons in the presence of Aβ. Furthermore, Aβ and proNGF promote BACE1/$p75^{NTR}$ interaction in neuron and cell line whereas NGF did not have influence on BACE1/$p75^{NTR}$ interaction. Aβ and proNGF are ligands for $p75^{NTR}$ (Chao and Hempstead 1995) and this bindings contributes to AD pathogenesis (Selkoe 1991, Longo and Massa 2005). Our data also confirmed that p75ECD recombinant protein and anti-p75ECD antibody could attenuate FRET signals from BACE1/$p75^{NTR}$ interaction. Thus, it is likely the Aβ and proNGF-enhance the BACE1/$p75^{NTR}$ interaction through a $p75^{NTR}$-dependent mechanism.

In the current study, we discovered that p75^{NTR} is a substrate of BACE1 and that the BACE1/p75^{NTR} interaction results in p75^{NTR} processing. p75^{NTR} consists of three domains including p75ECD, a trans- or juxta-membrane domain and p75ICD. Full length p75^{NTR} is generally cleaved by proteases such as TACE (ADAM17) and then p75ECD is produced to outside the cell (Weskamp, Schlondorff et al. 2004). Our data showed that the level of p75ECD in BACE$^{+/+}$ is higher than BACE$^{-/-}$ mouse brain, suggesting p75^{NTR} is partially cleaved by BACE1 to generate p75ECD. In addition, transfecting BACE1 into CHO^{APP695}cell line increased p75ECD generation in cell lysate. The co-transfection of BACE1 and p75^{NTR} in HEK-293T cells intracellularly process p75^{NTR} and generate more p75ECD. This data indicates that BACE1 is a key molecule to contribute to the shedding of p75^{NTR} and generate p75ECD. In order to detect whether p75ECD is secreted in the culture medium in the presence of BACE1, we conducted an ELISA assay as Western blot is not sensitive enough to detect p75ECD in culture medium. This ELISA data indicated that BACE1 reduced p75^{NTR} shedding on the cell surface and decreased the level of p75ECD in the culture medium. BACE1 contains two aspartic protease active site motifs on its extracellular domain, DTGS (residues 93–96) and DSGT (residues 289–292). These interact with APP (Kinoshita, Fukumoto et al. 2003) and initiate APP processing and Aβ production (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999). The optimum pH for TACE activity is around pH 7.5 and it is available at cell surface membrane (Wei, Kashiwagi et al. 2005). In contrast, an acidic pH (6.0) is required for the biological activity of BACE1 which is provided within the subcellular compartments of the endosomal pathway, including the TGN and endosomes (Koo and Squazzo 1994, Haass, Capell et al. 1995, Haass, Lemere et al. 1995, Vassar, Bennett et al. 1999). Our

data support the hypothesis that p75^{NTR} processing by BACE1 occurs within intracellular acidic compartments and this may explain why intracellular p75ECD is increased and why extracellular p75ECD is decreased in the presence of BACE1. This is analogous to the cleavage of APP by BACE1, in that BACE1 interacts with APP and mediates APP processing in the Early Endosome compartment, but not on the cell surface (Huse, Pijak et al. 2000, Kinoshita, Fukumoto et al. 2003). In addition, we previously (Chapter 2) reported that p75^{NTR}, in conjunction with Aβ, mediates BACE1 internalization from the cell surface. Based on other reports and our previous finding, our recent study suggests that the BACE1/p75^{NTR} association most likely induces BACE1 internalization and therefore promotes BACE1-induced p75^{NTR} processing.

Although, the physiological function of p75ECD has not been fully recognized, we have found that p75ECD recombinant protein attenuated Aβ, proNGF and proBDNF-induced neurite outgrowth impairment in cortical neurons. Previous studies from our laboratory showed that insoluble Aβ deposition in the brain is significantly increased after deleting a part of p75^{NTR} gene (ExonIII) that encodes p75ECD in APPswe transgenic mice. Moreover, p75ECD recombinant protein inhibited Aβ oligomerization in a dose-dependent manner and injection of p75ECD into the hippocampus of AD mice reduces local Aβ plaques (Wang, Wang et al. 2011). We also reported that p75ECD abolished Aβ and proBDNF-induced Sortilin upregulation (Saadipour, Yang et al. 2013). In addition, our previous data (Chapter 2) have shown that p75ECD inhibits the p75^{NTR}/APP interaction. Thus, our study indicates the cleavage of p75^{NTR} by BACE1 will reduce the level of full length p75^{NTR} and increase p75ECD which will protect

neurons from neurodegeneration triggered by proneurotrophins and Aβ. In addition, $p75^{NTR}$ as a substrate of BACE1 may compete with APP to reduce APP beta cleavage.

To sum up, BACE1 interacts with p75ECD and this interaction is enhanced by Aβ and proNGF, but not NGF. $p75^{NTR}$ is a substrate of BACE1 which intracellularly cleaves $p75^{NTR}$ at its extracellular domain. The significance of BACE1-mediated $p75^{NTR}$ processing is not fully known, but the cleavage of $p75^{NTR}$ to generate p75ECD may inhibit the neurodegenerative functions of $p75^{NTR}$.

CHAPTER 4: EFFECTS OF P75ECD-FC ON BEHAVIOURAL DEFICITS AND NEUROPATHOLOGY FEATURES IN ALZHEIMER’S DISEASE MOUSE MODELS

4.1 Abstract

Background: In AD, neurodegenerative signals such as Aβ and proNGF outweigh neurotrophic signals, causing synaptic dysfunction and neurodegeneration. $p75^{NTR}$ mediates both neurotrophic and neurodegenerative signals and its ectodomain shedding from the cell surface is tightly regulated. However, the function of p75ECD after shedding is not known. We have conducted an *in vivo* study to investigate the effects of p75ECD-Fc recombinant protein on cognitive functions and neuropathology features in a mouse model of AD. **Methods:** In the present study, p75ECD-Fc and Fc recombinant protein were injected intra peritoneally (i.p) in 7-month old APPswe/PS1DE9 (AD) and 3-month old PR5 transgenic mice for 3 months. Mice were then subjected to Morris Water Maze (MWM) to evaluate learning and memory performance. After this behavioural study, the brain was isolated from the mouse and subjected to IHC and Western blot in order to quantify Aβ and the other AD biomarkers. **Results:** Our data demonstrated that i.p delivery of p75ECD-Fc was not effective on cognitive function in APPswe/PS1DE9 (AD) mouse. p75ECD-Fc improved the process of learning ($p<0.05$), but not memory impairment in PR5 mouse model. In contrast to unchanged behavioural functions in AD mouse, p75ECD-Fc significantly decreased the BACE1 expression level ($p<0.05$) and subsequently decreased the size and number of Aβ plaques in AD mouse brain ($p<0.05$, $p<0.01$). Moreover, p75ECD-Fc significantly reduced GFAP levels in AD mouse. p75ECD-Fc was not effective in restoring the level of synaptic proteins, including the vesicle-associated membrane protein (VAMP2) and synaptosomal-associated protein 25 (SNAP-25) in AD mouse brain. p75ECD-Fc did not change ChAT levels, but it significantly reduced Tau phosphorylation ($p<0.01$) as well as the expression of BACE1 ($p<0.05$) in PR5 mouse brain. The levels of VAMP2 and

SNAP-25 were unchanged in PR5 mice groups treated with p75ECD-Fc and Fc. **Conclusion:** Our study suggests that p75ECD-Fc recombinant protein may be a desirable anti-AD agent. It facilitates peripheral Aβ clearance possibly through binding with peripheral Aβ and increasing the gradient concentration of Aβ toward the periphery. Following this, Aβ deposition decreases in the brain to subsequently protect neurons from Aβ and proNTs- induced neurotoxicity.

Keywords: p75ECD-Fc recombinant protein, Aβ, learning and memory, and Alzheimer’s disease.

4.2 Introduction

Many studies show that an imbalance between Aβ production and clearance in the brain and the formation of neurofibrillary tangles are hallmarks of AD and critical in AD pathogenesis (Hardy and Selkoe 2002). Although Aβ generation through amyloidogenic processing of APP plays a key role in AD, there are several studies to demonstrate regulation of Aβ catabolism (Iwata, Tsubuki et al. 2000), clearance (Ghersi-Egea, Gorevic et al. 1996, Shibata, Yamada et al. 2000), and aggregation (Wisniewski, Ghiso et al. 1997) critically mediate AD progression. For instance, the main function of ApoE in the development of AD, is not through Aβ production, in fact ApoE reduces Aβ clearance and enhances Aβ deposition, aggregation, plaque formation and increases neuritic degeneration in the brain (Strittmatter and Roses 1996, Bales, Verina et al. 1997, Du, Ni et al. 1997, Wisniewski, Ghiso et al. 1997, Russo, Angelini et al. 1998, Holtzman, Bales et al. 1999, Holtzman, Bales et al. 2000). There is a dynamic association between Aβ in the CNS and peripheral organelles, including blood, and disruption of the equilibrium of Aβ in these environments influences Aβ levels in the brain (Zlokovic 2004). For example, RAGE (Deane, Du Yan et al. 2003) and LRP1 (Shibata, Yamada et al. 2000) contribute to Aβ transportation across the BBB from the systemic circulation into the brain and brain to plasma, respectively. These molecules potentially involve regulating Aβ homeostasis in the body (Figure 1-3). Consistent with this, $A\beta_{40}$ is rapidly transported from CSF to plasma with a half-life of $\leq$30 min (Ghersi-Egea, Gorevic et al. 1996, Shibata, Yamada et al. 2000) and peripheral Aβ-binding compounds increase Aβ clearance and subsequently reduce the level of Aβ in the brain (DeMattos, Bales et al. 2001, Sagare, Deane et al. 2007). However, one report in an AD

mouse model shows that inhibition of $A\beta_{40}$ clearance in plasma did not alter elimination of $A\beta_{40}$ from the brain (Kandimalla, Curran et al. 2005). Our *in vitro* study results (Chapter 2 and 3) indicated that the p75ECD-Fc recombinant protein was potentially able to inhibit Aβ generation and AD pathogenesis in several pathways such as the inhibition of the $p75^{NTR}$/APP interaction and Aβ and proNTs induced neurite impairments. In the present study, we are conducting an *in vivo* study to elucidate whether if administration of the p75ECD-Fc recombinant protein attenuates cognitive deficits and neuropathology of AD in 2 AD mouse models.

4.3 Materials and methods

Antibodies, reagents and plasmids

In the present study, in addition to the antibodies, reagents and plasmids which were cited in Chapter 2, rabbit affinity purified anti-ChAT and rabbit anti-GFAP from Dr**.** John Oliver (Centre for Neuroscience, Department of Human Physiology, Flinders University), rabbit anti-VAMP2 (OSS00035W, Osenses, Australia), rabbit anti-SNAP25 (OSS00033W, Osenses, Australia) were used.

Animals

In the current study, APPswe/PS1DE9 (AD) transgenic mouse from Jackson Laboratory (explained in Chapter 2) (Jankowsky, Slunt et al. 2001) and PR5 mouse which expresses the longest human Tau isoform (h.Tau 40) from Prof. Jurgen Goetz (Queensland Brain Institute, The University of Queensland, Brisbane, Queensland, Australia) (Gotz, Chen et al. 2001) were used. All mice were bred in the University of South Australia (Animal House) and genotyping of mice was performed using primers and PCR following the supplier's instructions (Table 4-1).

Table 4-1: PCR primer pair sequences used for PR5 mice genotyping in this study

Gene	Forward primer	Reverse primer
PR5	5'- aagtcacccagcagggaggtg- 3'	5'- tgtctccaatgcctgcttcttc -3'

Animals were maintained under standard conditions at 22 °C and a 12 h light:dark cycle with *ad libitum* food and water. All mice husbandry procedures performed were

approved by the Animal Ethics Committee of South Australian Pathology (16b/12) in accordance with NHMRC guidelines.

p75ECD-Fc and Fc recombinant protein preparation

The synthesis and purification of p75ECD-Fc and Fc recombinant proteins are explained in the methodology section of Chapter 2.

Intra peritoneal (ip) injection of p75ECD-Fc and Fc protein

Seven-month old APP/PS1 (AD) transgenic (n=20) and 3-month old PR5 (n=12) mice were used in this study. Each mouse strain was randomly divided to two different groups with age matched wild type (C57bl) control which received 5 mg/kg p75ECD-Fc and 2.5 mg/kg Fc recombinant proteins twice/week in 150-200 µl volume PBS via ip injection for 3 months. In the first week 10 and 5 mg/kg of p75ECD-Fc and Fc recombinant proteins were administrated to mice, respectively, in order to provide a sufficient amount of the protein in the mice plasma.

Morris Water Maze (MWM) behavioural study

AD and Tau pathology-related tyrosine phosphorylation (PR5) mice received p75ECD-Fc and Fc recombinant protein for 3 months via ip injection. A day after the final injection, the mice were subjected to behavioural tasks and MWM test was employed according to a standard protocol with minor modification (Yau, McNair et al. 2007, Wang, Gao et al. 2010, Bromley-Brits, Deng et al. 2011). The test was conducted in a circular pool, 100 cm in diameter with a 60 cm-high wall, that was filled with opaque water to a depth of 21 cm containing white non-toxic dye (24 ± 1°C) and surrounded by

a set of spatial cues (Markowska, Long et al. 1993). The tank was imaginarily divided into four quadrants in software, and four start positions were chosen in the quadrants (**Error! Reference source not found.**) (Frick and Fernandez 2003).

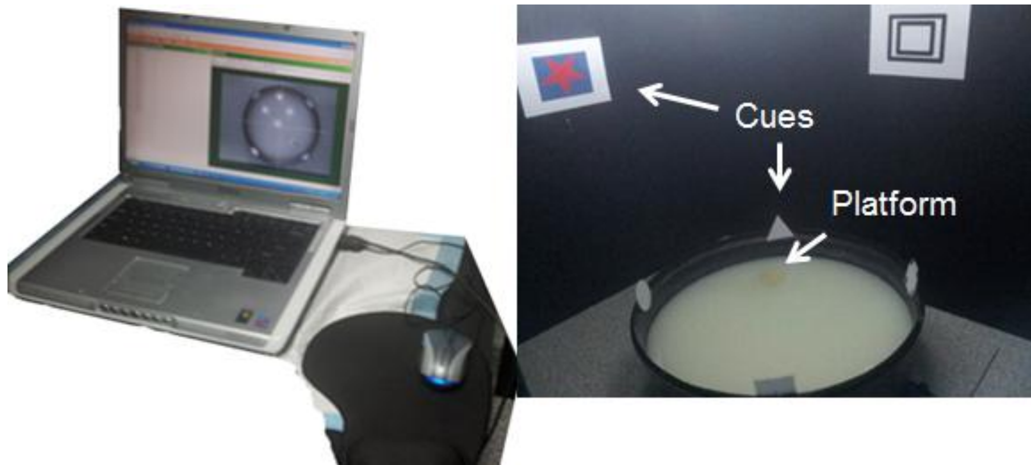


Figure 4-1: Presenting the Morris Water Maze apparatus

The MWM apparatus consists of a circular pool with 100 cm in diameter and a 60 cm-high wall, surrounded by a set of spatial cues. A digital camera was installed on the top of tank and was connected to a computer which equipped with image analysing software, ANY-maze.

The test consisted of a four platform trial per day and it continued for 5 days, followed by a probe trial. During the five days of platform trials, a 10 cm diameter platform (island) was hidden 1.5 cm under water surface in a constant position in a quadrant. Mice were placed in the tank in order to find platform. If the mouse could not find and locate the platform in 60 seconds, it was gently guided to the platform by a constant experimenter. Once the mouse found the platform, it remained on the platform for 20 seconds and then removed from the pool and dried with a towel and placed in cages. 24 hours after the last platform trial (on 6th day), all mice were given a single probe trial, in

which the platform was hidden, to evaluate the final strength of memory. The mice were allowed to swim freely for 90 seconds. All mice were placed into the water facing the pool wall at the position opposite to the target. Performance in all tasks was video-recorded and analysed by a computer-based video tracking system and image analysing software (ANY-maze, Stoelting). In platform trials, distance of path from the start location to the platform (in centimeters), latency of the time taken to reach the platform from the start location (in seconds) were measured, while in probe trials quadrant time (Total time spent in the platform quadrant) and platform crossings (the frequency that the mice crossed the exact location of the platform) were measured. For latency and swim distance in platform trials, lower scores present a better performance. For evaluation of quadrant time and platform crossings in probe trials, higher scores indicate a better performance (Frick and Fernandez 2003).

Tissue sample preparation for immunohistochemistry and Western blot assay

A day after, the behavioural study conducted, all animals were humanely killed by exposing with CO2 for 5 min, followed by perfusion of 50 ml cold PBS in intra cardially the right brain hemisphere was taken for histological analysis and fixed in 4% PF (pH 7.4) for 24 hours and then incubated in 30% sucrose for 48 hours for cryosection. Coronal sections of the brain were cut at 35 μm thickness with a cryosectioning microtome (Leica CM 3050 S) and stored at 4°C in PBS containing 0.1% sodium azide until use. Left brain hemisphere was snap frozen in liquid nitrogen and stored at −80°C for future biochemical analysis.

Histological staining of mouse brain sections

Brain sections were randomly selected and stained using free-floating immunohistochemistry for total Aβ and Glial fibrillary acidic protein (GFAP). Sections were incubated overnight with primary antibodies at 4°C and further developed with biotinylated secondary antibodies and ABC Kit using diaminobenzidine (DAB) and glucose oxidase (GO) as substrates. Images were taken by Olympus Microscope (CX40) using 4× magnification resolution and the number of DAB stained Aβ plaques and GFAP in neocortex and hippocampus areas in sections were counted by using Image J software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html). Following this quantitative analysis and data were reported as mean±SEM (Wang, Pollard et al. 2009, Wang, Wang et al. 2011).

Congo red staining for Aβ in mouse brain sections

In this method, a series of three equally spaced tissue sections for each mouse were treated with working sodium chloride solution (containing sodium chloride saturated in 80% alcohol and 0.01% sodium hydroxide) at room temperature for 20 minutes, then placed directly into working Congo red solution (containing saturated Congo red in working sodium chloride solution) for 45 minutes, and dehydrated rapidly in absolute alcohol. Images were taken by Olympus Microscope (CX40) using 4× magnification resolution and the number of Congo red stained Aβ plaques in neocortex and hippocampus areas in sections were counted using Image J software and followed by quantitative analysis. Data were reported as mean±SEM (Wang, Pollard et al. 2009, Wang, Wang et al. 2011).

Western blot assay

Protein purification from mice brain and Western blot analysis were applied according to the method explained in Chapter 2. Briefly, the mice brain from left hemisphere was snap frozen and homogenated in RIPA buffer including 1 mM PMSF, antipain, pepstatin, and leupeptin (Roche, Australia). After sonication and protein extraction, 40 μg of total protein was separated by SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked for an hour in skim milk and after overnight incubation with primary and (HRP)-linked secondary antibodies respectively, the membrane was developed by Image Quant LAS 4000 (GE Healthcare, US) and Image J software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html) was used for quantitative analysis and data were normalized to 100% and reported as mean±SEM (Saadipour, Yang et al. 2013).

Statistical analysis

All statistical analyses were performed using SPSS 22 software package (IBM, provided by Flinders University). Variables between groups were determined by either one-way ANOVA following Tukey's post-hoc test or Student's t-test. Values of $p<0.05$ were considered statistically significant. Data is presented as mean±SEM.

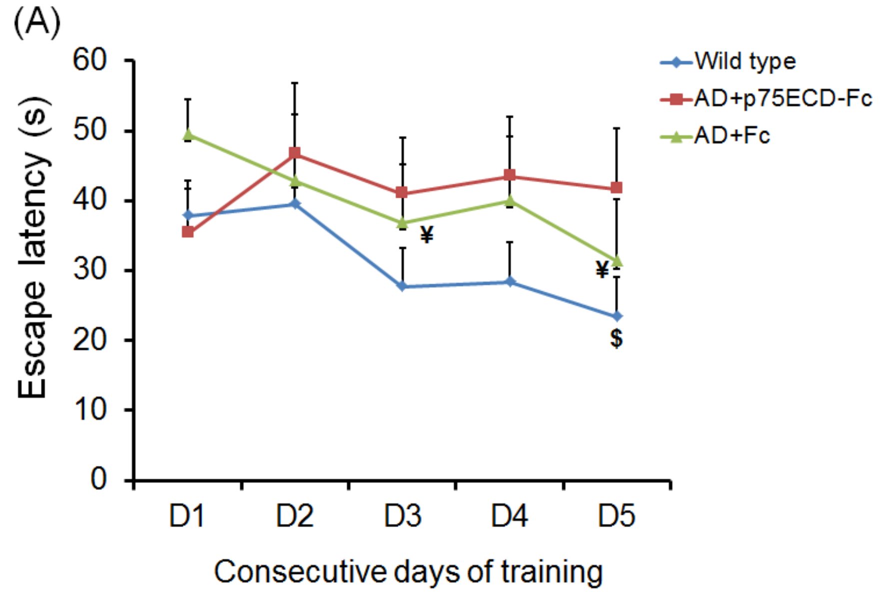
4.4 Results

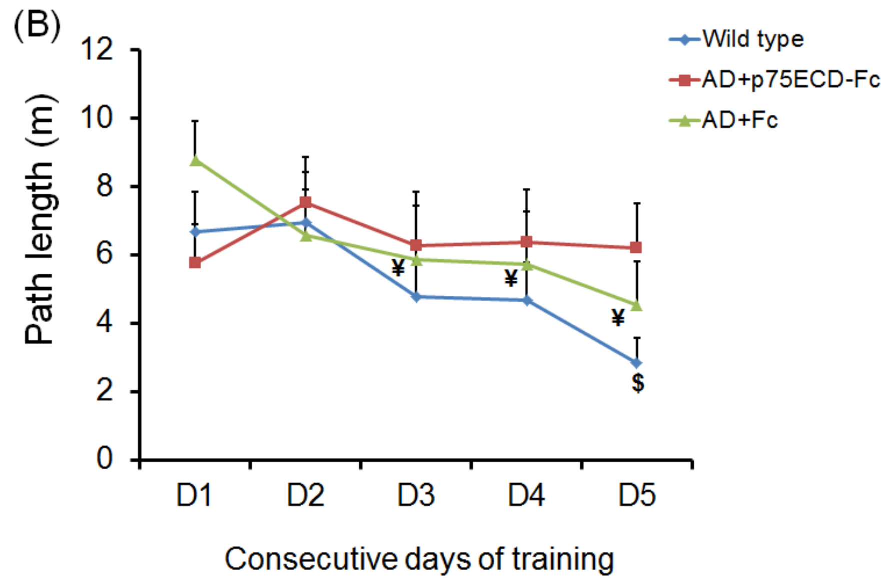
In the current study, the effect of p75ECD-Fc recombinant protein on cognitive function in two different AD mouse models, APPswe/PS1dE9 (AD) and PR5, has been investigated. Following this the mouse brains were extracted and subjected to IHC and Western blot assays in order to quantify critical AD biomarkers.

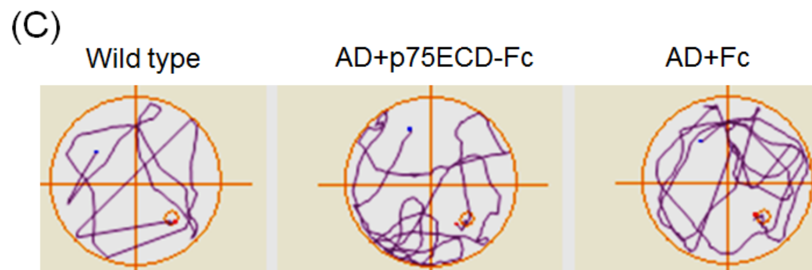
Effects of p75ECD-Fc on cognitive deficits in APPswe/PS1dE9 (AD) mice

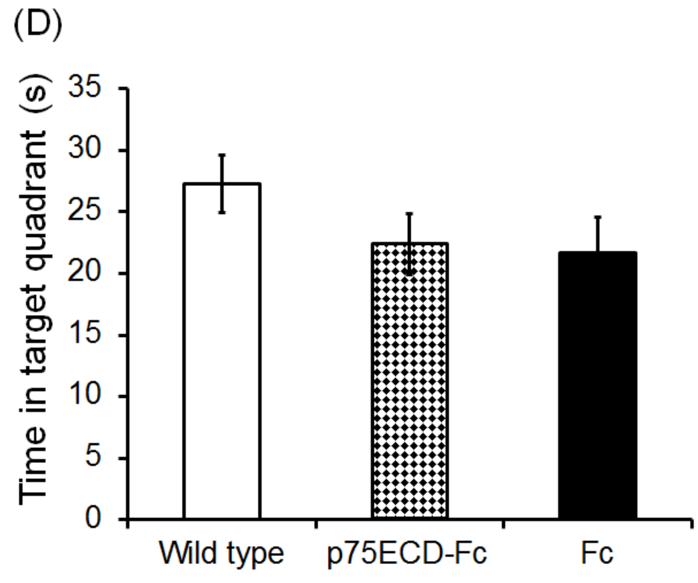
Seven-month old AD mice were randomly divided into two groups (each group n=10) and received p75ECD-Fc and Fc recombinant proteins for 3 months. A group of age matched wild type mice was used as a control for the AD mice. In the wild type goup, an improvement in the learning curve was seen and the latency to find the platform decreased with the time. However, in AD mice either treated with p75ECD-Fc or Fc, no significant changes were seen in the escape latency time (Figure 4-2 A) and travel distance (Figure 4-2 B and C) within progressive platform learning trials. A significant reduction of escape latency and travel distance in day of 5th vs 1st was seen during the learning process in wild type group (Figure 4-2 A). The escape latency in the Fc group was decreased at day 3rd and 5th vs 1st and travel distance in Fc group was reduced at day 3rd, 4th and 5th during learning process (Figure 4-2 B). No significant changes of learning process in p75ECD-Fc group was detected (Figure 4-2 A and B). In addition, there was no significant difference among groups in the time spent in the target quadrant (Figure 4-2 D). The frequency of entries in the platform quadrants in the Fc group is significantly lower than wild type, but there is no change in the p75ECD-Fc group

compared with wild type or Fc (Figure 4-2 E). The frequency of crossing on the withdrawn platform site in the probe trials was unchanged between p75ECD-Fc and Fc, but a significant reduction in p75ECD-Fc and Fc compared with wild type was detected (Figure 4-2 F). So based on this data, the i.p injection of p75ECD-Fc did not significantly improve spatial learning and memory in 10-month old AD mice.


(A)
Escape latency (s)
60
50
40
30
20
10
0
D1
D2
D3
D4
D5
Consecutive days of training
Wild type
AD+p75ECD-Fc
AD+Fc
¥
¥
$


(B)
Path length (m)
12
10
8
6
4
2
0
D1
D2
D3
D4
D5
Consecutive days of training
Wild type
AD+p75ECD-Fc
AD+Fc
¥
¥
¥
$


(C)
Wild type
AD+p75ECD-Fc
AD+Fc


(D)
Time in target quadrant (s)
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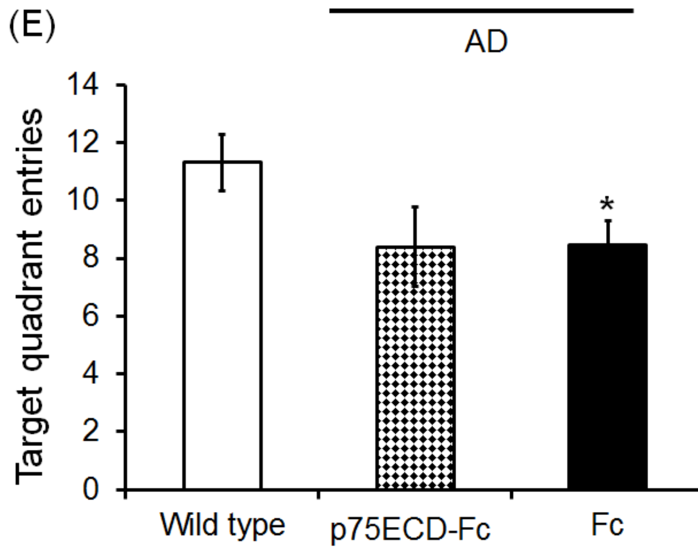
30
25
20
15
10
5
0
Wild type
p75ECD-Fc
Fc
AD


(E)
Target quadrant entries
14
12
10
8
6
4
2
0
*
Wild type
p75ECD-Fc
Fc
AD

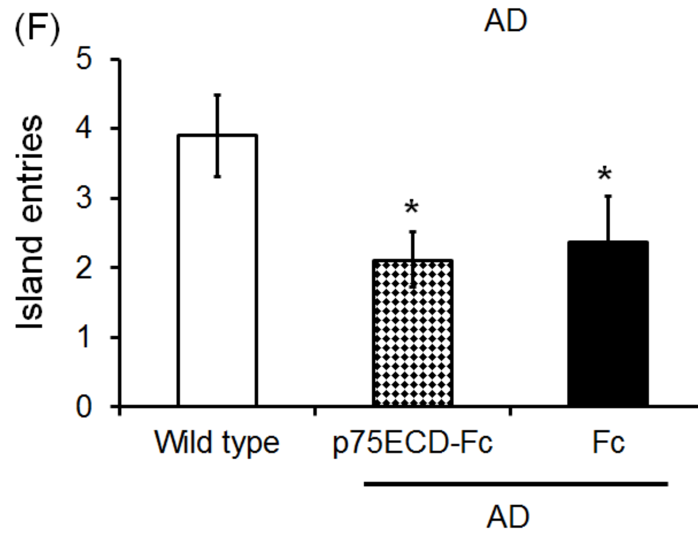
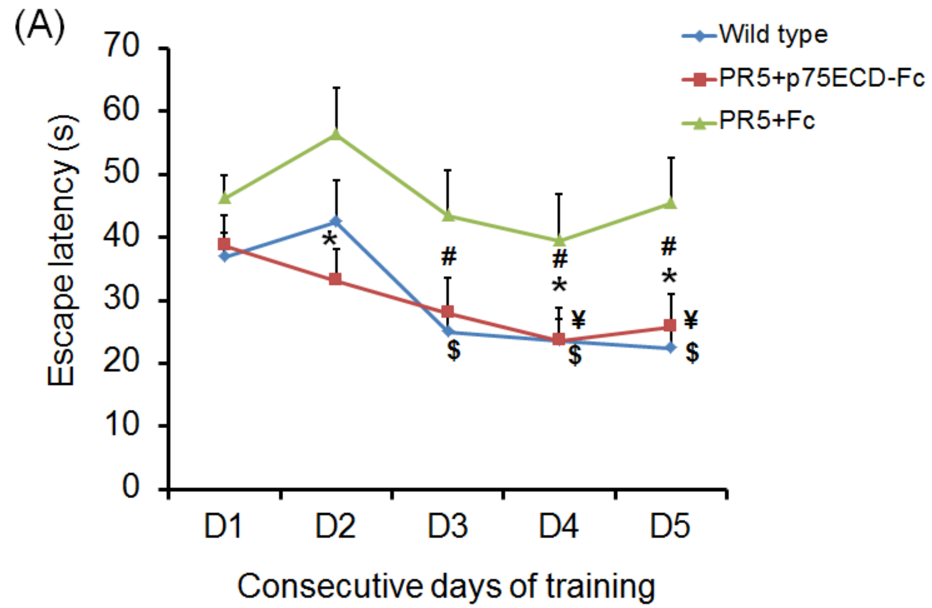

(F)
Island entries
5
4
3
2
1
0
*
*
Wild type
p75ECD-Fc
Fc
AD

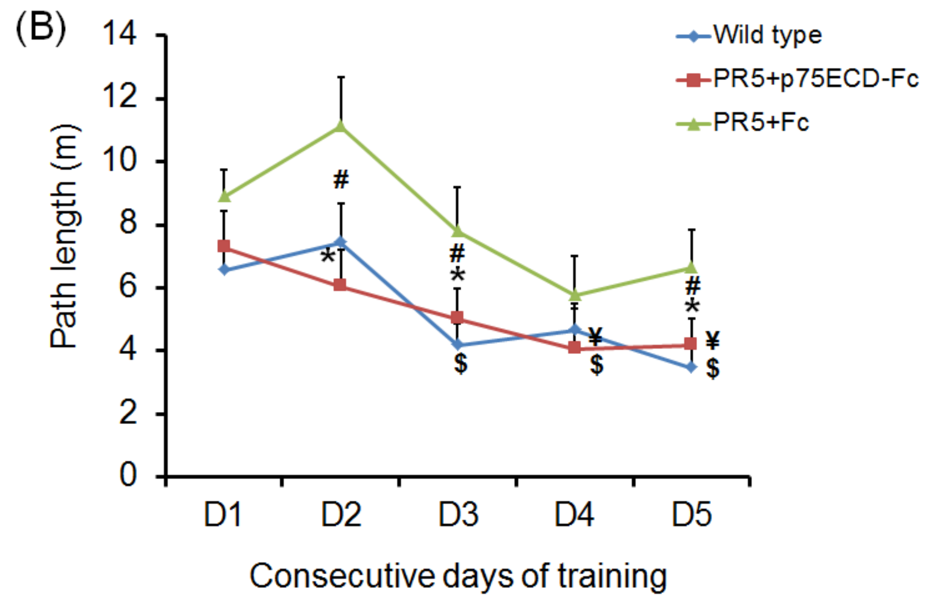
Figure 4-2: Effects of p75ECD-Fc on learning and memory functions in APPswe/PS1dE9 (AD) mouse

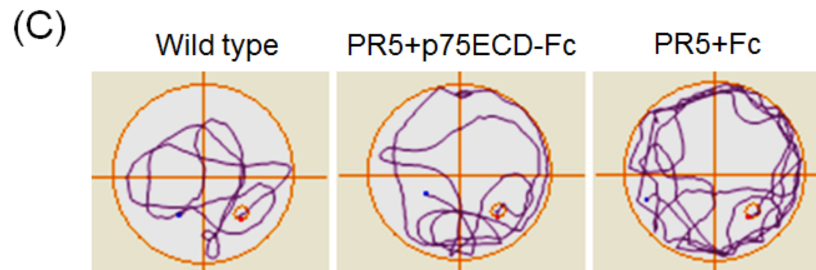
The MWM data presenting the effects of p75ECD-Fc recombinant protein on escape latency time (A), travel distance to find platform (B) within progressive platform learning trials in AD mouse. C) Topographic image for an average travel distance in each group of AD mouse. The plot showing time spent (D), the numbers of entries in the platform quadrants (E), and the numbers of crossing the hidden platform site in the probe trials (F) (*$p<0.05$ vs wild type; $$p<0.05$ in wild type during learning process compared with 1st day of learning; ¥$p<0.05$ in p75ECD-Fc during learning process compared with 1st day of learning *$p<0.05$) (n=10 per group, mean±SEM, Tukey post-hoc).

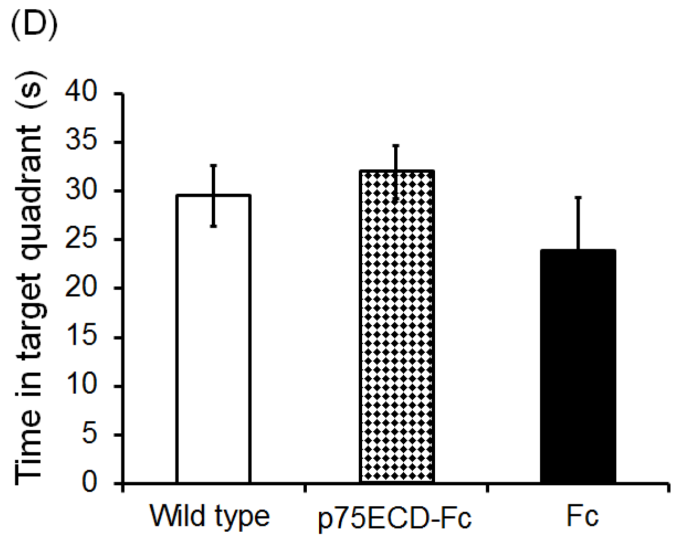
Effects of p75ECD-Fc on cognitive deficits in PR5 mice model

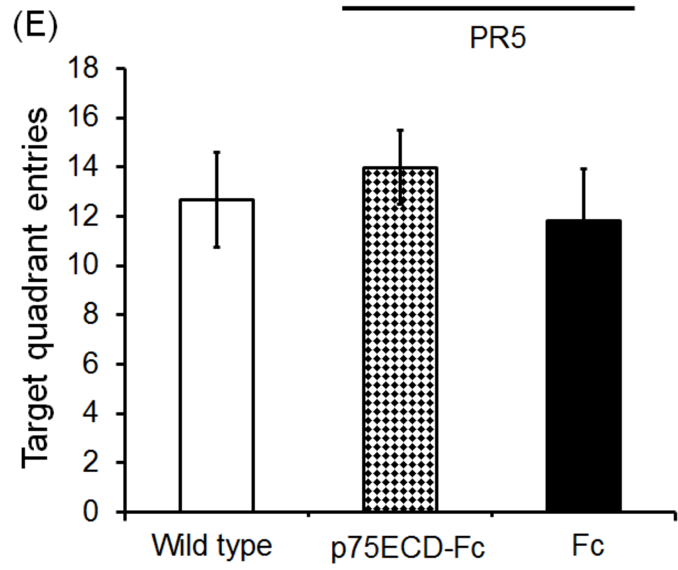
In order to evaluate the effects of p75ECD-Fc on learning and memory functions in PR5 mice, 12 3-months old mice were divided to two groups and received p75ECD-Fc and Fc recombinant proteins for 3 months, respectively. A group of age and sex matched wild type mice were used as control. MWM test for PR5 mice showed that p75ECD-Fc significantly reduced escape latency time (Figure 4-3 A) during 2nd, 4th, and 5th day of learning trials and travel distance (Figure 4-3 B,C) during 2nd, 3rd, and 5th day of the learning trials in comparision with the Fc group. The learning process in wild type and p75ECD-Fc groups are improved. A significant reduction of escape latency and travel distance within day of 3rd, 4th, and 5th vs 1st are seen during learning process. In p75ECD-Fc group escape latency and travel distance were decreased within day of 4th and 5th vs 1st during learning process whereas no significant improvement of learning was seen in Fc group during learning process. p75ECD-Fc did not change the mean time in the target quadrant (Figure 4-3 D), number of entries in the platform quadrants (Figure 4-3 E) in the probe trial session. The number of crossing the platforn in the probe trial was unchanged between groups (Figure 4-3 F). The results suggest that p75ECD-Fc could improve the process of learming, wherase it was not effective on memory impairment in PR5 mouse.


(A)
Wild type
PR5+p75ECD-Fc
PR5+Fc
Escape latency (s)
0
10
20
30
40
50
60
70
D1
D2
D3
D4
D5
Consecutive days of training
*
#
$
¥


(B)
Wild type
PR5+p75ECD-Fc
PR5+Fc
Path length (m)
0
2
4
6
8
10
12
14
D1
D2
D3
D4
D5
Consecutive days of training
*
#
$
¥


(C)
Wild type
PR5+p75ECD-Fc
PR5+Fc


(D)
Time in target quadrant (s)
40
35
30
25
20
15
10
5
0
Wild type
p75ECD-Fc
Fc
PR5


(E)
Target quadrant entries
18
16
14
12
10
8
6
4
2
0
Wild type
p75ECD-Fc
Fc
PR5

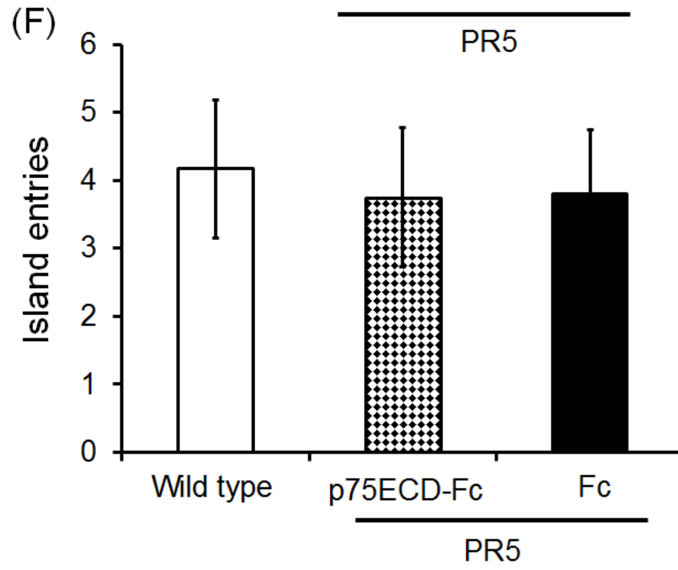

(F)
Island entries
6
5
4
3
2
1
0
Wild type
p75ECD-Fc
Fc
PR5

Figure 4-3: Effects of p75ECD-Fc on learning and memory function in PR5 mouse

The MWM test demonstrating the effects of p75ECD-Fc on escape latency time (A) and travel distance to find platform (B,C) within progressive platform learning trials in PR5 mouse. The graphs showing time spent (D) and the number of entries in the platform quadrants (E) and the numbers of crossing the hidden platforn site in the probe trials between groups (*$p<0.05$ for p75ECD-Fc vs Fc group, #$p<0.05$ for wild type vs Fc group; \$$p<0.05$ in wild type during learning process compared with 1st day of learning; ¥$p<0.05$ in p75ECD-Fc during learning process compared with 1st day of learning *$p<0.05$) (n=6 per group, mean±SEM, ANOVA, Tukey's post hoc test).

p75ECD-Fc prevents BACE1 expression in APPswe/PS1dE9 (AD) mouse brain

After carrying out the behavioural study, we first investigated the effects of p75ECD-Fc on BACE1 expression in AD mouse. The Western blot data indicated that p75ECD-Fc recombinant protein inhibits BACE1 expression in AD mice brain ($p<0.05$) (Figure 4-4).

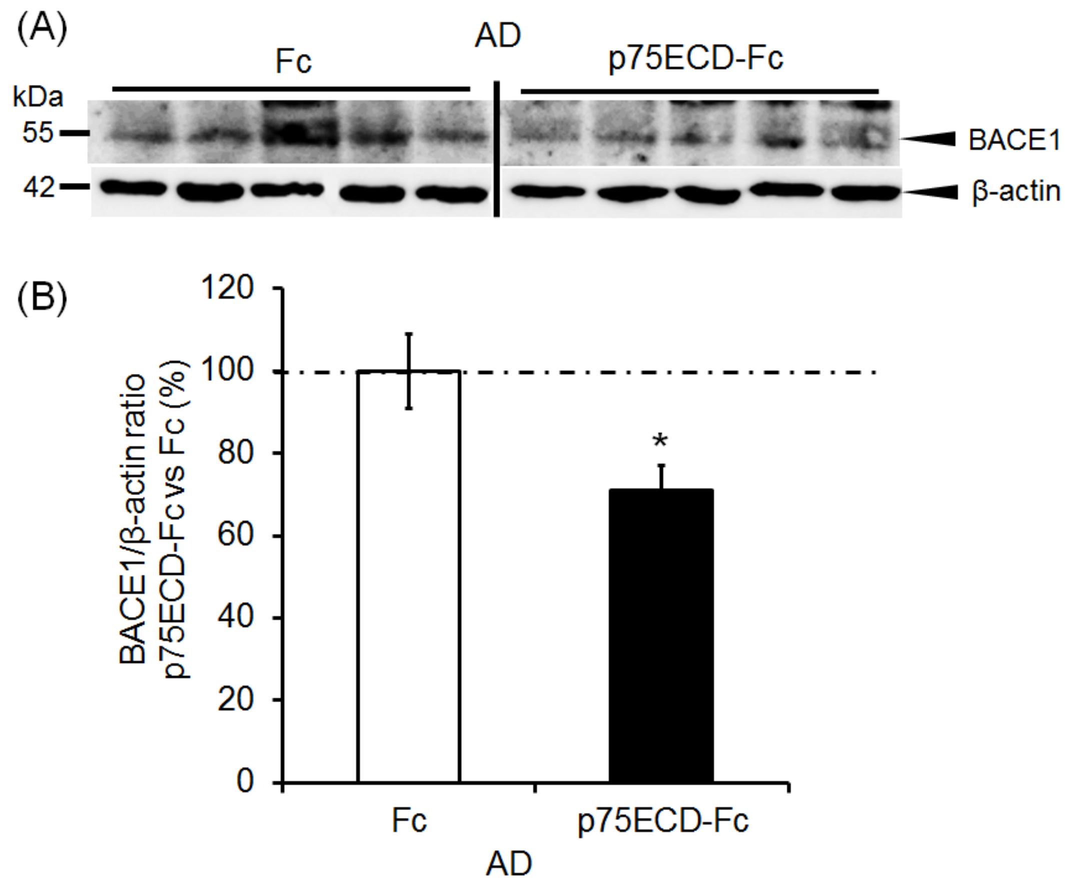
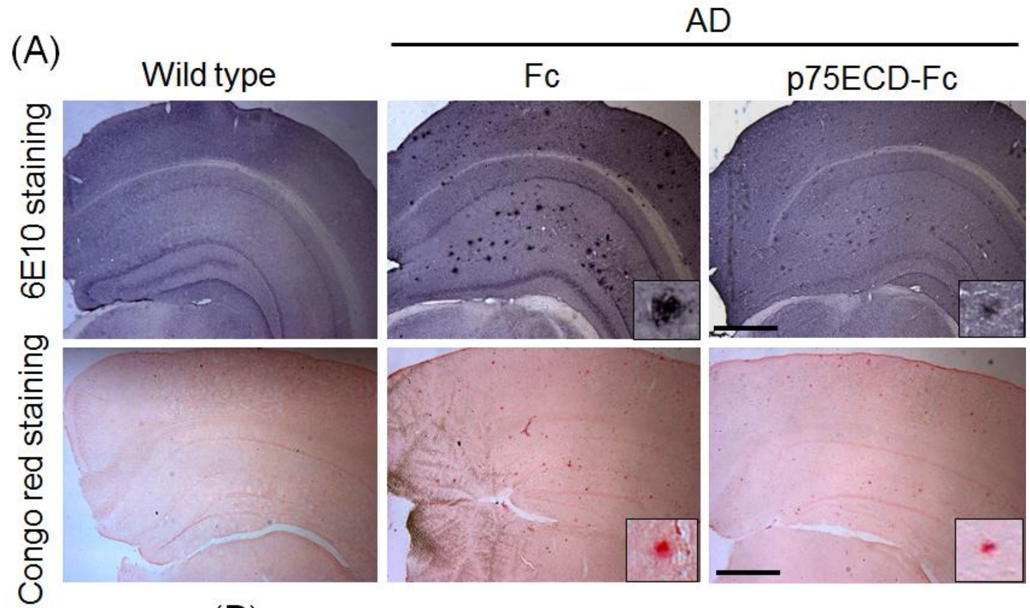


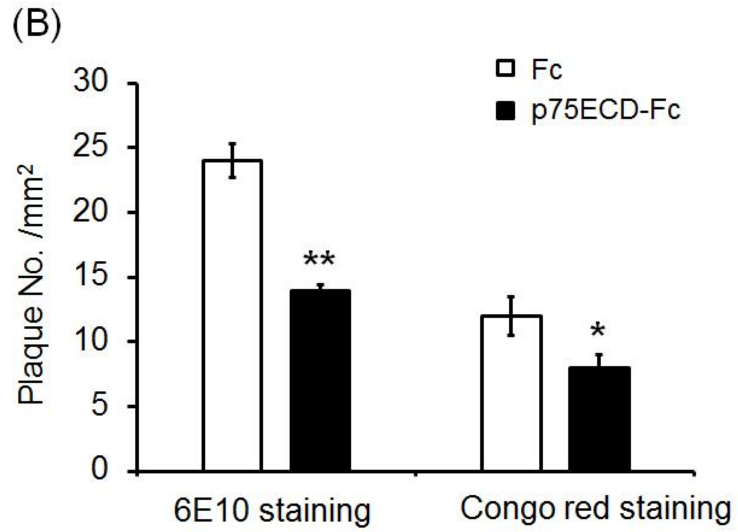
Figure 4-4: p75ECD-Fc inhibited BACE1 expression in AD mouse brain

A) Western blot analysis for BACE1 and β-actin in the brains of AD mouse in p75ECD-Fc and Fc groups B) The graph showing the statistical analysis of BACE1 protein level normalized to β-actin in p75ECD-Fc and Fc groups in AD mouse (*$p< 0.05$ vs Fc group) (n=10 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

p75ECD-Fc attenutes the plaque size and depossiton of Aβ in AD mouse brain

Following BACE1 expression analysis in AD mice, we investigated whether Aβ production was influenced by BACE1 downregulation caused by p75ECD-Fc. Both 6E10 ($p<0.01$) and Congo red ($p<0.05$) staining methods demonstrated that the number of Aβ was reduced by p75ECD-Fc in hippocampus (HC) and neo-cortex (NC) regions of mouse brain. Aβ staining by 6E10 also indicated that the size of Aβ plaque in mouse brain was significantly decreased by p75ECD-Fc recombinant protein ($p<0.01$). However, Congo red did not show a significant change for Aβ plaque size between AD-p75ECD-Fc and AD-Fc group (Figure 4-5).


(A)
AD
Wild type
Fc
p75ECD-Fc
6E10 staining
Congo red staining


(B)
Fc
p75ECD-Fc
Plaque No. /mm²
30
25
20
15
10
5
0
**
*
6E10 staining
Congo red staining

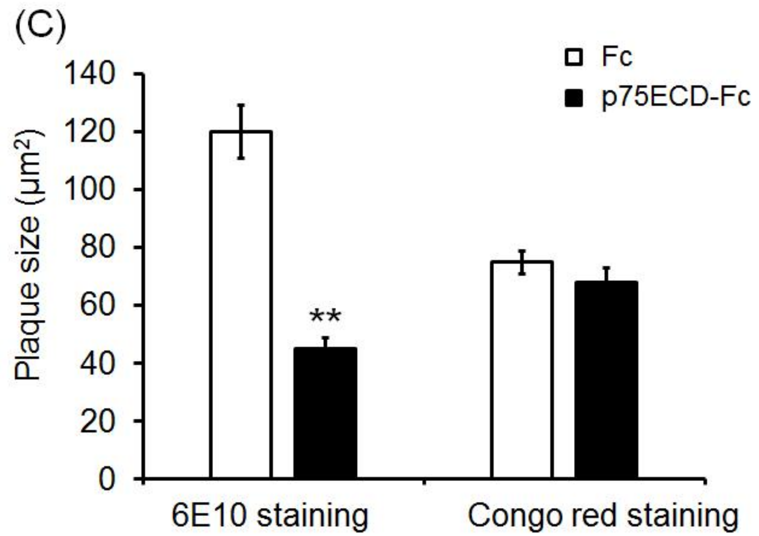

(C)
Fc
p75ECD-Fc
Plaque size (μm²)
140
120
100
80
60
40
20
0
**
6E10 staining
Congo red staining

Figure 4-5: p75ECD-Fc decreased Aβ plaques size and depossiton in APPswe/PS1dE9 (AD) mice brain

A) IHC using either primary antibody against Aβ (6E10) or Congo red staining to detect Aβ plaque in hippocampus (HC) and neo-cortex (NC) of wild type, AD-p75ECD-Fc and AD-Fc groups. The wild type group was used as a negative control. The graphs presenting the statistical analysis of the number of Aβ plaque per millimetre square (mm^2) (B) and the size of Aβ plaque based on square micrometre (μm^2) (C) in p75ECD-Fc group compared with Fc group in AD mice (*$p<0.05$, **$p<0.01$ vs Fc group) (n=10 per group, mean±SEM, Student's t-test).

p75ECD-Fc reduced astrogliosis in AD mouse brain

GFAP is expressed in astrocytes in the CNS and is considered to be a highly specific marker for glia. The expression of GFAP as a marker for astrogliosis in neurons is associated with AD pathology (Jacque, Vinner et al. 1978, Hol, Roelofs et al. 2003). Thus, in this study, we applied IHC to stain GFAP in AD mice brain in p75ECD-Fc and Fc groups. The results demonstrated that p75ECD-Fc decreased astrogliosis in AD brain ($p<0.01$) (Figure 4-6).

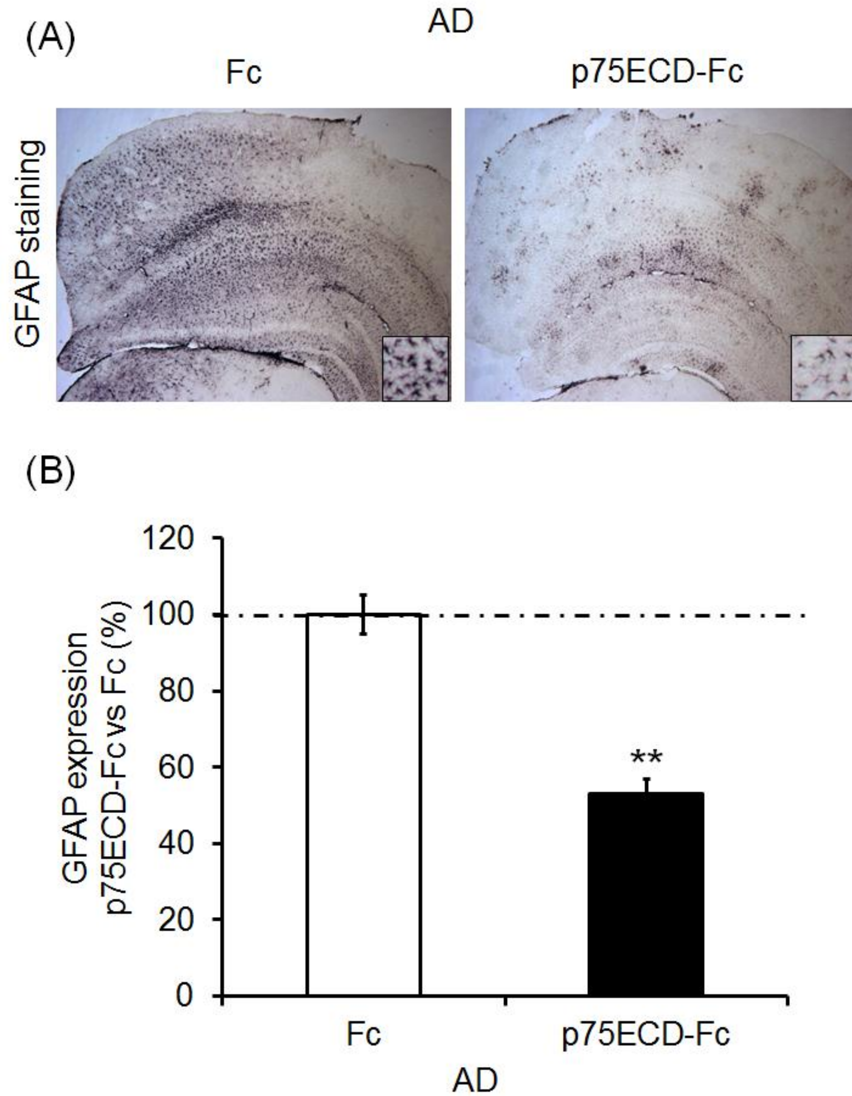


Figure 4-6: Effects of p75ECD-Fc on astrogliosis in AD mouse brain

A) IHC for GFAP staining in p75ECD-Fc and Fc groups in AD mouse brain, B) The graph presenting the statistical analysis for GFAP between the p75ECD-Fc and Fc groups (**p<0.01) (n=10 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

Effects of p75ECD-Fc on the levels of synaptic proteins in AD mouse brain

Next, we quantified the levels of synaptic proteins, including the VAMP2 and SNAP-25 in p75ECD-Fc and Fc groups in AD mouse by Western blot analysis. The data indicated that the levels of synaptic proteins, were unchanged in the brain of p75ECD-Fc in comparison with Fc group (Figure 4-7).

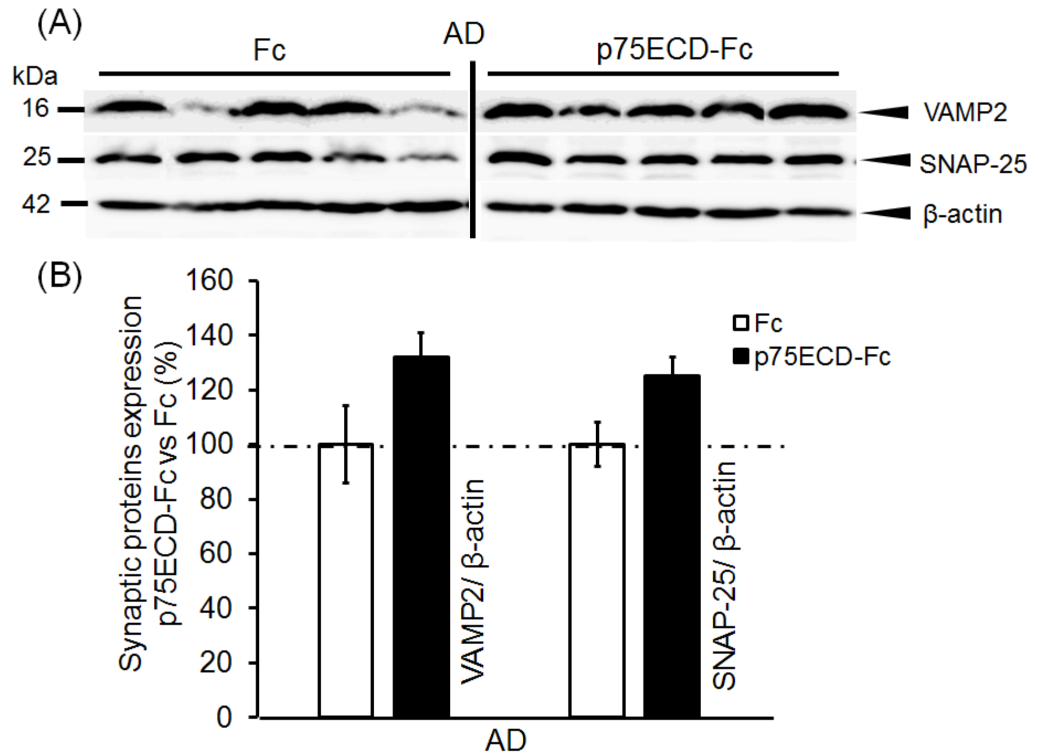


Figure 4-7: Effects of p75ECD-Fc on the levels of synaptic proteins in AD mouse brain

A) Western blot image for VAMP2 and SNAP-25 in p75ECD-Fc and Fc groups in AD mouse brains. B) The graph presenting the statistical analysis of VAMP2 and SNAP-25 levels in p75ECD-Fc and Fc groups in AD mouse (n=10 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

p75ECD-Fc reduced the expression of BACE1 in PR5 mouse brain

In order to evaluate the effect of p75ECD-Fc on AD hallmarks in PR5 mouse, the brain lysate from both groups, p75ECD-Fc and Fc, were subjected to Western blot and incubated with different primary antibodies for measuring AD biomarkers proteins. We first quantified the BACE1 and sAPPβ expression levels in PR5 mouse brain and the Western blot data demonstrated that p75ECD-Fc significantly reduced BACE1 expression ($p<0.05$) (Figure 4-8 A and B). However, the levels of sAPPβ was not changed in p75ECD-Fc group vs Fc in PR5 mouse (Figure 4-8 A and C).

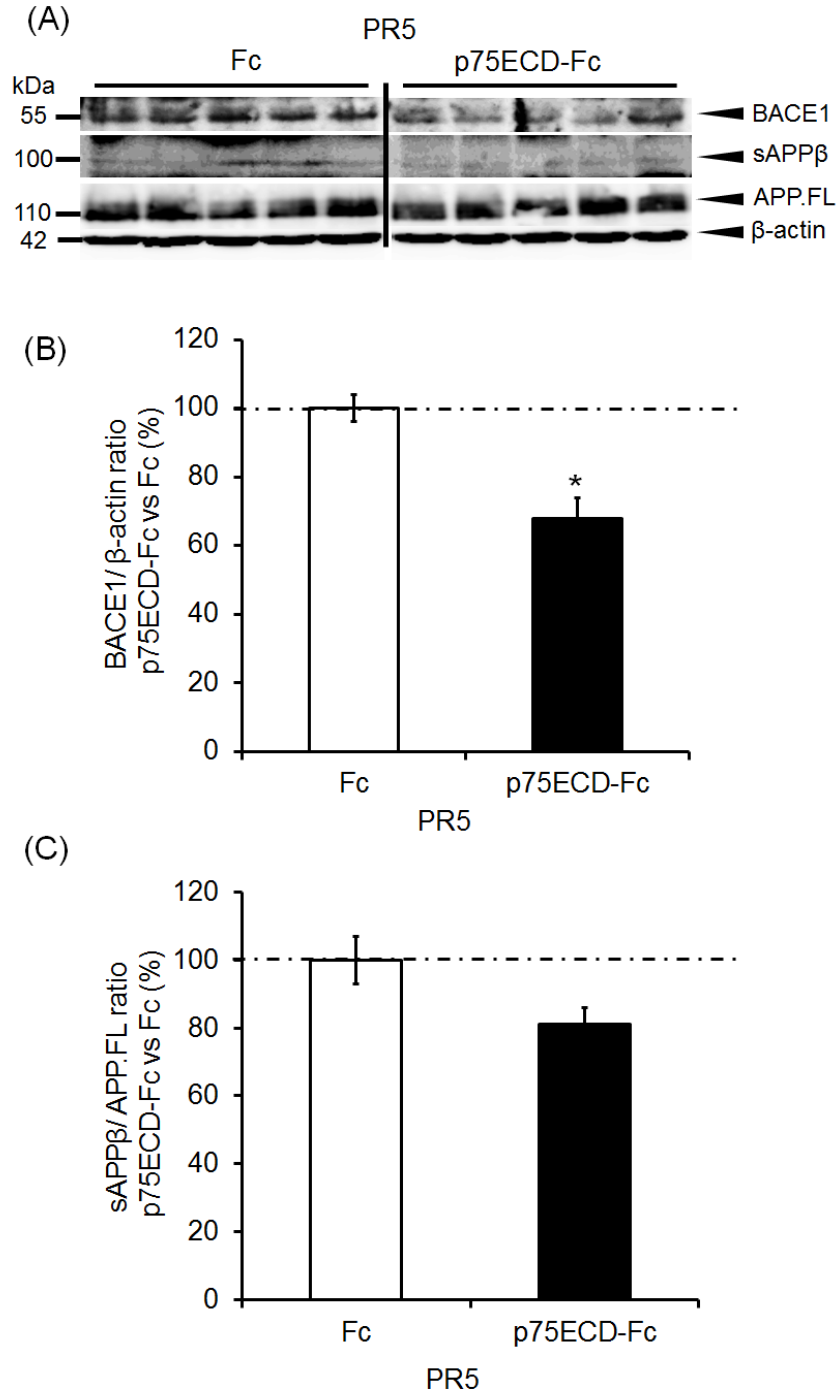


Figure 4-8: p75ECD-Fc reduced BACE1 expression in PR5 mouse brain

A) Western blot illustrating the expression of BACE1, sAPPβ, full length of APP (APP.FL) and β-actin in p75ECD-Fc and Fc groups in PR5 mouse brain. The graphs are presenting the expression ratio of BACE1/β-actin (B), sAPPβ/β-actin (C) in p75ECD-Fc and Fc groups in PR5 mouse (n=5 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

p75ECD-Fc attenuated Tau phosphorylation (p.Ser202 and Thr205) in PR5 mouse brain

Next, we tested whether p75ECD-Fc inhibits Tau phosphorylation in PR5 mouse. The brain lysate from PR5 mouse was sujected to Western blot analysis in order to detect Tau-phosphorylated protein using mouse phosphor-PHF-Tau pSer202+Thr205 monoclonal (AT8) antibody. Data showed that p75ECD-Fc significantly reduced Tau (Ser202 and Thr205) phosphorylation in PR5 mouse ($p<0.01$) (Figure 4-9).

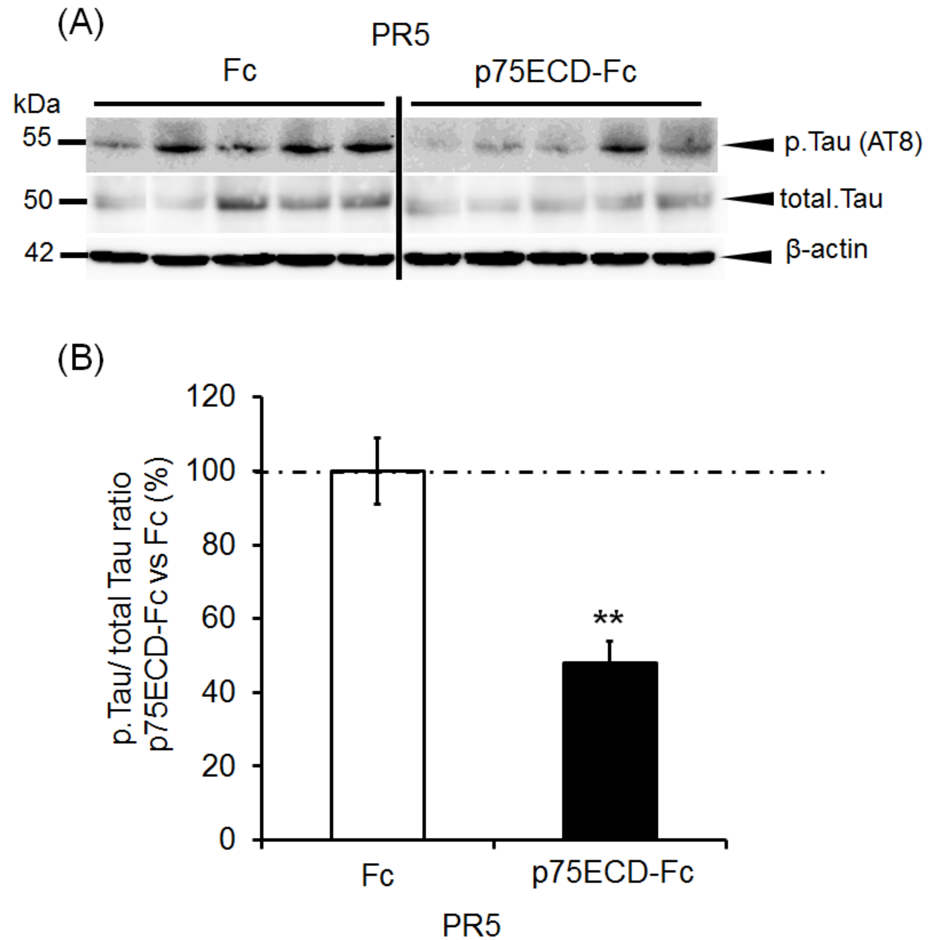


Figure 4-9: p75ECD-Fc inhibited the phosphorylation of Tau at Ser202 and Thr205 in PR5 mouse brain

A) Western blot image showing the phosphorylated Tau at Ser202 and Thr205 AA using mouse phospho-PHF-Tau pSer202+ Thr205 monoclonal (AT8) antibody, total Tau, and β-actin in p75ECD-Fc and Fc groups in PR5 mice brain. C) The statistical analysis for phosphorylated Tau at Ser202 and Thr205 AA in p75ECD-Fc and Fc groups in PR5 mouse brain (**$p<0.01$ vs Fc) (n=5 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

Effects of p75ECD-Fc on degeneration of cholinergic neurons in PR5 mouse brain

Loss of BFCN contributes to learning and memory deficits and is considered a hallmark of AD pathogenesis (Bartus, Dean et al. 1982). Thus, here we performed a Western blot assay to measure the levels of ChAT in PR5 mouse which received p75ECD-Fc and Fc recombinant proteins. The results indicated that the p75ECD-Fc did not significantly change ChAT levels in PR5 mouse (Figure 4-10).

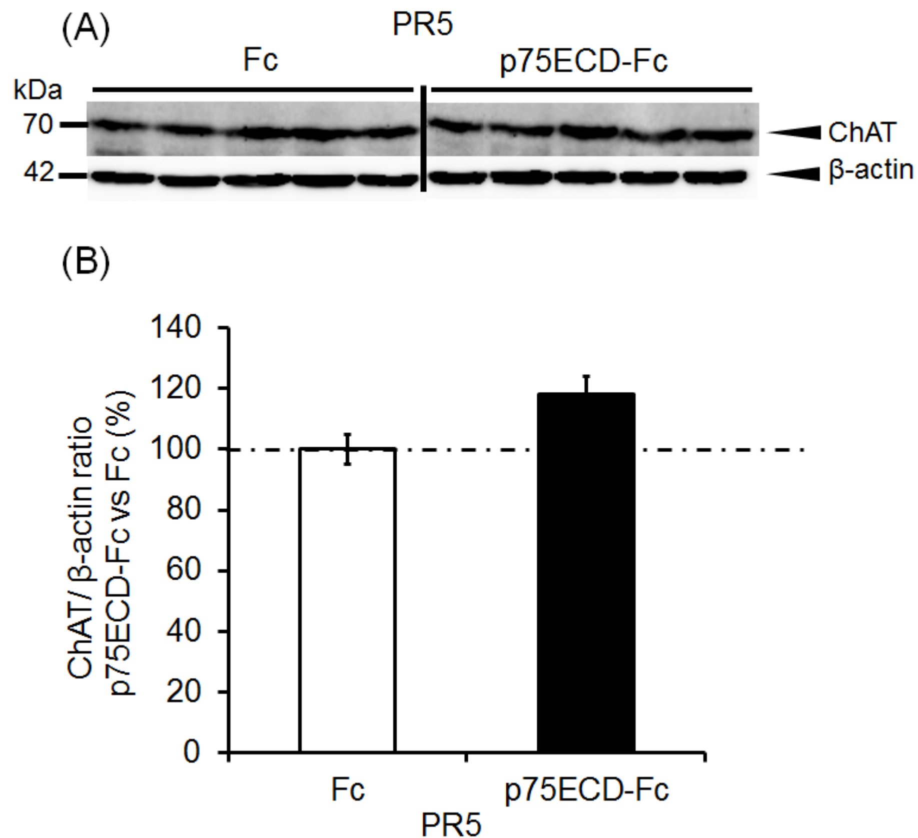


Figure 4-10: Effects of p75ECD-Fc on the levels of ChAT protein in PR5 mouse brain

A) Western blot image demonstrating ChAT and β-actin expression in p75ECD-Fc and Fc groups in PR5 mouse brain B) The graph presenting the statistical analysis of ChAT/β-actin ratio in p75ECD-Fc and Fc groups in PR5 mouse (n=5 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

Effects of p75ECD-Fc on the levels of synaptic proteins in PR5 mouse brain

The levels of synaptic proteins including VAMP2 and SNAP-25 in both p75ECD-Fc and Fc in PR5 groups were quantified by Western blot analysis. The results did not show any significant changes of VAMP2 and SNAP-25 between groups (Figure 4-11).

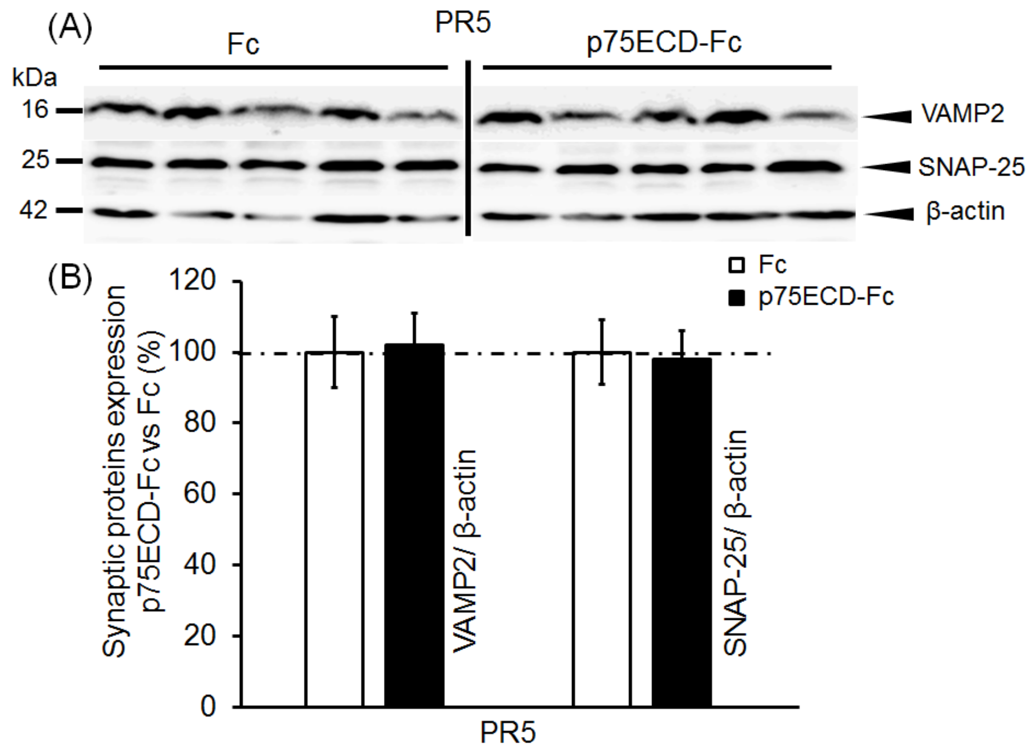


Figure 4-11: Effects of p75ECD-Fc on VAMP2 and SNAP-25 levels in PR5 mouse brain

A) Western blot to detect VAMP2 and SNAP-25 in p75ECD-Fc and Fc groups in PR5 mouse brains, B) The graph presenting the statistical analysis of VAMP2 and SNAP-25 in p75ECD-Fc and Fc groups in PR5 mouse brains (n=5 per group, mean±SEM, Student's t-test; "Fc" was normalized to 100%).

4.5 Discussion

In the current study, we have elucidated the effect of 3 months administration of p75ECD-Fc recombinant protein on cognitive function and AD biomarkers in two AD mouse models. We first elucidated the effect of p75ECD-Fc recombinant protein on learning and memory in AD mouse brain. Our data indicated that p75ECD-Fc did not change the escape latency time and travel distance within progressive platform learning trials in AD mouse model. In addition, the time spent in the target quadrant was not significantly changed in AD groups receiving p75ECD-Fc and Fc. The frequency of entries in the platform quadrants in the AD-Fc group is significantly lower than wild type, but there was not any changes in the p75ECD-Fc group compared with wild type. The frequency of crossing on the withdrawn platforn site in the probe trials was unchanged between AD-p75ECD-Fc and AD-Fc, but a significant reduction in AD-p75ECD-Fc and AD-Fc compared with wild type was detected. The data of this experiment demonstrated that the i.p injection of p75ECD-Fc did not improve spatial learning and memory in 10-month old AD mice. The MWM test for PR5 mice demonstrated that p75ECD-Fc significantly reduced escape latency time during 2nd, 4th, and 5th day of learning trials and travel distance during 2nd, 3rd, and 5th day of the learning trials in comparision with the Fc group. This is indicating that the learning process in wild type and p75ECD-Fc groups are improved. A significant reduction of escape latency and travel distance within day of 3rd, 4th, and 5th vs 1st are seen during learning process in wild type group. In p75ECD-Fc group escape latency and travel distance were decreased within day of 4th and 5th vs 1st during learning process whereas no significant improvement of learning was seen in Fc group during the learning process. p75ECD-Fc did not change the mean of time in the target quadrant or number

of entries in the platform quadrants in the probe trial session. The number of crossing the platform in the probe trial was unchanged between PR5 groups. To summerize our behavioral data, p75ECD-Fc recombinant protein did not change learning process and memory in AD mice. p75ECD-Fc was able to improve the learning process in PR5, but it did not alter memrory of the PR5 mouse in probe trial session. We do not exactly know why there is not any significant different of learning process between AD groups compared to wild type. We also do not know why there is not any significant changes of memory in PR5-p75ECD-Fc and PR5-Fc groups wherase our data revealed that p75ECD-Fc enhanced learning process. We assume these may be related to a technical issue in this experiment as I have not experienced MWM before and I was first exprimenter who installed MWM at University of South Australia therefore this may affect on my result. We also have not designed a sham control which would be helpful to rule out the effect of solvent and injection procedure on mouse cognitive fuction. In contrast to behavioral result, we found that p75ECD-Fc recombinant protein could reduce BACE1 expression and Aβ deposition in AD mouse. Moreover, p75ECD-Fc significantly decreased GFAP levels in AD mouse. Our result suggests that p75ECD-Fc decreased Aβ production through BACE1 inhibition and prevented astrogliosis in AD. The age of AD mice was 10 months which is considered an old age in mouse when we applied cognitive function assay and biochemical study. This may affect on the cognitive and biochemical results. The matter of age and over production of Aβ in AD mice may cause that p75ECD-Fc has less effects on cognitive funstion rather than AD biomarkers in AD mouse.

Our data presented that the levels of synaptic proteins including VAMP2 and SNAP-25 in AD mice were not significantly changed by p75ECD-Fc recombinant protein. We also did not find a significant effect of p75ECD on other AD biomarkers such as ChAT and the level of phosphorylated Tau (data were not shown). Our data indicated that p75ECD-Fc significantly inhibited BACE1 expression in PR5 mice, but it was not effective on APP processing. In addition, p75ECD-Fc recombinant protein was able to inhibit the phosphorylation of Tau at Ser202 and Thr205 in PR5 mouse brain. Western blot analysis did not show a significant change of ChAT level between p75ECD-Fc and Fc in PR5 mouse. p75ECD-Fc did not change the levels of VAMP2 and SNAP-25 in PR5 mouse. We did not quantify the level of AD biomarkers in AD and PR5 age matched wild type control therefore we do not know for example whether p75ECD-Fc could completely or partialy restore AD biomarkers in mouse brain. We also have tried to detect ChAT in brain slice to count the number of cholinergic neurons using several commercial antibodies and different techniques, but our antibodies did not work for IHC. We also were not successful in detecting phosphorylated Tau by IHC assay in brain slice.

APP and Aβ are generated by a variety of cells in peripheral tissues including platelets and lymphocytes, as well as by neurons in the CNS (Li, Fuller et al. 1999). In addition, regulation of Aβ catabolism (Iwata, Tsubuki et al. 2000), clearance (Ghersi-Egea, Gorevic et al. 1996, Shibata, Yamada et al. 2000), and aggregation (Wisniewski, Ghiso et al. 1997) contribute to the development of AD. There is a dynamic equilibrium between the CNS and peripheral Aβ levels. The interruption of this balance results in disruption of Aβ homeostasis in these environments and therefore influences Aβ deposition in the brain (Zlokovic 2004, Wang, Zhou et al. 2006). Given the relatively

large volume of distribution of the peripheral (circulation) compared with central compartments, the addition of anti-Aβ or other anti-peptide antibodies to block Aβ could serve to quickly and efficiently alter Aβ clearance and block its pathological activity in the brain (DeMattos, Bales et al. 2001, Sagare, Deane et al. 2007). Thus, a strategy to increase peripheral Aβ clearance and to reduce the Aβ concentration gradient between the brain and the periphery in order to transport Aβ from the brain to the periphery would help to inhibit development of AD by reducing Aβ deposition in the CNS. Aβ binds to the extracellular domain of $p75^{NTR}$ and induces cell death (Yaar, Zhai et al. 1997, Sotthibundhu, Sykes et al. 2008). The $p75^{NTR}$ antagonistic peptide (CATDIKGAEC) attenuated Aβ toxicity in brain (Yaar, Arble et al. 2008). Our studies demonstrated that $p75^{NTR}$ has a double faced function in AD pathogenesis. The full length of $p75^{NTR}$ promotes Aβ generation whereas p75ECD shedding fragment is able to block the biological activity of Aβ. For example, p75ECD-Fc recombinant protein is able to prevent Aβ aggregation and disaggregate preformed Aβ fibrils (Wang, Wang et al. 2011). p75ECD-Fc also attenuates Aβ-induced Sortilin upregulation in human neuroblastoma cells (Saadipour, Yang et al. 2013). So based on other people reports and our laboratory findings, we applied an *in vivo* study to elucidate whether i.p delivery of p75ECD-Fc recombinant protein for 3 months can restore cognitive function and AD biomarkers in APPswe/PS1dE (AD) and PR5 mice. There are some controversial reports due to adverse consequences of the effects of peripheral Aβ reduction on cognitive functions in AD. On one hand, direct targeting of Aβ in the brain is not the only way to reduce Aβ level and recently researchers tried to reduce peripheral Aβ levels in order to prevent AD pathogenesis (Bard, Cannon et al. 2000, Wilcock, Rojiani et al. 2004, Levites, Das et al. 2006). On the other hand, it was reported that peripheral reduction of

Aβ by Aβ degrading enzyme, neprilysin, does not attenuate AD pathogenesis (Walker, Pacoma et al. 2013, Henderson, Andersson et al. 2014). Thus, the lack of effect of p75ECD on cognitive function and some AD biomarkers in old AD mice also may be analogous to the failure of human clinical trial of Aβ antibodies (Lemere 2013) due to the over production of Aβ in the older brain and the resistance to the reversal of AD pathology in the transgenic strain.

In AD pathogenesis, APP is sequentially cleaved firstly by BACE1 followed by γ-secretase cleavage to generate Aβ peptides. It is known that the biological activity of BACE1 increases with aging in human, monkey and mice brain and thereby promotes APP processing and Aβ generation (Fukumoto, Rosene et al. 2004). In addition, the expression and activity of BACE1 are increased in association with an increased level of Aβ in different regions of sporadic and familial AD brain (Fukumoto, Cheung et al. 2002, Holsinger, McLean et al. 2002, Tyler, Dawbarn et al. 2002, Yang, Lindholm et al. 2003, Li, Lindholm et al. 2004). BACE1 has been an important therapeutic target for treatment of AD since it was discovered over 14 years ago (Hussain, Powell et al. 1999, Sinha, Anderson et al. 1999, Vassar, Bennett et al. 1999, Yan, Bienkowski et al. 1999, Lin, Koelsch et al. 2000). Many BACE1 inhibitors have been introduced in clinical trials (Ghosh and Osswald 2014). In our present study, we have discovered that p75ECD-Fc recombinant protein inhibited BACE1 expression in the brain and reduced Aβ production and sAPPβ in AD mice brain. Consistent with our finding, it was reported that a monoclonal antibody (m266) against Aβ reduced the Aβ concentration in plasma in AD transgenic mice therefore decreased Aβ deposition in the CNS (DeMattos, Bales et al. 2001). Moreover, it was shown that recombinant Low-density lipoprotein receptor-

related protein cluster IV (LRP-IV) can binds Aβ in the plasma of AD transgenic mice and reduced the Aβ accumulation in the brain (Sagare, Deane et al. 2007). These studies support the peripheral sink hypothesis in AD. Accumulating evidence indicate that Aβ stimulates BACE1 expression and enhances Aβ generation and subsequently promotes a vicious cycle in sporadic AD brain (Buggia-Prevot, Sevalle et al. 2008, Guglielmotto, Monteleone et al. 2011, Chami, Buggia-Prevot et al. 2012). Our previous study revealed that p75ECD-Fc inhibited Aβ-induced Sortilin over expression in SH-SY5Y cells. In addition, our *in vitro* results (Chapter 3) indicate that p75ECD-Fc rescued Aβ and proNTs- neurite outgrowth impairment. Thus, our data suggest that p75ECD-Fc most likely inhibits BACE1 expression through inhibition and clearance of peripheral Aβ and reduction of Aβ deposition in the CNS. Aβ induces Tau hyper-phosphorylation in rat hippocampal (Ma, Yang et al. 2009) and septal cholinergic neurons and therefore promotes neuronal death and the development of AD through disrupting microtubule structure and the neuronal cytoskeleton (Zheng, Bastianetto et al. 2002). We discovered that p75ECD-Fc recombinant protein could significantly prevent Tau phosphorylation in PR5 mice brain. Cholinergic neurons are severely degenerated during the late stage of AD (Whitehouse, Price et al. 1981, Bartus, Dean et al. 1982, Allen, Dawbarn et al. 1988, Vogels, Broere et al. 1990, DeKosky, Harbaugh et al. 1992). SNAP-25 binds to VAMP2 in neurotransmitter-containing vesicles (Bhalla, Chicka et al. 2006) and it is reported that the level of SNAP-25 is down regulated in patients with DS and AD (Greber, Lubec et al. 1999). Our data showed that p75ECD was not significantly effective on ChAT, VAMP2 and SNAP-25 in AD and PR5 mice.

Brain inflammation including reactive microgliosis and astrogliosis are secondary hallmarks of AD (Eikelenboom, Veerhuis et al. 2008). Our results indicate that the fractional area of GFAP (astrogliosis) staining in the cortex and hippocampus of AD mice was significantly reduced in p75ECD-Fc group in comparison with Fc group. Consistent with our finding, Aβ-binding compounds in the periphery increase Aβ clearance and subsequently reduces the level of Aβ in the brain (DeMattos, Bales et al. 2001, Sagare, Deane et al. 2007). p75ECD-Fc can bind to Aβ and attenuates Aβ neurotoxicity (Wang, Wang et al. 2011, Saadipour, Yang et al. 2013). In addition, p75ECD can block neurite collapse mediated by proNGF and proBDNF (Wang, Valadares et al. 2010, Sun, Lim et al. 2012). According to a complex structure of p75ECD-Fc, it should not be able to pass through the BBB, therefore we assume that p75ECD-Fc binds peripheral Aβ and reduces Aβ deposition in the brain and restores AD biomarkers; however, we did not quantify the level of p75ECD-Fc in the mouse brain and plasma after i.p injection therefore we are quite sure whether the positive effects of p75ECD-Fc on some AD biomarkers and cognitive behavioural in AD and PR5 mice are due to peripheral or central effect of p75ECD-Fc on Aβ clearance.

Taken together, our study suggests that p75ECD recombinant protein may be a desirable anti-AD agent to facilitate peripheral Aβ clearance and increases a concentration gradient in concentration of Aβ towards the periphery and subsequently reduces Aβ and proNTs- induced neurotoxicity in the CNS (Figure 4-12).

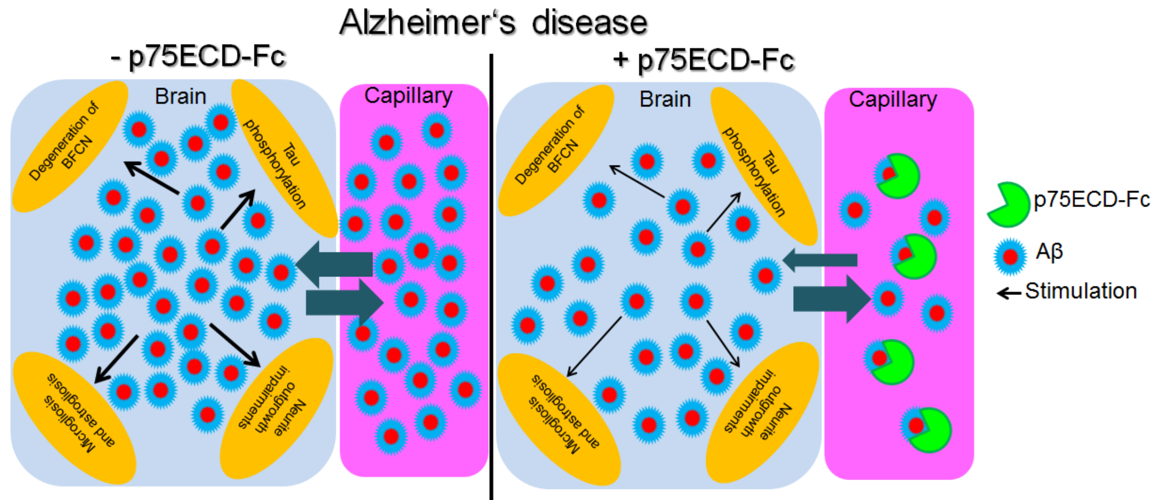


Figure 4-12: Graphical summary presenting the effects of p75ECD-Fc recombinant protein on deposition of Aβ in the brain

There is a dynamic homeostasis of Aβ concentration between the CNS and periphery. The interruption of this balance influences on Aβ deposition in the brain. Here, peripheral administration of p75ECD-Fc protein binds Aβ in circulation, increases Aβ clearance and the gradient of Aβ concentration toward circulation, reduces Aβ deposition in the brain and consequently attenuates Aβ-induced neurotoxicity and AD pathogenesis.

CHAPTER 5: AMYLOID BETA$_{1-42}$ (AB$_{42}$) UP-REGULATES THE EXPRESSION OF SORTILIN VIA THE P75^{NTR}/RHOA SIGNALLING PATHWAY

5.1 Abstract

Background: Sortilin, a Golgi sorting protein and a member of the Vps10p family, is the co-receptor for proneurotrophins, regulates protein trafficking, targets proteins to lysosomes and regulates low density lipoprotein metabolism. The aim of this study was to investigate the expression and regulation of Sortilin in AD. **Methods:** In this study, we used SH-SY5Y cells to investigate effects of Aβ in dose- and time- dependent manner on the expression Sortilin gene and protein which were measured by real time PCR and Western blot respectively. In addition we quantified Sortilin expression in 6-month old APPswe/PS1dE9 (AD) transgenic and age-matched wild type (c57bl) mice brain. **Results:** A significantly increased level of Sortilin was found in human AD brain and in brains of 6-month old APPswe/PS1dE9 transgenic mice. Aβ_{42} enhanced the protein and mRNA expression level of Sortilin in a dose and time-dependent manner in SH-SY5Y cells but had no effect on sorLA. In addition, proBDNF also significantly increased the protein and mRNA expression of Sortilin in these cells. p75ECD-Fc recombinant protein or the antibody against the p75ECD blocked the upregulation of Sortilin induced by Aβ, suggesting that Aβ_{42} increased the expression level of Sortilin protein and mRNA in SH-SY5Y via $p75^{NTR}$. Inhibition of ROCK, but not JNK, suppressed constitutive and Aβ_{42}-induced expression of Sortilin. **Conclusion:** In conclusion, this study shows that Sortilin expression is increased in the AD brain in human and mice and that Aβ_{42} oligomer increases Sortilin gene and protein expression through $p75^{NTR}$ and RhoA signalling pathways, suggesting a potential physiological interaction of Aβ_{42} and Sortilin in AD.

Keys words: Sortilin, Aβ_{42}, $p75^{NTR}$, RhoA signalling pathway and Alzheimer's disease

5.2 Introduction

AD is the most common cause of dementia which leads to a marked impairment of cognition and learning and contributes to a reduced lifespan (Katzman 1986, Selkoe 2002, Blennow, de Leon et al. 2006). AD is pathologically characterized by the progressive accumulation of amyloid plaques in the brain and blood vessels and neurofibrillary tangles in neurons (Glenner and Wong 1984). Aβ peptide is generated by sequential cleavage of APP by β- and γ-secretase in sub-cellular compartments. The TGN and endosomes have been identified as major sites for β -secretase activity on APP processing (Skovronsky, Moore et al. 2000). Aβ interacts with and activates several membrane receptors, including TrkA and $p75^{NTR}$ (Bulbarelli, Lonati et al. 2009), APP itself (Verdier, Zarandi et al. 2004), RAGE (Yan, Bierhaus et al. 2009), insulin receptor (Townsend, Mehta et al. 2007), acetylcholine receptors (Parri and Dineley 2010), contributing to the pathogenesis of AD potentially through MAPK, NF-κB, JNK and other pathways. Aβ up-regulates the expression of the $p75^{NTR}$ through IGF-1R phosphorylation in SH-SY5Y human neuroblastoma cells and AD transgenic mice (Chakravarthy, Gaudet et al. 2010, Chakravarthy, Menard et al. 2012, Ito, Menard et al. 2012). In our previous studies, we also observed that the expression of $p75^{NTR}$ in the brain of APPswe transgenic mice is increased, illustrating Aβ as a crucial factor in the regulation of $p75^{NTR}$ over expression in AD (Wang, Wang et al. 2011). It has been reported that Aβ up-regulates the expression of BACE1 through JNK pathway (Guglielmotto, Monteleone et al. 2011). Sortilin is a Golgi sorting protein and a member of the family of Vps10p domain receptors and the co-receptor for proneurotrophins in different cells (Nykjaer, Lee et al. 2004, Jansen, Giehl et al. 2007, Nakamura, Namekata et al. 2007). Sortilin plays an important role in the regulation of neuronal viability and

function (Petersen, Nielsen et al. 1997, Nykjaer and Willnow 2012) and is expressed in neurons of the CNS and the PNS, and also in non-neural tissues like liver and fat (Petersen, Nielsen et al. 1997, Morris, Ross et al. 1998, Sarret, Krzywkowski et al. 2003, Kjolby, Andersen et al. 2010). As a process of aging, proNGF and $p75^{NTR}$ are up-regulated and activate the Sortilin receptor to promote neuronal cell death (Nykjaer, Lee et al. 2004, Jansen, Giehl et al. 2007). Sortilin interacts with BACE1 and regulates the trafficking of BACE1and results in overproduction of Aβ (Finan, Okada et al. 2011). Sortilin also directly interacts with APP (Gustafsen, Glerup et al. 2013) and negatively regulates APP lipid raft targeting (Yang, Virassamy et al. 2013). Furthermore, Sortilin interacts with $p75^{NTR}$ to induce neuronal apoptosis and increase AD severity (Skeldal, Sykes et al. 2012). However, the role of Sortilin in the pathogenesis of AD remains unclear and controversial, thus this study has sort to understand its role in AD development.

5.3 Materials and methods

Cell culture and treatment

SH-SY5Y and SH-SY5Y^{APP695} human neuroblastoma cells were provided by Prof. Richard Lewis (Institute of Molecular Biosciences, University of Queensland, Australia) and Prof. Nigel Hooper (Institute of Molecular & Cellular Biology, University of Leeds). Cells were grown in DMEM (Invitrogen, Mulgrave, VIC, Australia) supplemented with 10% FBS and 2 mM L-glutamine and 1% penicillin/streptomycin and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2. After overnight seeding of SH-SY5Y cells in 6-well plates (10^6/well) (Invitrogen, Mulgrave, VIC, Australia), the SH-SY5Y cells were treated with varying doses (0.1, 0.25, 0.5, 1, 2.5, 5, 10 μM) of oligomer form of Aβ_{42} and scrambled Aβ_{42} over a time-course (3, 6, 12, 24 hours). In order to see whether Sortilin upregulation occurs through the p75^{NTR} receptor, SH-SY5Y cells were incubated with 10 μg/ml human p75ECD-FC recombinant peptide (Wang, Wang et al. 2011) and 10 μg/ml rabbit polyclonal anti-human p75^{NTR}-ECD antibody (generous gift from Prof. Moses V. Chao, Department of Cell Biology, Skirball Institute, New York, US) in conjunction with 1 μM Aβ_{42} for 24 hours. Human and rabbit IgG (Sigma-Aldrich, St Louis, MO, US) were used as negative control. In order to quantify the level of Sortilin expression in presence of mature and premature form of neurotrophoin, SH-SY5Y cells were incubated with 50 ng/ml of proBDNF (Virovek, US) a mutant recombinant protein which is resistant against proBDNF processing enzymes (Fan, Wu et al. 2008) and BDNF (Amgen, USA) for 24 hours. To investigate which signalling pathway plays a role in upregulation of Sortilin by Aβ_{42}, ROCK inhibitor (5, 10 μM; Y27632); (Sigma-Aldrich, St Louis, MO, US), and

JNK inhibitor (2 μM; SP600125); (Sigma-Aldrich, St Louis, MO, US) (Guglielmotto, Monteleone et al. 2011) were used to block the RhoA and JNK signalling pathways respectively.

Preparation of oligomer form of Aβ_{42}

Aβ_{42} oligomer was prepared according to the instruction which is described in Chapter 2.

Transgenic AD mice

In this study, the brain of 6-month old APPswe/PS1dE9 (AD) transgenic mice as described in Chapter 2, were extracted and subjected to Western blot in order to detect Sortilin expression.

The brain tissue of human

Human brain tissue was obtained from the South Australia Brain Bank at Flinders University. Post-mortem brain tissue from four normal female subjects (mean age 85.6 years) and four female patients (mean age 81 years) neuropathologically diagnosed with AD was snap-frozen and stored at -80 °C. Temporal cortical samples were homogenized in RIPA buffer containing 2 mM PMSF, antipain, pepstatin, and leupeptin. Homogenate was centrifuged at 2500 g for 10 min at 4 °C. The protein concentration of the lysates was determined using BCA protein assay Kit (Thermo Scientific, Rockford, US). Supernatant containing 30 μg of protein was subjected to SDS-PAGE for Western blot analysis. All procedures involving human subjects/patients were approved by the Flinders Clinical Research Ethics Committee.

Protein extraction from cell line and the brain tissue of mice

Total protein from cell lines and brain tissue of mice brain was obtained to subject to western blotting and real-time PCR. Briefly, culture medium was removed and cells were gently washed twice with chilled PBS and lysed by suspending in RIPA buffer containing 2 mM PMSF, antipain, pepstatin, and leupeptin. 6-month-old APPSwe/PS1dE9 and control mice were killed by overdosing with pentobarbital and the cortex of brain was extracted and homogenised in RIPA buffer containing 2 mM PMSF, antipain, pepstatin, and leupeptin. The cells and brain lysates were sonicated and then centrifuged at 16,000 g for 10 min at 4°C and total protein concentration of supernatants were determined using BCA protein assay Kit (Thermo Scientific, Rockford, US). All lysates were stored at -80°C until use.

Western blot analysis

Protein extracts (30 µg) from cell lines and the brain of mice and human were separated by 4–12% precast SDS-PAGE gels (Bio-Rad, US) which were run at 80-120 V using Tris-glycine running buffer and transferred to 0.2 mm pore size nitrocellulose membrane (GE Healthcare, Uppsala, CA, US). Membrane was then blocked for 1 hour at room temperature (25°C) in PBS containing 5% skim milk and incubated with rabbit anti-Sortilin (Osenses, Adelaide, SA, Australia), rabbit polyclonal anti-Sortilin (Abcam, Cambridge, MA, US), sheep anti-GAPDH (Osenses, Adelaide, SA, Australia) and mouse monoclonal anti β-actin (Sigma-Aldrich, St Louis, MO, US) as internal controls, mouse monoclonal anti-Aβ_{42} (6E10) (Covance, Dedham, MA, US), mouse monoclonal anti-RhoA-GTP (New-East Biosciences, Malvern, Pennsylvania, US) and rabbit polyclonal anti- total Rho (New-East Biosciences, Malvern, Pennsylvania, US) in 2%

skim milk in PBS for overnight, respectively, followed by incubation with the HRP-linked secondary antibodies in 1:3000 in 2% skim milk in PBST (Sigma-Aldrich, St Louis, MO, US) for 1 hour at room temperature (25°C). Membranes were developed by Image Quant LAS 4000 (GE Healthcare Bio Science AB, Uppsala, CA, US) and Image J software (Research Service Branch; National Institute of Health, http://rsbweb.nih.gov/ij/index.html) was used for quantitative analysis.

Real-time quantitative RT-PCR

SH-SY5Y and SH-SY5Y^{APP695} were seeded in 6-well plates (10^6/well) (Invitrogen, Mulgrave, VIC, Australia) and after treatment, culture medium was removed and cells were gently washed twice with PBS and cells lysed in lysis buffer (QIAGEN, Doncaster, VIC, Australia). Total RNA (400-600 ng/well) was extracted using an optimized RNA extraction protocol based on the RNeasy mini Kit isolation system (QIAGEN, Doncaster, VIC, Australia) according to the manufacturer's protocol and immediately afterward, cDNA was synthesized using iScripTM cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. As a negative control, a cDNA synthesis reaction was also carried out without the addition of reverse transcriptase. All PCR primers (Table 5-1) were obtained from GeneWorks (Australia) and were designed using human DNA sequences and NCBI PRIMER BLAST software (Bethesda, MD, US), whereby forward and reverse primers were designed in different exons, 200-300 bp in length, CG content 40-50%, and Tm 2 at 55–60. For quantitative RT-PCR of genes of interest, a reaction volume of 10 ml was found adequate using iTaq™ Universal SYBR® Green **s**upermix (Bio-rad). For each cDNA sample, quantitative PCR assays were run on a CFX Connect Real-Time PCR System (Bio-Rad) in duplicate. From the

amplification curves, relative expression was calculated using the comparative Ct ($2^{-\Delta Ct}$) method, with GAPDH serving as the endogenous control.

Table 5-1: Real-time PCR primer pair sequences used in this study

Gene	**Forward primer**	**Reverse primer**
Sortilin	5’- ccgtcctatcaatgtgattaag- 3’	5’- ccatatggtatagtccttctc -3’
SorLA	5’- aataccacatcattgtccaactg-3’	5’- tgtaattatgacccatcttcag-3’
GAPDH	5’- acaactttggtatcgtggaagg-3’	5’- catatttggcaggtttttctag -3’

Cell viability assay

The vitality of the SH-SY5Y cell line treated with $A\beta_{42}$ oligomer of was assessed by Methyl Thiazoly Blue Tetrazolium Bromide (MTT) Assay Kit (Sigma, US) following the manufacturer’s instructions. Briefly, the SH-SY5Y cells were cultured at a density $2x10^4$ cells per well in growth medium for 12 hour in 96-well plates, and then exposed to different concentrations of $A\beta_{42}$ oligomer (0.1, 0.25, 0.5, 1, 2.5, 5, 10 μM) for 24h. 10 μl of MTT solution (5mg/ml) was added to each well and incubated at 37°C for 4h. Absorbance was recorded at 490 nm with a micro plate reader WALLAC 1420 (PerkinElmer, Waltham, Massachusette, US). In all cases, test substances were added containing medium alone to determine whether they interfered with the assay. The data is expressed as mean percent viable cells compared to respective control cultures.

Statistical analysis

All statistical analyses were performed using SPSS 22 software package (IBM, provided by Flinders University). Variables between groups were determined by either one-way ANOVA following Tukey's post-hoc test or Student's t-test and values of $p<0.05$ were considered statistically significant. Data is presented as mean±SEM.

5.4 Results

Sortilin expression is up-regulated in brain of human AD and APPswe/PS1dE9 (AD) mice

As Sortilin interacts with APP and BACE1, it is likely that Sortilin plays an important role in AD. To examine functions of Sortilin in AD, we first investigated whether the level of Sortilin expression in AD brain and in the brain of APPswe/PS1dE9 (AD) transgenic mice is altered. A pilot study of n=4 AD brain and n=4 controls was performed and illustrate that the expression of Sortilin in human post mortem AD brain cortex was significantly increased compared to control brain ($p<0.05$) (Figure 5-1 A and B). We also examined the level of Sortilin in the brain of 6-month old AD transgenic mice. These results show that Sortilin protein in the brain tissue of AD mice was increased compared with WT (C57BL/6j) ($p<0.01$) (Figure 5-1 C and D).

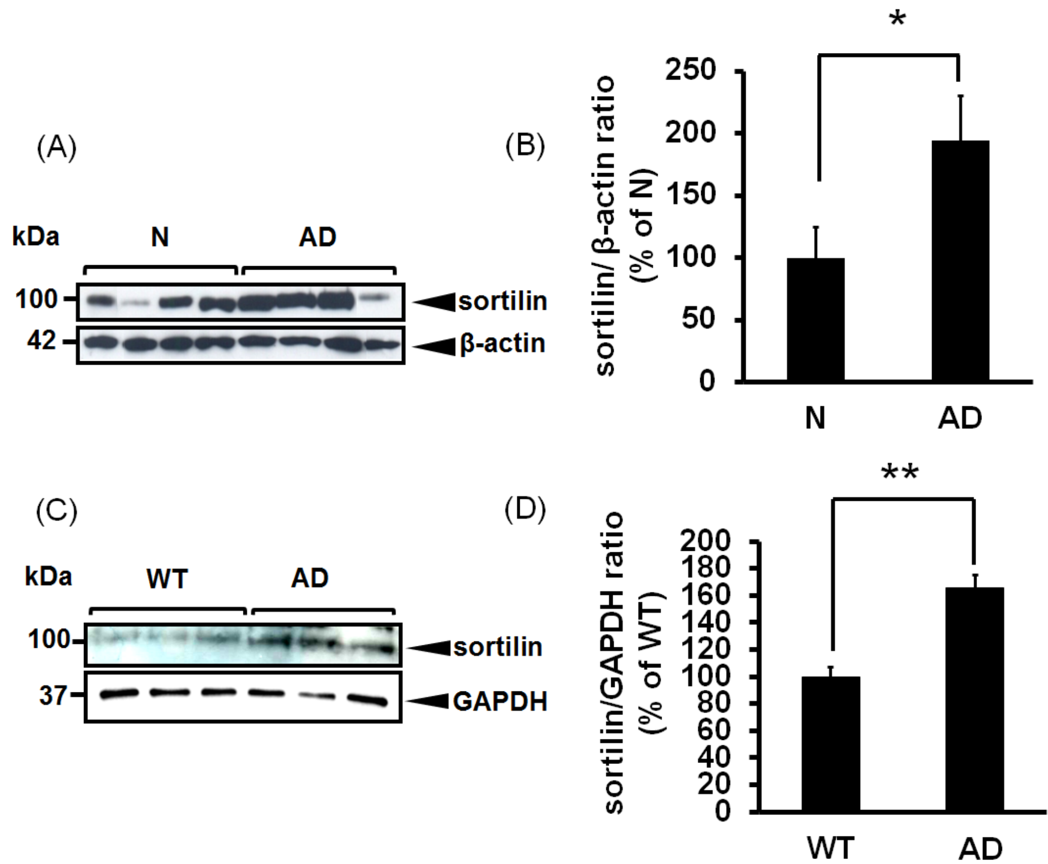


Figure 5-1: Sortilin protein expression is increased in brains from human AD patients and APPswe/PS1dE9 (AD) transgenic mice

A and B) Sortilin protein was found to be higher in AD brain cortex lysate relative to β-actin when compared to normal (*$p<0.05$) (n=4 for each group, Mean±SE; Student's t-test; "N" was normalized to 100%). C and D) A remarkable increase in Sortilin relative to GAPDH in the brain of 6-month old AD transgenic mice compared with WT (**$p<0.01$) (n=9 for each group, mean±SEM, Student's t-test; "WT" was normalized to 100%).

The expression level of Sortilin in SH-SY5Y^{APP695} is higher than in SH-SY5Y

To further examine Sortilin expression in AD models, we tested whether SH-SY5Y human neuroblastoma cells express Sortilin and that they are a suitable model to investigate the effects of Aβ_{42} on Sortilin expression in an *in vitro* study. 30 µg/well of total SH-SY5Y cell protein of was used on a Western blot and Sortilin protein was detected by rabbit anti-Sortilin antibody (Figure 5-2 A). Recent studies have highlighted the potential importance of soluble oligomeric forms of Aβ_{42} in AD (Kim, Chae et al. 2003, Walsh and Selkoe 2007, Glabe 2008, Reddy, Manczak et al. 2010). Since Aβ_{42} is more likely to form oligomers than Aβ_{40}, and is more critical in AD (Jarrett, Berger et al. 1993, Iwatsubo, Odaka et al. 1994), we used oligomer form of Aβ_{42} (Figure 2-2) in this study for any further experiments. To determine whether there is any difference in the expression level of Sortilin mRNA and protein in SH-SY5Y and SH-SY5Y^{APP695}, we applied quantitative real-time PCR and Western blot to analyse respectively mRNA and protein level of Sortilin in normal and transgenic cell line of human neuroblastoma cells. We found a significant increase in mRNA and protein levels of Sortilin in SH-SY5Y^{APP695} compared with SH-SY5Y ($p<0.05$, $p<0.01$) (Figure 5-2 C-E).

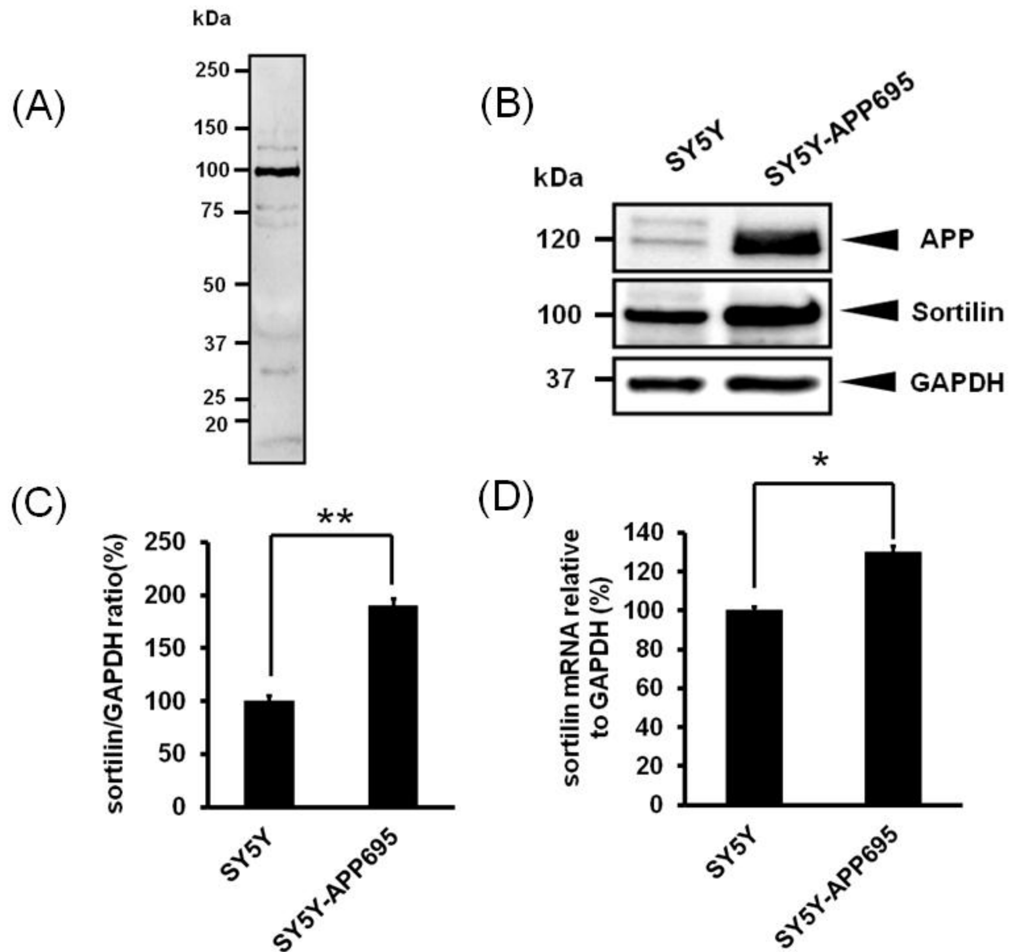


Figure 5-2: Sortilin protein and mRNA expression in SH-SY5Y cell line

A) The endogenous expression of Sortilin. Thirty micro gram of total protein of SH-SY5Y cell lysate was analysed by Western blot and probed with rabbit anti-Sortilin (Osenses, Adelaide, SA, Australia). B-D) Significant increase in protein and mRNA level of Sortilin relative to GAPDH in SH-SY5Y^{APP695} compared with SH-SY5Y respectively (*$p<0.05$, **$p<0.01$) (Each bar represents as mean±SEM of 4 individual experiments, Student's t-test; "SY5Y" was normalized to 100%)

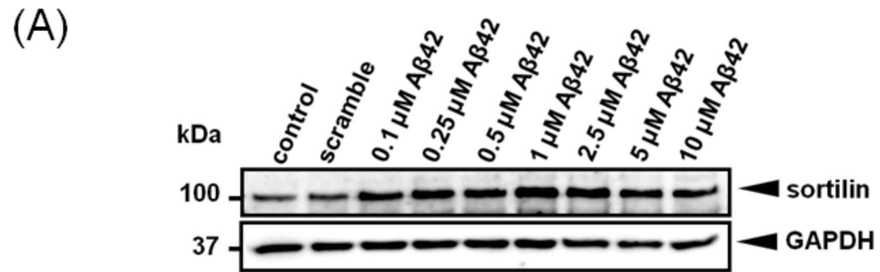
A dose-dependent effect of Aβ_{42} oligomers on Sortilin expression in SH-SY5Y cells

Based on the data above, we hypothesized that Aβ_{42} may regulate the expression of Sortilin. To test the hypothesis, we treated SH-SY5Y cells with different concentrations of Aβ_{42} oligomers for 24 hours and examined the gene and protein expression of Sortilin. Aβ_{42} oligomer up-regulated the expression of Sortilin in SH-SY5Y human neuroblastoma in a dose-dependent manner($p<0.05$, $p<0.01$) (Figure 5-3 A and B) up to 1 μM. Quantitative real time PCR showed that Aβ_{42} oligomer enhanced the mRNA level of Sortilin ($p<0.05$) (Figure 5-3 C).

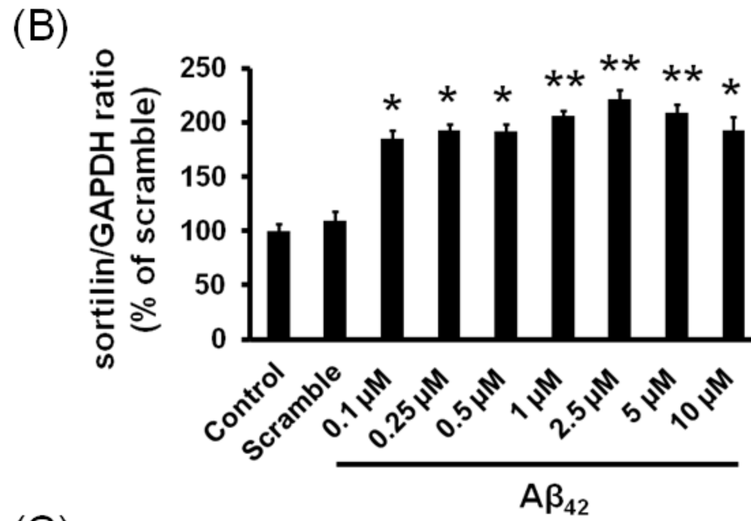
MTT assay was conducted to check cell viability in the presence of different concentrations of Aβ_{42}, and observed a dose-dependent toxicity of Aβ_{42} on cell viability in SH-SY5Y cells ($p<0.05$) (Figure 5-3 D). Based on this finding, 1μM of Aβ_{42} was found to be optimal and was used in the following experiments described herein.

As Aβ mediates neurotoxicity via the $p75^{NTR}$ receptor (Coulson, May et al. 2009), it is likely that other $p75^{NTR}$ ligands also regulate Sortilin expression. To understand whether neurotrophins and proneurotrophins alter the protein and gene expression of Sortilin, SH-SY5Y cells were incubated with 50ng/ml of proBDNF and BDNF for 24 hours for western blot and quantitative real-time PCR (Figure 5-3 E-G). The results showed that proBDNF but not mature BDNF significantly elevated the levels of both protein (Figure 5-3 E-F) and mRNA (Figure 5-3 G) of Sortilin.

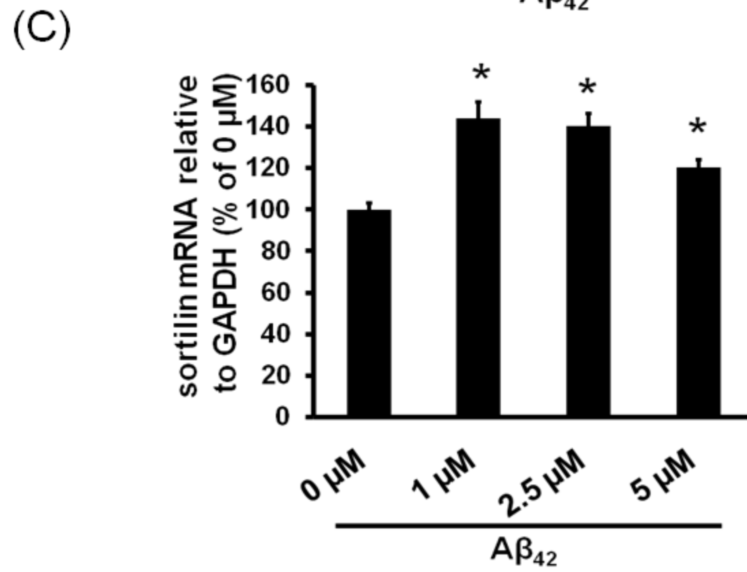
(A)

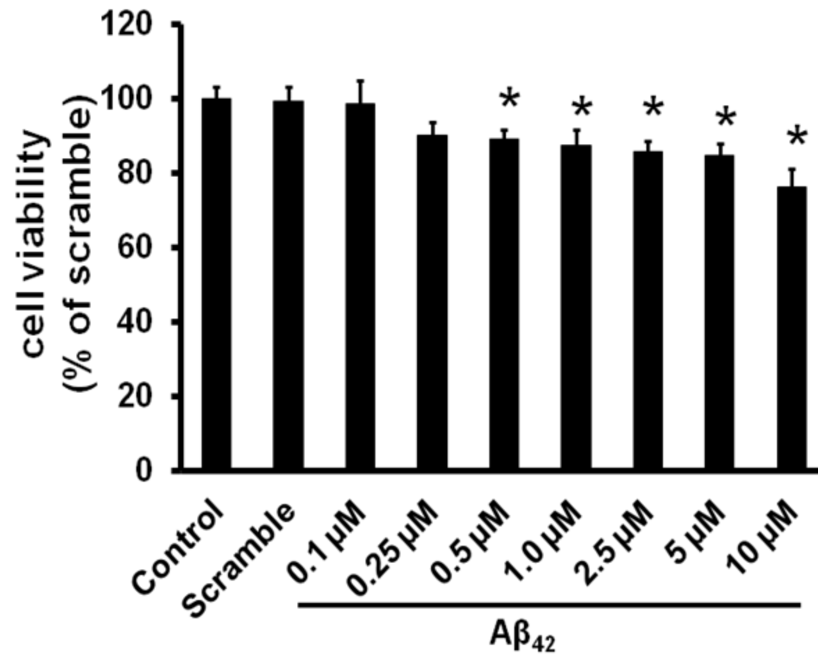


(B)



(C)




(D)
cell viability
(% of scramble)
120
100
80
60
40
20
0
*
*
*
*
*
Control
Scramble
0.1 μM
0.25 μM
0.5 μM
1.0 μM
2.5 μM
5 μM
10 μM
Aβ42

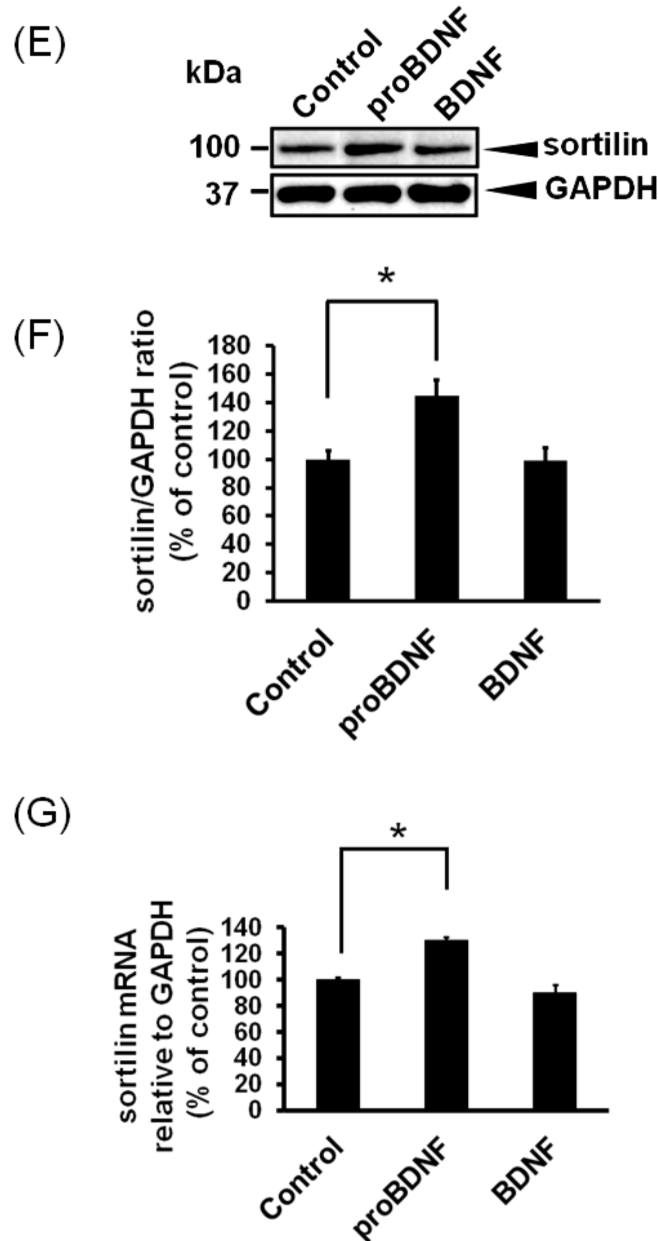


Figure 5-3: Dose-response of Aβ_{42} on Sortilin protein and mRNA expression by Western blot and quantitative RT-PCR

A-C) Effect of Aβ_{42} oligomer on protein (A and B) and mRNA (C) expression level of Sortilin in SH-SY5Y. Cells were treated with different concentration of Aβ_{42} for 24 hours and were subjected to Western blot (A and B) and RT-PCR (C) (*$p<0.05$, **$p<0.01$) (n=4, mean±SEM, ANOVA, Tukey's post hoc test; "Control" and "0 µM Aβ_{42}" were normalized to 100%). D) Effect of dose-dependent cell viability of Aβ_{42}

oligomer in SH-SY5Y cell line. MTT assay was applied to quantify the effect of varying concentrations of Aβ_{42} oligomer for 24 hours on cell viability in SH-SY5Y cell line ; (*$p<0.05$) (n=3, mean±SEM, ANOVA, Tukey's post hoc test; "Control" was normalized to 100%). E-G) Effect of proBDNF and BDNF on Sortilin expression in SH-SY5Y. proBDNF (50ng/ml) for 24 hours significantly increased protein (E and F) and mRNA (G) expression of Sortilin in SH-SY5Y. BDNF (50 ng/ml) did not change the protein (E and F) and mRNA (G) level of Sortilin when compared to control (50 ng/ml of BSA) (*$p<0.05$) (n=4, mean±SEM, ANOVA, Tukey's post hoc test; "Control" was normalized to 100%).

Time-dependent effect of $A\beta_{42}$ oligomers on Sortilin expression in SH-SY5Y cells

To test the effect of $A\beta_{42}$ time-dependent Sortilin gene and protein expression, SH-SY5Y cells incubated with 1µM $A\beta_{42}$ over a time course of 3, 6, 12, 24 hours and the cell lysate was subjected to Western blot and real-time PCR for quantification of Sortilin protein and gene expression, respectively. Interestingly, 12 and 24 hours incubation with 1µM $A\beta_{42}$ significantly up-regulated the protein and gene expression of Sortilin in SH-SY5Y cells however a dramatic overexpression of Sortilin mRNA was observed after 6 hours incubation with $A\beta_{42}$ ($p<0.05$, $p<0.01$) (Figure 5-4 A-C). Since sorLA is another member of the Vps10p family of receptors and has been linked to the development of AD (Rogaeva, Meng et al. 2007, Gustafsen, Glerup et al. 2013), we investigated whether $A\beta_{42}$ enhances the gene expression of sorLA in SH-SY5Y. These cells were incubated with 1µM $A\beta_{42}$ for 3, 6, 12, 24 hours but $A\beta_{42}$ oligomers did not elevate the gene expression of sorLA (Figure 5-4 D).

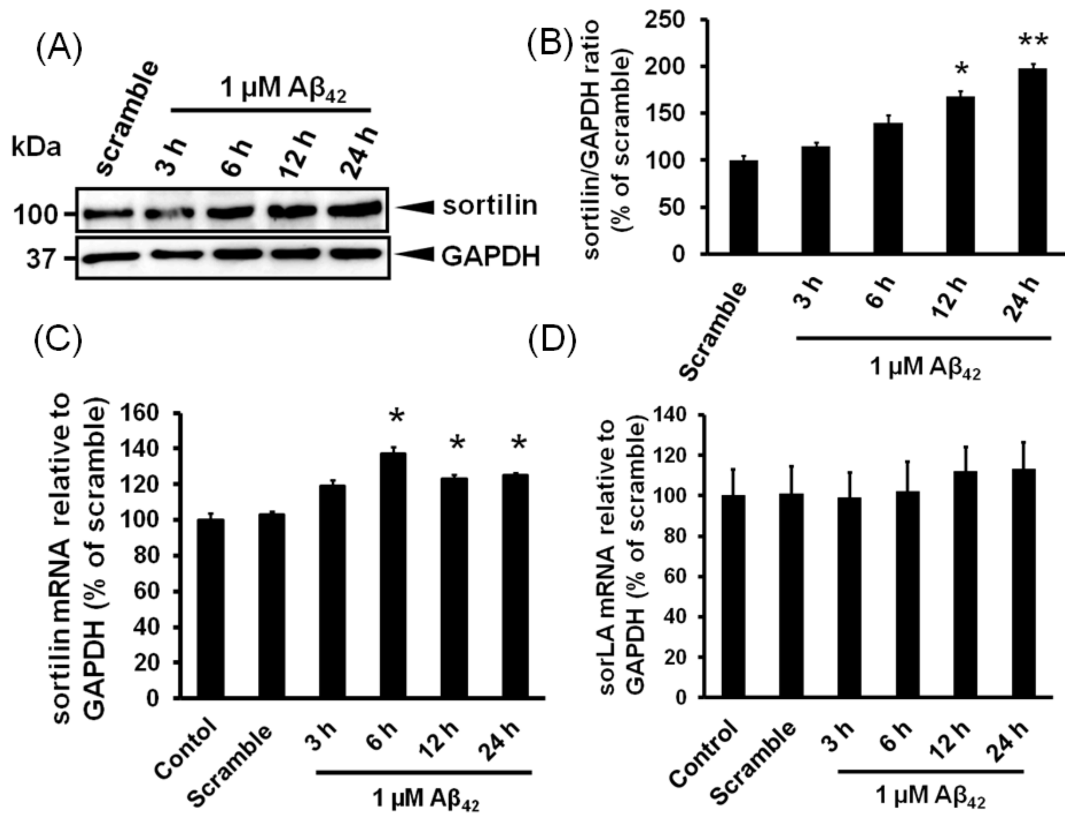


Figure 5-4: Effect of optimized standard 1μM $A\beta_{42}$ over a 24h time-course on protein and mRNA expression of Sortilin in SH-SY5Y by Western blot and quantitative RT-PCR respectively

A-C) 12 and 24 hours incubation with 1μM $A\beta_{42}$ significantly up-regulates Sortilin protein expression in SH-SY5Y cells (A and B), while a dramatic overexpression of Sortilin mRNA (C) was seen after 6 hours treatment with $A\beta_{42}$ (* $p<0.05$, ** $p<0.01$) (n=4, mean±SEM, ANOVA, Tukey's post hoc test; "Control" for B and "Scramble" for C were normalized to 100%). D) Effect of 1μM $A\beta_{42}$ for 3, 6, 12, 24 hour incubation on mRNA expression level of sorLA in SH-SY5Y (n=4, mean±SEM, ANOVA, Tukey's post hoc test; "Control" was normalized to 100%). In spite of Sortilin, the expression SorLA was not increased in the presence of $A\beta_{42}$ oligomer.

Aβ_{42} enhances Sortilin expression via p75^{NTR} receptor in SH-SY5Y cells

To elucidate whether p75^{NTR} is involved in Aβ-induced Sortilin overexpression, we blocked p75^{NTR} with 10 μg/ml p75ECD-Fc recombinant peptide or 10 μg/ml anti-p75ECD antibody for 24 hours in conjunction with 1 μM Aβ_{42}. Interestingly, Aβ_{42}-induced Sortilin protein and mRNA overexpression appears to occur via the p75^{NTR}, as the p75ECD-Fc and anti-p75ECD blocked the gene and protein upregulation of Sortilin in the presence of Aβ_{42} ($p<0.05$, $p<0.01$) (Figure 5-5 A-C).

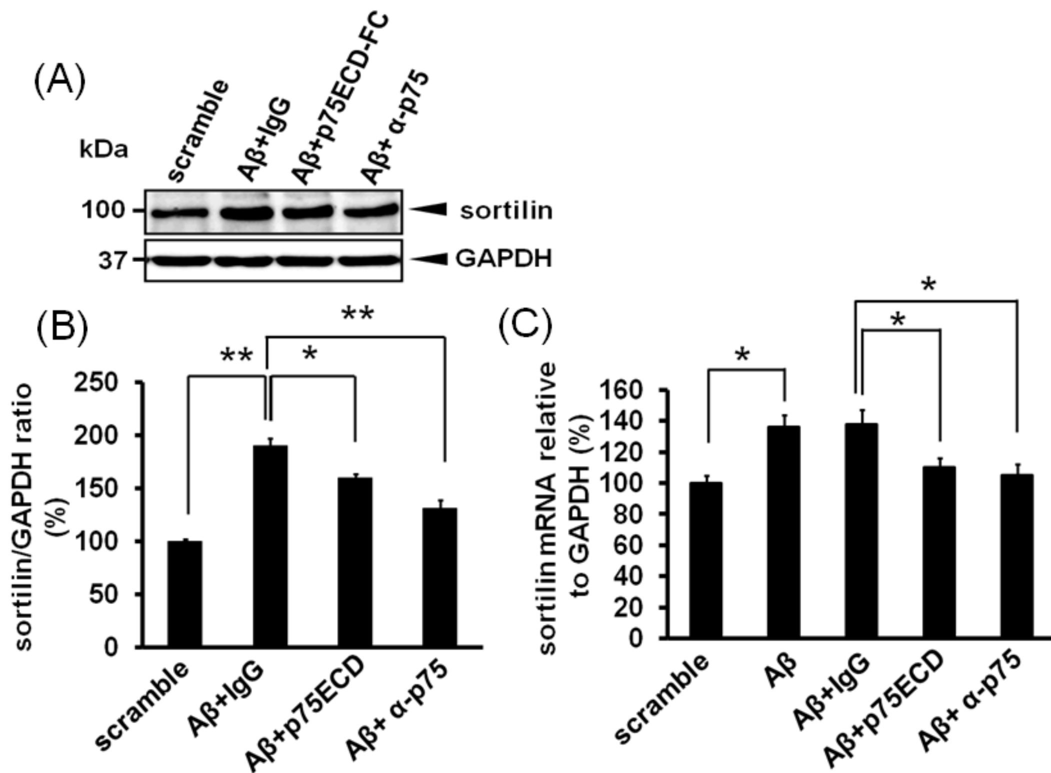


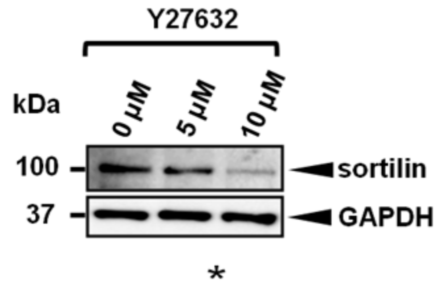
Figure 5-5: Aβ_{42} functions through the p75^{NTR} receptor in SH-SY5Y cells

p75ECD-Fc recombinant protein and an antibody against p75ECD blocked the Aβ_{42}-induced protein (A and B) and gene (C) overexpression of Sortilin in SH-SY5Y cell line (*$p<0.05$, **$p<0.01$) (n=4, mean±SEM, ANOVA, Tukey's post-test; "Scramble" was normalized to 100%).

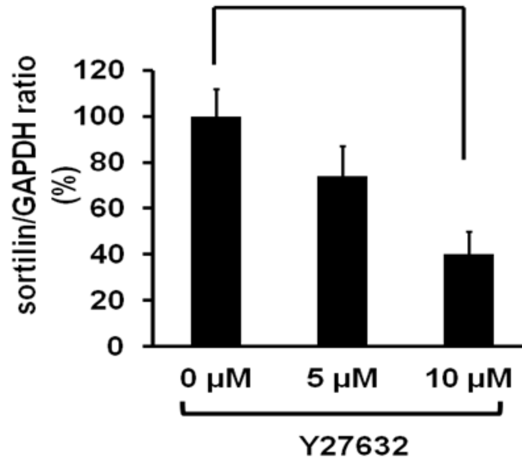
Aβ_{42} up-regulates Sortilin expression through the RhoA signalling pathway in SH-SY5Y cells

Several signalling pathways are involved in the signal transduction of p75^{NTR}. Aβ and proneurotrophins can activate JNK and RhoA via the p75^{NTR} receptor (Yao, Nguyen et al. 2005, Skeldal, Matusica et al. 2011, Sun, Lim et al. 2012). Next, we asked whether RhoA is involved in Sortilin overexpression by Aβ_{42}. Y27632 (a well characterized ROCK inhibitor) was used to inhibit ROCK. When 5 and 10 μM Y27632 were incubated with SH-SY5Y cells for 24 hours, 10μM Y27632 resulted in a significant down-regulation of endogenous Sortilin protein ($p<0.05$) (Figure 5-6 A and B). This indicates that RhoA is involved in Sortilin regulation, however, to elucidate whether Y27632 reduces Aβ-induced Sortilin upregulation, SH-SY5Y cells were treated with 1 μM Aβ_{42} and 10 Y27632 for 24 hours. It appeared that Y27632 suppressed Sortilin expression induced by Aβ_{42} ($p<0.05$, $p<0.01$) (Figure 5-6 C and D). We also observed that 1 μM Aβ_{42} significantly increased the ratio of active RhoA-GTP relative to total Rho ($p<0.05$) (Figure 5-6 E and F).

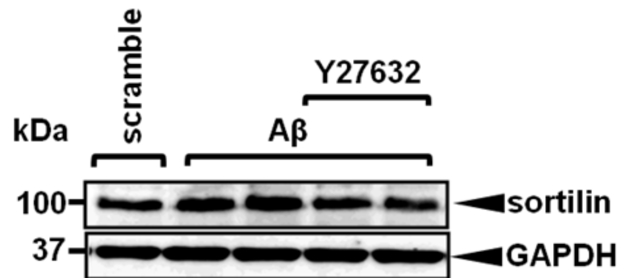
(A)



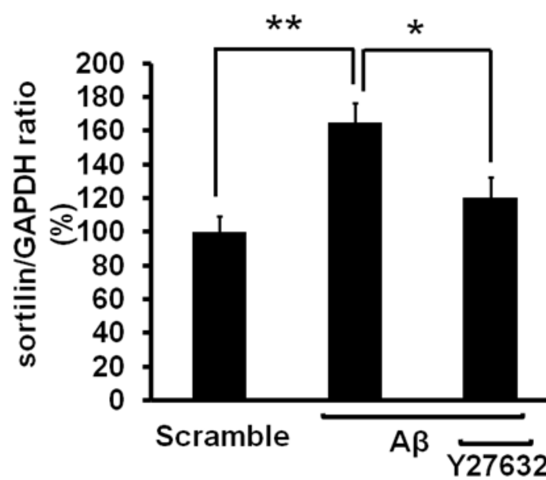
(B)



(C)



(D)



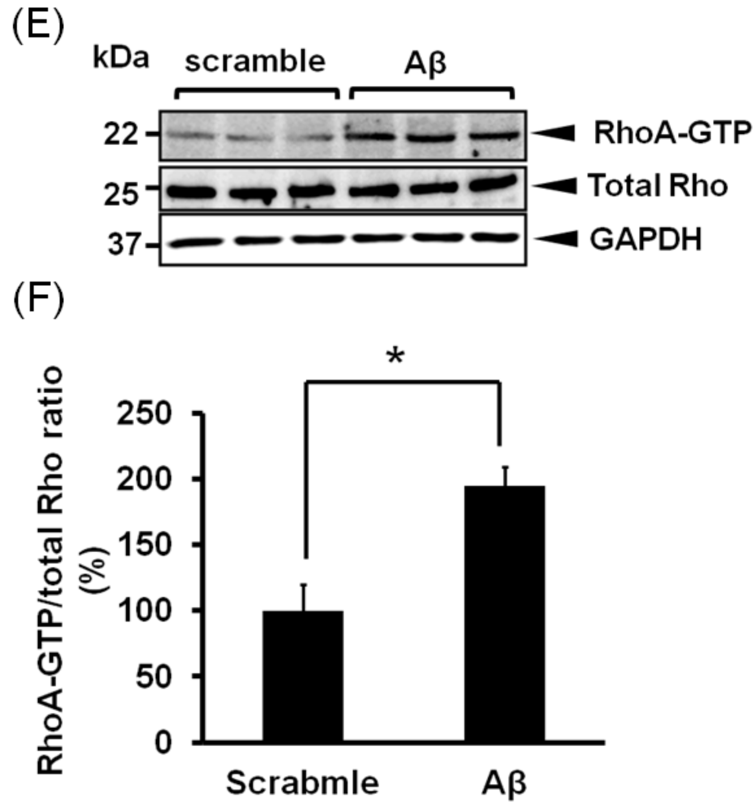


Figure 5-6: Involvement of RhoA signalling pathway in Sortilin expression by SH-SY5Y cell line assessed by Western blot

A and B) ROCK inhibitor (Y27632) down-regulates the endogenous expression of Sortilin in SH-SY5Y in dose-dependent manner after 24 hours incubation, with the effect of 10 μM Y27632 was significant on Sortilin expression compare with control (*$p<0.05$) (n=3, mean±SEM, ANOVA, Tukey's post hoc test; "0 μM Y27632" was normalized to 100%). C and D) SH-SY5Y cells were treated with 10 μM Y27632 and 1 μM $A\beta_{42}$ in incubation for 24 hours indicating that Y27632 suppressed overexpression of Sortilin in presence of $A\beta_{42}$. Results are represented as mean±SEM of 3 individual experiments (*$p<0.05$, **$p<0.01$) (ANOVA, Tukey's post hoc test; "Scramble" was normalized to 100%). E and F) the results also show that 1 μM $A\beta_{42}$ increased the level of RhoA-GTP (Activated) relative to total Rho (*$p<0.05$) (n=3, mean±SEM, ANOVA, Tukey's post hoc test; "Scramble" was normalized to 100%).

$A\beta_{42}$ does not activate the overexpression of Sortilin receptor through JNK signalling pathway in SH-SY5Y cells

JNK signalling pathway is one potential mechanism regulating the transcriptional activity of $A\beta_{42}$ and mediates the upregulation of essential proteins such as BACE1 in AD (Kadowaki, Nishitoh et al. 2005, Yao, Nguyen et al. 2005, Ma, Yang et al. 2009, Guglielmotto, Monteleone et al. 2011, Ramin, Azizi et al. 2011). Therefore, we speculated that JNK may play a role in the regulation of Sortilin expression in the presence of $A\beta_{42.}$ In order to investigate this, a JNK inhibitor (SP600125, 2 μM) (Guglielmotto, Monteleone et al. 2011) was incubated with SH-SY5Y cells for 24 hours and the cell lysate collected to assess Sortilin protein expression. SP600125 did not alter the endogenous expression of Sortilin (Figure 5-7 A and B). We also tested if SP600125 prevents Sortilin upregulation in the presence of $A\beta_{42}$. Thus, SH-SY5Y cells were incubated together with 1 μM $A\beta_{42}$ and 2 μM SP600125 for 24 hours, however, SP600125 did not block the overexpression of Sortilin protein in the presence of $A\beta_{42}$ (Figure 5-7 C and D).

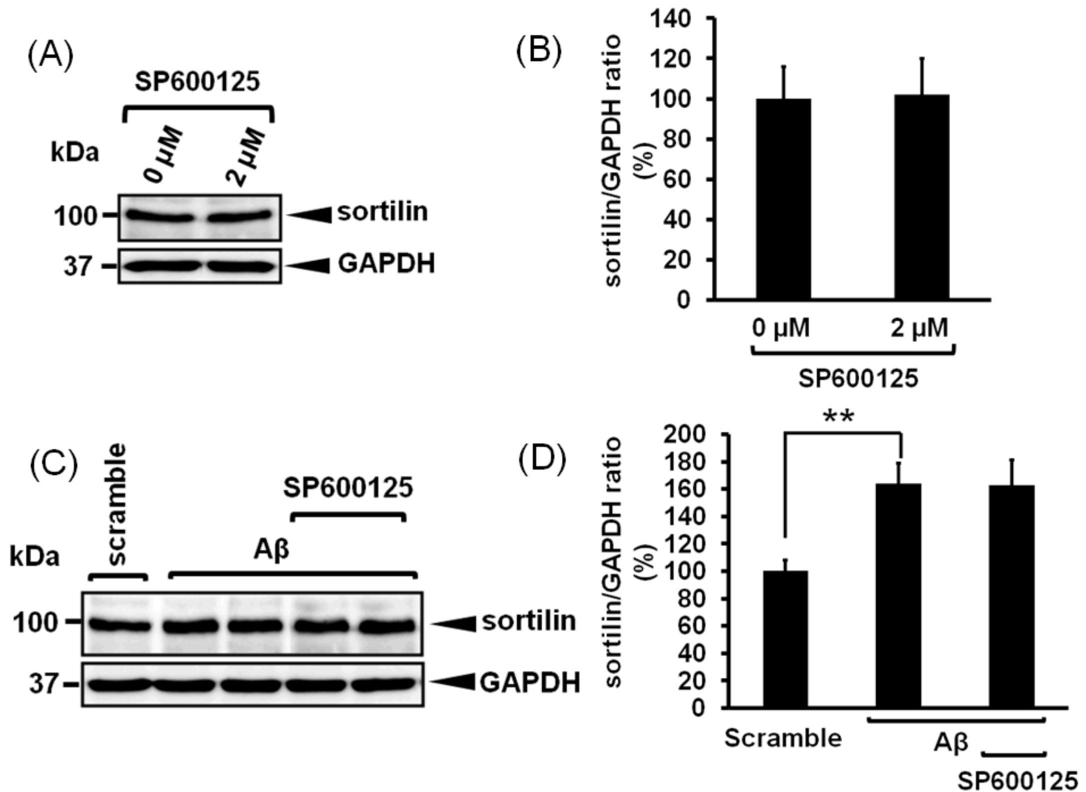


Figure 5-7: Potential involvement of the JNK pathway in the expression of Sortilin by $A\beta_{42}$ in SH-SY5Y cell line

A and B) SH-SY5Y cells were exposed with 2 μM JNK inhibitor (SP600125) (Guglielmotto, Monteleone et al. 2011) for 24 hours, whereby SP600125 did not block the endogenous expression of Sortilin (n=3, mean±SEM, Student's t-test; "0 μM SP600125" was normalized to 100%). C and D) SH-SY5Y cells were treated with 1 μM $A\beta_{42}$ and 2 μM SP600125 for 24 hours, whereby the above data demonstrates that SP600125 did not block Aβ-induced upregulation of Sortilin (**$p<0.01$) (n=3, mean±SEM, Student t-test; "Scramble" was normalized to 100%).

5.5 Discussion

Our study has uncovered a novel functional effect of Aβ_{42} on Sortilin expression in AD. We demonstrated that Aβ_{42} stimulates the expression of the Sortilin receptor in SH-SY5Y cells and that the level of Sortilin expression in human AD brain is higher than controls. Protein expression data indicates that Sortilin is also up-regulated in the brain of 6-month old APPswe/PS1dE9 (AD) transgenic mice when compared with aged-matched wild type controls. We also found that Aβ_{42} activates Sortilin expression through the p75^{NTR}/ RhoA signalling pathway.

The mammalian Vps10p sorting receptors, consisting of 5 members of type-I transmembrane protein (Sortilin, SorLA, and SORCS1-3), may play differential roles in the pathogenesis of AD. They are involved in mediating a variety of intracellular sorting and trafficking functions between endosome and TGN compartments and subsequently increase the progress of neurodegenerative diseases like AD (Nyborg, Ladd et al. 2006, Lane, St George-Hyslop et al. 2012). For example, sorLA and SORCS1 mediate retrograde trafficking of APP from the cell surface to Golgi compartments and reduce the Amyloidogenic processing of APP and production of Aβ and decrease the risk of AD (Nyborg, Ladd et al. 2006, Rogaeva, Meng et al. 2007, Gustafsen, Glerup et al. 2013). The function of sorLA and SORCS1 on APP processing occurs via their interactions with the retromer complex (Small, Kent et al. 2005, Lane, Raines et al. 2010, Fjorback, Seaman et al. 2012). Sortilin, on the other hand, plays an important role in the regulation of neuronal viability and function and it interacts with p75^{NTR} and proNGF (Nykjaer, Lee et al. 2004) and is required for the toxic action of Aβ oligomers (Takamura, Sato et

al. 2012). The functions of Sortilin in APP trafficking and Aβ production are not fully understood. Sortilin interacts with BACE1 regulates its retrograde trafficking and accelerates production of $A\beta_{42.}$ On the other hand, Sortilin binds APP, regulates dendritic and axonal targeting of APP and mediates the processing of APP toward the non-Amyloidogenic pathway and increases the production of sAPPα (Gustafsen, Glerup et al. 2013). We recently found that Sortilin interacts with APP in a head to head and tail to tail manner, in which the FLVHRY motif of Sortilin interacts with the APP NPTYKFFE motif, regulating lysosomal targeting of APP and reducing production of Aβ (Yang, Virassamy et al. 2013).

It has been shown that the high-affinity, pro-survival NGF receptor TrkA is reduced in AD (Mufson, Wuu et al. 2010, Finan, Okada et al. 2011). As Sortilin interacts with APP and BACE1, it is thought that Sortilin plays an important role in the progression of AD. In support of this hypothesis, we first found that the level of Sortilin expression in AD brain and in the brain of 6-month old APPswe/PS1dE9 (AD) transgenic mice to be higher than age-matched normal brain of human and wild type mice. The finding from human AD brain, while being from a small sample size, is consistent with the results from the transgenic mice and AD cell line models. In particular, we demonstrated that $A\beta_{42}$ oligomer caused upregulation of the Sortilin receptor at the gene and protein level. Interestingly, proBDNF, but not mature BDNF, also stimulates gene expression of Sortilin. It appears that the role in Sortilin expression is specific, as sorLA gene expression is not altered in the presence of $A\beta_{42}$.

Oligomeric forms of Aβ_{42} play a critical role in progress of AD as Aβ_{42} is more likely to form oligomers rather than Aβ_{40} (Kim, Chae et al. 2003, Walsh and Selkoe 2007, Glabe 2008, Reddy, Manczak et al. 2010). The oligomers disrupt cognitive functions in the brain and potently inhibit long-term potentiation (LTP), enhance long-term depression (LTD) and reduce dendritic spine density in normal rodent hippocampus (Chromy, Nowak et al. 2003, Lacor, Buniel et al. 2004, Cleary, Walsh et al. 2005). Interestingly Aβ oligomers activate differential gene expression in the human brain (Sebollela, Freitas-Correa et al. 2012). For example, it has been shown that Aβ_{42} up-regulates the expression of $p75^{NTR}$ neurotrophin receptor in SH-SY5Y human neuroblastoma cells and AD transgenic mice through IGF-1 (Chakravarthy, Gaudet et al. 2010, Chakravarthy, Menard et al. 2012, Ito, Menard et al. 2012). In our previous study, we reported that the expression of $p75^{NTR}$ in AD mouse brain is higher than wild type (Wang, Wang et al. 2011). It is also suggested that Aβ_{42} triggers upregulation of BACE1 through the JNK pathway and induces physiological transcriptional activation of BACE1 (Guglielmotto, Monteleone et al. 2011, Piccini, Borghi et al. 2012). It is also known that Aβ can activate RhoA causing neurite collapse (Petratos, Li et al. 2008), but the mediating receptor is not known. Elucidation of the receptor pathway involved in upregulation of Sortilin in the presence of Aβ_{42} is crucial for understanding the significance of this biological phenomenon. Previous studies have shown that some gene expression is predominantly regulated by proneurotrophins via $p75^{NTR}$ (Nakamura, Namekata et al. 2007, Mufson, Wuu et al. 2010). In addition, Aβ can bind $p75^{NTR}$ mediating the apoptosis and degeneration of cholinergic neurons (Coulson, May et al. 2009). Therefore, we investigated whether Aβ_{42} up-regulates Sortilin via $p75^{NTR}$. Three

pieces of evidence support the notion that p75^{NTR} mediates the gene and protein upregulation of Sortilin in the presence of Aβ_{42}. First, the diffusible p75ECD peptide can block the upregulation of Sortilin induced by Aβ. Secondly, the antibody to the extracellular domain of p75^{NTR} also blocks the response. Thirdly, an alternate p75^{NTR}/Sortilin ligand proBDNF but not p75^{NTR}/TrkB ligand mature BDNF triggers the response. These data indicate that neurodegenerative ligands of p75^{NTR}/Sortilin receptors up-regulate the expression of Sortilin. It is likely that such a regulatory mechanism operates *in vivo* in the brain of AD mice and human patients as Sortilin is clearly increased in brains of AD mice. What are the down-stream signals of p75^{NTR} responsible for the upregulation of Sortilin in response to Aβ and proBDNF? It is well known that JNK3 is one of most common signalling pathways to be activated by proNTs and through Sortilin/p75^{NTR}, resulting in neuronal cell death (Reichardt 2006, Volosin, Song et al. 2006, Koshimizu, Hazama et al. 2010, Skeldal, Matusica et al. 2011). It also reported that Aβ_{42} induces BACE1 expression through JNK (Guglielmotto, Monteleone et al. 2011). Therefore, it is of interest to investigate whether the upregulation of Sortilin in the presence Aβ_{42} occurs through JNK. To our surprise, a JNK inhibitor did not influence Aβ-induced Sortilin upregulation. The neurodegenerative signals of proBDNF, proNGF and Aβ cause neurite collapse via activating p75^{NTR} and RhoA GTPase/ROCK pathway (Petratos, Li et al. 2008, Sun, Lim et al. 2012). We propose that this pathway may be involved in the regulation of Sortilin expression. Consistent with this idea, we found that the ROCK inhibitor, Y27632, blocked the response induced by Aβ. In addition, we also demonstrated that Aβ_{42} increased the level of active RhoA-GTP, confirming the previous finding (Petratos, Li et al. 2008). This data suggests that the

upregulation of Sortilin is positively regulated by Aβ, proBDNF and $p75^{NTR}$ via activation of the RhoA-GTPase/ROCK signal pathway.

In summary, in contrast to sorLA, the expression of Sortilin is increased in the AD brain of mice and humans and in an AD cell line model. The increased expression of sortlin is regulated by Aβ oligomers by activating the RhoA GTPase/ROCK pathway through the neurotrophin receptor $p75^{NTR}$. As Sortilin is important in neuronal cell death induced by the proneurotrophin/$p75^{NTR}$ pathway and Aβ production, this novel regulation mechanism may play a role in AD pathogenesis (Figure 5-8).

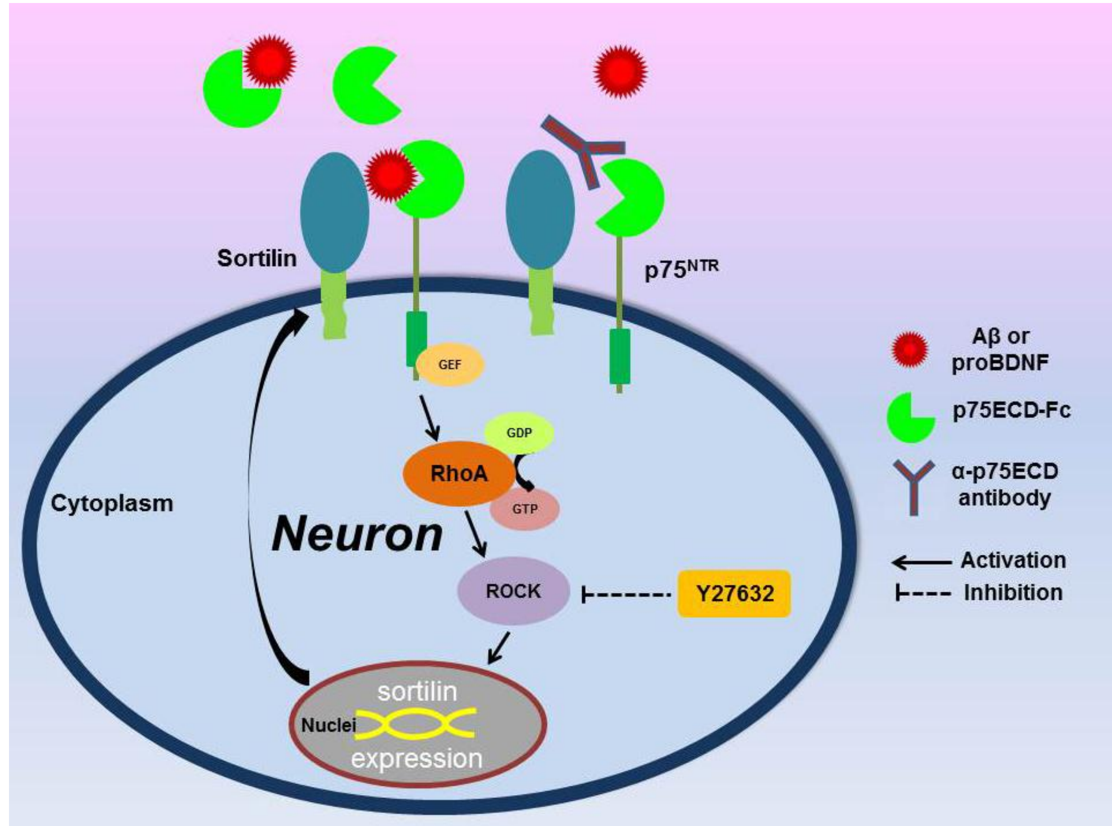


Figure 5-8: Graphical summary for the mechanism of Aβ-induced Sortilin upregulation in neurons

Sortilin is the co-receptor of $p75^{NTR}$ which signals the cell death induced by Aβ and proneurotrophins. We found that Sortilin is increased in the AD brain and up-regulated by Aβ and proBDNF. Aβ-induced upregulation of Sortilin is mediated by $p75^{NTR}$ and the down-streaming RhoA-ROCK signalling pathway. The Aβ/Sortilin/$p75^{NTR}$ signalling may play a role in the pathogenesis of AD.

CHAPTER 6: GENERAL DISCUSSION

6.1 Summary

Neuronal degeneration is a common feature of AD pathogenesis and contributes to cognitive impairment. Aβ, as a pathological hallmark of AD, promotes the progression of neurite degeneration in AD patients, but the mechanism of this phenomenon still is not fully known (Petratos, Li et al. 2008). The neurotrophins and their receptors regulate neurite degeneration and the subsequent pathological procession of sporadic AD. There is some evidence to show that Aβ mediates neuronal degeneration through interactions with $p75^{NTR}$ and the activation of the c-Jun pathway, a common downstream signalling pathway of $p75^{NTR}$ (Morishima, Gotoh et al. 2001, Hashimoto, Kaneko et al. 2004, Yaar, Zhai et al. 2007). For example, Aβ induces neuronal apoptosis of cholinergic neurons in the BF through interaction with $p75^{NTR}$ (Sotthibundhu, Sykes et al. 2008, Knowles, Rajadas et al. 2009). In contrast, $p75^{NTR}$ promotes sympathetic innervation in AD mouse brain and suggesting $p75^{NTR}$ not only contributes to neuronal degeneration via association with Sortilin and Aβ, but promotes neuronal survival via interaction with the TrkA-NGF signalling pathway (Chao 2003, Bengoechea, Chen et al. 2009). This study has been focused or addressing how $p75^{NTR}$ modulates amyloidogenic processing of APP and subsequently accelerates AD progression. In addition, since Sortilin, a close co-receptor of $p75^{NTR}$, interacts with BACE1 and modulates BACE1 trafficking and Aβ production (Finan, Okada et al. 2011), we investigated whether Aβ can up-regulate Sortilin expression.

Our study indicated that $p75^{NTR}$ interacts with APP and promotes amyloidogenic processing of APP in the CHO^{APP695} cell line. Furthermore, Aβ induces BACE1 and APP expression through interaction with $p75^{NTR}$ and subsequently promotes APP

processing in AD/p75$^{+/+}$, but not in AD/p75$^{-/-}$ cortical neurons. Aβ_{42} mediates APP and BACE1 internalization through p75^{NTR}. Moreover, the BACE1/APP interaction is enhanced in the presence of proNGF and Aβ. Aβ_{42} promotes APP-Thr668 and Tau phosphorylation in mouse cortical neurons via a p75^{NTR}-dependent mechanism. Therefore our study suggests that full length p75^{NTR} is a key receptor to involve in the Aβ-driven AD pathogenesis.

Our study demonstrated that BACE1 associates with p75ECD and Aβ and that proNGF increased the BACE1/p75^{NTR} interaction. BACE1 proteolytically cleaves p75^{NTR} within subcellular compartments and subsequently releases p75ECD intracellularly. To examine the significance of p75ECD in AD, we found that p75ECD-Fc recombinant protein restores the neurite outgrowth impairment induced by Aβ and proNTs in cortical neurons. This suggests p75ECD plays a protective function for in AD and can mitigate Aβ-induced neuronal degeneration.

Our *in vivo* study indicated that i.p. delivery of p75ECD-Fc recombinant protein was not effective on learning and memory in APPswe/PS1DE9 (AD) mouse. p75ECD-Fc improved the learning process, but not memory in PR5 mouse. In addition, p75ECD-Fc was able to prevent BACE1 upregulation and subsequently reduce Aβ plaque deposition in AD mice brain through the inhibition of BACE1 expression. p75ECD-Fc significantly reduced astrogliosis, hallmark of AD pathogenesis, in AD mice. In PR5 mice we found that p75ECD-Fc was able to inhibit Tau phosphorylation as well as BACE1 expression.

As Sortilin co-operates with p75^{NTR} in neuronal degeneration, we investigated the expression and regulation of Sortilin in AD patients and mouse brain. Our study showed

an increased level of Sortilin in human AD brain and in brains of 6-month old APPswe/PS1dE9 (AD) transgenic mouse in comparison with aged-matched normal controls. $A\beta_{42}$ and proBDNF increase the gene and protein expression level of Sortilin in SH-SY5Y cells. In addition, we discovered that the inhibition of $p75^{NTR}$ and ROCK, but not JNK, suppressed both the constitutive and $A\beta_{42}$-induced expression of Sortilin.

6.2 Future directions

Based on our studies, we have shaped and defined our future directions in order to better understand the function of $p75^{NTR}$ and its associated downstream signalling pathways in the AD pathology. Amyloidogenic processing of APP occurs mostly within intracellular organelles as BACE1 activity increases in the acidic environment (Daugherty and Green 2001), but how BACE1 and APP converge into the acidic environment is not clear. The convergence of APP and BACE1 is essential for the production of Aβ. Whilst Aβ production is activity- and endocytosis-dependent (Cirrito, Kang et al. 2008), the extracellular signals that regulate the endocytosis of APP and BACE1 are not known. Identification of extracellular signals regulating the convergence and endocytosis of BACE1/APP may shed light on how the amyloidogenic pathway is regulated. We show that $p75^{NTR}$ interacts with BACE1 via the p75ECD (Figure 3-2). We and others (Fombonne, Rabizadeh et al. 2009) showed $p75^{NTR}$ also interacts with APP. However, the significance of these interactions is not clear. Our study indicated Aβ/$p75^{NTR}$ mediates APP and BACE1 internalization from the cell surface (Figure 2-12). We propose that binding of neurodegenerative ligands to $p75^{NTR}$ will trigger the endocytosis of $p75^{NTR}$ together with BACE1 and APP where BACE1 may cleave both APP and $p75^{NTR}$ and increase Aβ production. It would therefore be logical to next examine whether $p75^{NTR}$ and its neurodegenerative ligands regulate the convergence of BACE1 and APP in subcellular compartments such as early endosomes. To address this question, we would quantify the co-localization of endogenous APP and BACE1 in subcellular compartments in $p75^{+/+}$ and $p75^{-/-}$ mouse cortical neurons. To further investigate this, $p75^{+/+}$ and $p75^{-/-}$ mouse cortical neurons would be transfected with

BACE1-CFP and APP-YFP in the presence and absence of Aβ and proNTs and then subjected to FRET acceptor bleaching assay in order to evaluate the BACE/APP interaction. This experiment will reveal whether Aβ and proNTs influence on BACE1/APP interaction through the $p75^{NTR}$ receptor. We could also use live imaging using confocal microscopy to demonstrate the spatial and temporal convergence of BACE1/APP regulation in $p75^{+/+}$ and $p75^{-/-}$ mouse cortical neurons in response to $p75^{NTR}$ ligands Aβ and proNGF. The results would be compared between ligand-treated and non-treated in $p75^{+/+}$ and $p75^{-/-}$ mouse neurons. The data would address whether $p75^{NTR}$ directly regulates BACE1/APP association in response to $p75^{NTR}$ ligands.

The majority of BACE1 substrates are type I transmembrane molecules which form an alignment of membrane with BACE1. $p75^{NTR}$ is also a type-I transmembrane protein and shares with APP the same ectodomain shedding β-secretase and ADAM17 to generate p75ECD (Weskamp, Schlondorff et al. 2004, Parkhurst, Zampieri et al. 2010) and the same intra-membrane proteolysis machinery γ-secretase to generate CTF and ICD (Parkhurst, Zampieri et al. 2010). We have shown that the level of p75ECD in the brain of $BACE1^{+/+}$ mice is higher than and $BACE1^{-/-}$ (Figure 3-5). We also presented that BACE1 cleaves $p75^{NTR}$ and generate both, 50 kDa and 26 kDa, p75ECD fragments in two different cell lines (Figure 3-6 and Figure 3-7), but it remains necessary to define the cleavage site of $p75^{NTR}$ by BACE1. We could use biochemical approaches to address this question, we will use. We could perform digestion assays on $p75^{NTR}$ recombinant protein with commercial BACE1. The digested fractions would be purified and subjected for sequencing to identify the cleavage site by Prof. Moses V. Chao who mapped the $p75^{NTR}$ cleavage site by TACE (Zampieri, Xu et al. 2005).

Our *in vitro* data presented that p75ECD-Fc recombinant protein prevents Aβ and proNTs-induced neurite outgrowth impairment (Figure 3-9, Figure 3-10 and Figure 3-11). In order to address the effects of $p75^{NTR}$ processing by BACE1, we would carry out an *in vivo* study to see whether the cleavage of $p75^{NTR}$ by BACE1 is neuroprotective. Our subcellular fractionation results in $p75^{+/+}$ and $p75^{-/-}$ mice brain indicated that $p75^{NTR}$ contributes to APP distribution in subcellular compartments which are involved in APP processing (Figure 2-11). In order to address whether Aβ stimulates the subcellular distribution of APP and BACE1 via $p75^{NTR}$ in cortical neurons, we would use a biochemical approach to separate different organelles from the membrane fraction of neurons isolated from $p75^{+/+}$ and $p75^{-/-}$ mice treated with or without Aβ in high density neuron cultures. Neurons would be treated with or without Aβ, then subjected to membrane isolation (Das, Scott et al. 2013) and the membrane would be subjected to sucrose gradient separation (Widmann, Dolci et al. 1995, Uldry, Steiner et al. 2004). The fractions would be probed with BACE1, APP and EEA1 antibodies with the non-separated fraction as input controls. This experiment would confirm whether there is convergence of APP and BACE1 in endosomal fractions regulated by $p75^{NTR}$ and its ligands.

Our *in vitro* study revealed that Aβ induces Tau phosphorylation through $p75^{NTR}$ in mouse cortical neurons (Figure 2-16). To further investigate this, we will apply an *in vivo* study to investigate the effects of $p75^{NTR}$ on cognitive function and AD biomarkers in PR5 mouse. To address this question we will cross PR5 mice with $p75^{-/-}$ mice in order to produce $Tau^{+}/p75^{-/-}$ and $Tau^{+}/p75^{+/+}$ strains. These mice would be injected with Aβ into the hippocampi for 1 week and then the spatial learning and memory function

quantified by MWM test. Following this, the brain would be isolated and subjected to IHC and Western blot analysis in order to evaluate AD biomarkers including different isoforms of hyper-phosphorylated Tau, ChAT, TUNEL, activated caspase-3, $p75^{NTR}$, MAP-2, synaptophysin, SNAP25, and VAMP2.

To conclude, multiple mechanisms are involved the development of AD. Overproduction of Aβ, reduction of Aβ clearance, OS, inflammation as well as genes all contribute to AD pathogenesis in the brain. To find a suitable method to control and prevent the causative mechanisms of Aβ over-production and feed-forward in AD is a great ambition for scientists. A number of treatments have been trialled that target Aβ removal or inhibition of Aβ generation from its precursor. For instance, Aβ plaque deposition in the brain of APP transgenic mouse was reduced after immunization with $A\beta_{42}$ (Schenk, Barbour et al. 1999). The inhibition of β- and γ-sectretase was another strategy to prevent Aβ production in AD (Ghosh, Gemma et al. 2008, Imbimbo 2008). However, there has a complete failure to develop any therapy targeted of Aβ. For instance, Wischik et al demonstrates more than 25 AD clinical trials aimed at Aβ have failed (Wischik, Harrington et al. 2014). Also, Ittner LM et al showed that Tau can drive Aβ toxicity but without Tau hyperphosphorylation, Aβ has no toxicity. Thus the Aβ theory of AD may be wrong (Ittner, Ke et al. 2010). There is no effective drug that can halt the progression of AD. One of the reasons for this failure of drug development is due to a lack of understanding of the pathogenesis of the disease despite Aβ being known to be the central player. How Aβ is excessively produced and accumulated in sporadic AD brain is not fully understood. Our discoveries within this study give us the opportunity to analyse new mechanisms into how $p75^{NTR}$ and its ligands and co-

receptors such as Sortilin are involved in the pathogenesis of sporadic AD. This research not only addresses the important signalling pathways leading to amyloidogenesis and AD pathogenesis, but also answers the important general question of how different ligands of $p75^{NTR}$ have different biological outcomes. In our study, we found that $p75^{NTR}$ has dual effects and plays a changing role in the brain which can be considered as a potential therapeutic target for AD (Figure 6-1 andFigure 6-2)

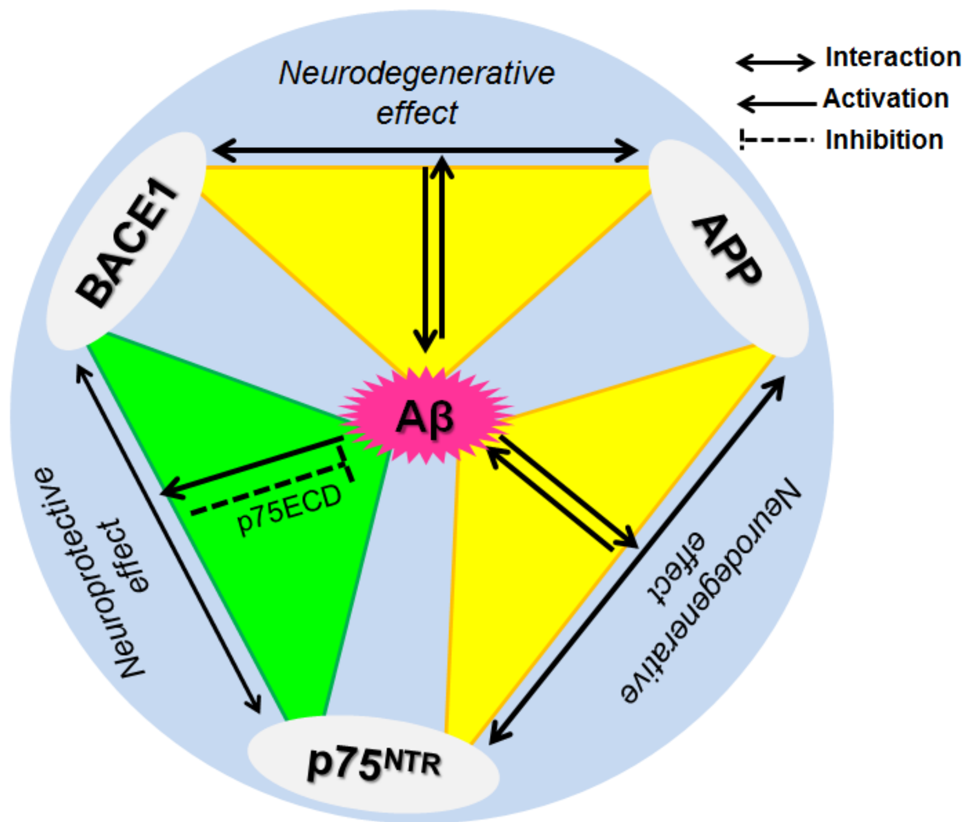


Figure 6-1: Graphical summary presenting a complex p75^{NTR}/BACE1/APP interaction in neuron

BACE1 interacts with p75^{NTR} and mediates p75^{NTR} processing to release p75ECD which has neuroprotective effects through the inhibition of Aβ activity. The interaction between APP with BACE1 and p75^{NTR} results in more Aβ production and Aβ-driven neurodegeneration. Aβ stimulates interactions between BACE/p75^{NTR}, BACE1/APP, and p75^{NTR}/APP and p75^{NTR} is required for the interaction of APP and BACE1.

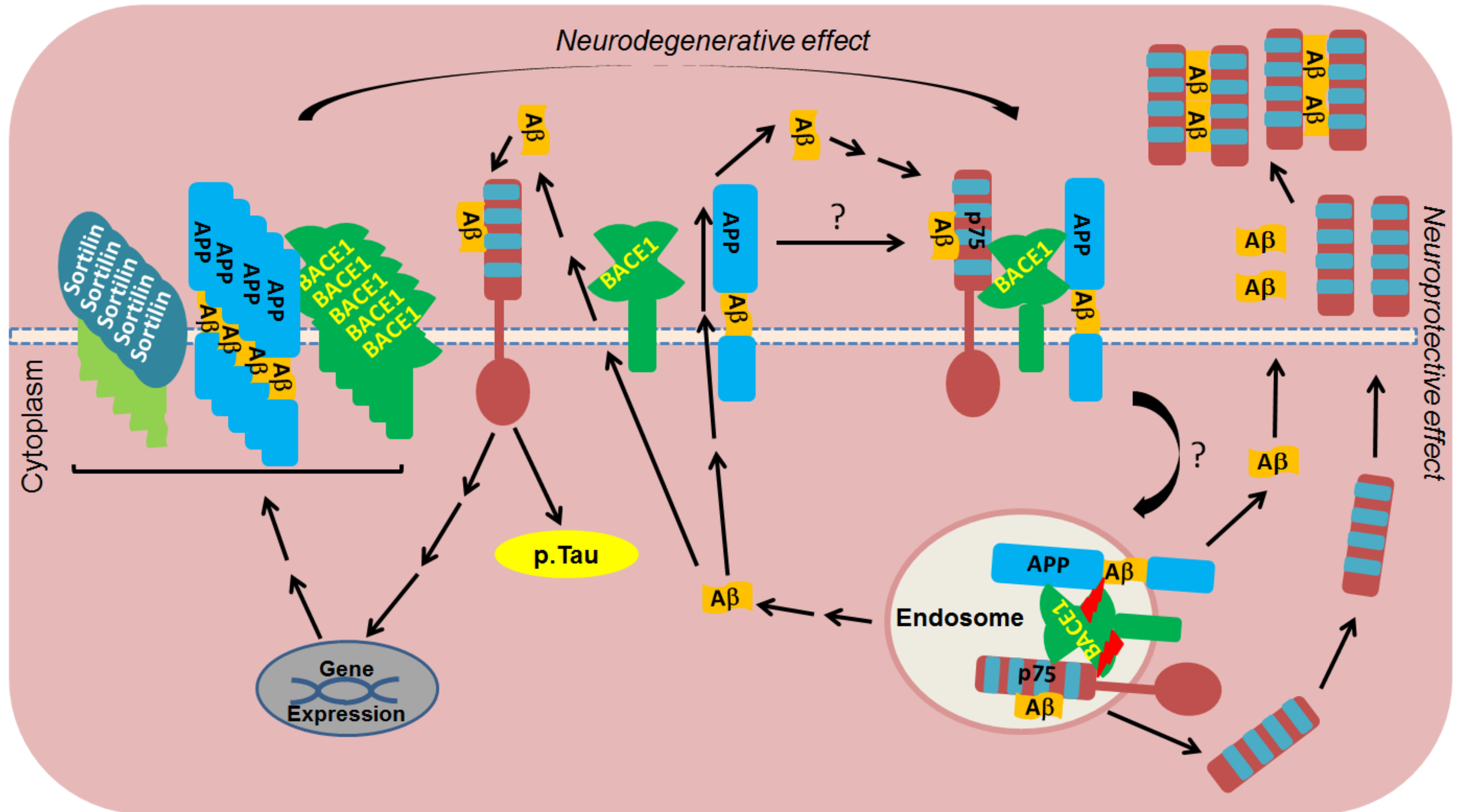

Neurodegenerative effect
Neuroprotective effect
Cytoplasm
Sortilin
APP
BACE1
Aβ
p75
?
p.Tau
Gene
Expression
Endosome

Figure 6-2: Graphical summary presenting dual roles of $p75^{NTR}$ in the development of AD

Aβ promotes APP and BACE internalization and possibly mediates BACE1/APP convergence in the early endosome via $p75^{NTR}$-dependent signalling mechanism. In addition, Aβ promotes APP and BACE1 expression and the phosphorylation of tau protein through a $p75^{NTR}$-dependent mechanism. All these phenomena enhance APP processing and lead to neuronal degeneration in AD brain. On the other hand, the internalization of BACE cleaves $p75^{NTR}$ to generate intracellular p75ECD which has a neuroprotective function in the brain extracellulary.

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